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Drugs of Abuse: Chemistry, Pharmacology, Immunology, and AIDS

U. S. DEPARTMENT OF HEALTH AND HUMAN SERVICES • Public Health Service • Alcohol, Drup Abuse, and Mental Health Administration

Drugs of Abuse: Chemistry, Pharmacology, Immunology, and AIDS

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Introduction

Phuong Thi Kim Pham

Thousands of pharmaceutical drugs line the shelves of apothecaries across the Nation. They are designed to treat a host of diseases and ailments ranging from heart disease and diabetes to allergies and strep throat. But, of all these drugs, only two (methadone and naltrexone) are available to treat narcotic addiction. The cocaine epidemic of the last few years, coupled with the spread of acquired immunodeficiency syndrome (AIDS) in the drug-abusing population, make the development of effective pharmacological treatments for drug addiction more critical than ever before.

While the prevalence of human immunodeficiency virus (HIV) seropositivity in the United States is highest for homosexual males (ranging from 20 percent to 70 percent), the incidence of seropositivity among intravenous (IV) drug abusers may be even greater, with prevalence in certain areas reaching 75 percent. Of all seropositives, IV drug abusers are generally regarded as posing the most substantial risk of spreading the disease to heterosexuals, with the risk being greater in urban areas. Also at risk are infants born to parents who are IV drug users. Current statistics suggest that one out of two children born to seropositive women develop and almost certainly die of AIDS before the age of 5. Therefore, does the high rate of seroprevalence among drug abusers reflect some factor predisposing them to AIDS?

Both clinical and preclinical studies suggest that chronic morphine treatment depresses the immune function, predisposing an individual to higher incidence of infections. By altering immunocompetence, abused drugs, such as morphine, heroin, and possibly cocaine, represent a potential source of immunodeficiency independent of HIV infection, thus making the addicts susceptible to HIV and the development of AIDS.

The first direct evidence that heroin addiction may influence the immune system came from the observation that lymphocyte mitogenesis was depressed in heroin addicts. Opiate addiction produced a significant depression in the absolute number of T lymphocytes in peripheral blood. The immune modulatory capacity of the opioid receptor agonist morphine led investigators to hypothesize the existence of opioid receptors on cells of the

immune system. Recent studies have now identified such receptors. Binding sites for both classical opioids, i.e., naloxone-reversible agonists, and nonclassical opioids, insensitive to naloxone reversibility, have been detected on lymphocytes. In addition, recent observations describing the production of opioid peptides by cells of the immune system have led to the hypothesis of bidirectional pathways shared between the immune and neurcendocrine systems based on the commonality of ligands and receptors.

The intention of this technical review was to bring together a group of internationally recognized medicinal chemists, pharmacologists, and immunologists working on both drug addiction and AIDS to discuss recent advances. Researchers from areas of pharmacology, medicinal chemistry, and immunology presented their work, interacted, and discussed several new ideas as well as areas of emphasis for future research.

It is hoped that investigations of the effects of drug abuse on the immune system may facilitate our understanding of the basic mechanisms underlying the AIDS syndrome. This in turn will help us to determine the feasibility of treatment modalities for AIDS and AIDS-related syndromes.

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The Bivalent Ligand Approach in the Design of Highly Selective Opioid Receptor Antagonists

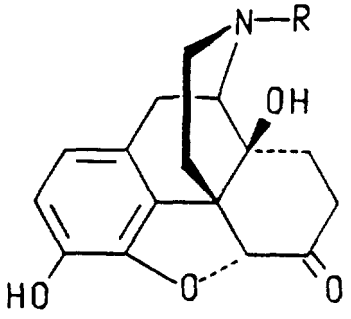
Philip S. Portoghese

INTRODUCTION

Significant advances in opioid research rely heavily on the use of selective ligands as tools. Although the opioid antagonists naloxone *1* and naltrexone 2 have been employed extensively for this purpose (Sawynok et al. 1979; Gold et al. 1982) their usefulness in sorting out the effects mediated by different opioid receptor types is limited because they are not highly selective. The multiplicity of opioid receptors, their varied distribution in neural tissue, and the array of physiologic and pharmacologic effects produced by endogenous and exogenous opioid ligands have created a need for highly selective antagonists for use as research tools (Herz 1987). In this regard, the opioid antagonist should be potent, highly selective, and be useful both *in vitro* and *in vivo* for optimal utility as receptor probes. Also, such antagonists should be relatively stable to enzymatic degradation and be capable of penetrating the central nervous system (CNS). All of these properties are desirable in the same molecule so that the *in vivo* pharmacology can be more reliably correlated with *in vitro* studies. This chapter reviews two convergent variations of the bivalent ligand approach (Portoghese 1987) that have been employed in the design of highly selective opioid antagonists.

DESIGN CONSIDERATIONS AND STRUCTURE-ACTIVITY RELATIONSHIP (SAR) STUDIES

The rationale employed to design selective opioid antagonists emanated from two different concepts that bear certain elements of similarity. In the first approach, there was a possibility of bridging two vicinal opioid receptors by means of ligands that contain two opioid antagonist pharmacophores linked through a spacer (Portoghese 1987). The second approach (Portoghese et al. 1988a) utilized the "message-address" concept (Schwyzer 1977) that involved the linkage of an opioid antagonist pharmacophore (the "message")



1, R=$CH_2CH=CH_2$; and *2,* R=$CH_2CH(CH_2)_2$

through a spacer to a nonpharmacophore recognition unit (the "address"). These two different approaches are covered separately, and their convergence under certain conditions is discussed in this chapter. The two approaches share the feature of simultaneous occupation of two vicinal recognition sites by the ligand. In this regard, the general term "bivalent ligand" has been given to ligands that are involved in such interactions.

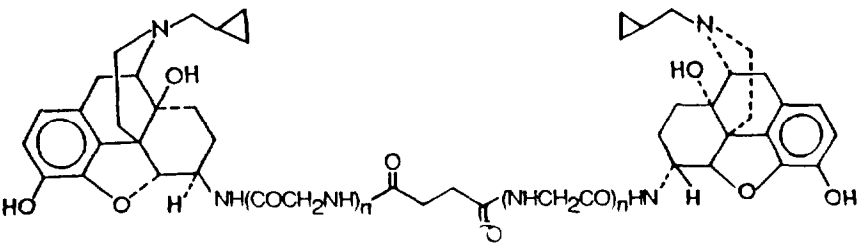
The opioid antagonist pharmacophore component of bivalent ligands based on the double pharmacophore approach and the message-address concept was derived from the potent opioid antagonist naltrexone *2.* This antagonist is selective for the μ opioid receptor type, but it also blocks κ and δ receptors (Takemori and Portoghese 1984). It was chosen as the pharmacophore because it (a) is compact, (b) is conformationally defined, (c) is relatively stable to enzymatic degradation, (d) has a ketone group that can be modified for attachment of a spacer, and (e) can penetrate the blood-brain barrier.

Double Pharmacophore Approach

The basic premise of this approach was that enhanced potency or selectivity may be conferred by simultaneous occupation of proximal recognition sites (bridging) by both recognition units in a single bivalent ligand (Portoghese 1987). These sites may be (a) two neighboring opioid receptors or (b) two different sites on a single opioid receptor. The author's initial studies were of a range-finding nature in order to evaluate the feasibility of changing selectivity and enhancing affinity through this approach. Subsequent studies were designed in an effort to distinguish between cases a and b.

The ligands 3 and 4 were synthesized in initial studies contained glycyl units (Portoghese et al. 1986a; Portoghese et al. 1986b). This permitted varying spacer length by changing the number of glycyl units, and it provided easy access to different-length spaces using standard peptide

chemistry. Another reason for selecting glycyl units rather than a hydrocarbon chain was the desire to avoid incremental increases in hydrophobic properties upon lengthening the spacer. Symmetry was introduced into the spacer by a succinyl or fumaryl group. Both groups were employed in order to study the relationship between spacer flexibility and antagonist activity.



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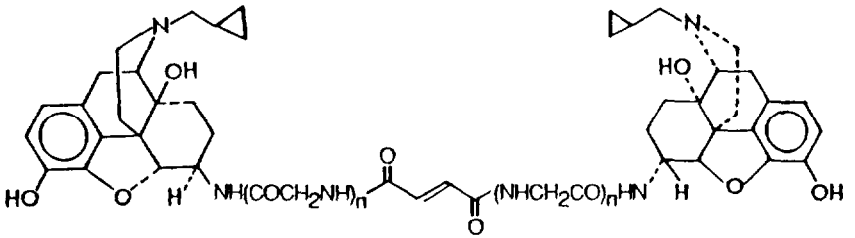
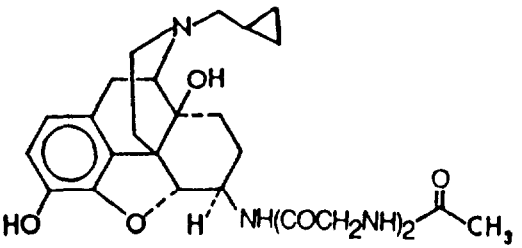


Series 4

In order to factor out the effect of the spacer on potency, ligands *5* and *6* containing a single pharmacophore and an appropriate spacer were synthesized as reference compounds.

The structure-activity profiles of series *3* and *4* are presented in figure 1. These studies were carried out on the guinea pig ileum (GPI) preparation that contains μ and κ receptors. The graphs illustrate the relative effectiveness of members of the series to antagonize either morphine (μ-selective agonist) or ethylketazocine (κ--selective agonist) as a function of the number of glycyl units (n) in the spacer.



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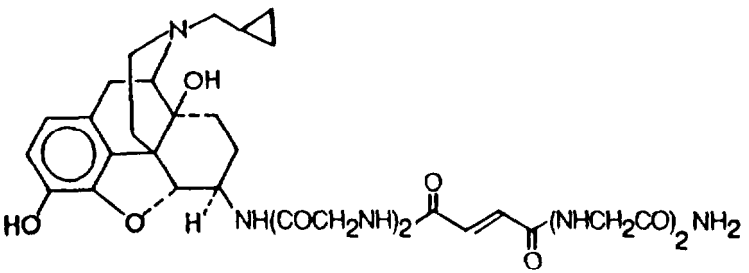

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Significantly, the SAR profile of the succinyl series 3 for antagonism of morphine was substantially different form that of ethylketazocine (figure 1, panel A); peak antagonism of morphine was observed at n=2, whereas maximum antagonism of ethylketazocine was seen with the bivalent ligand having the shortest spacer length (n=0). The fact that antagonism of the agonist effect of morphine and ethylketazocine was greatly enhanced at specific spacer lengths is consistent with bridging of either receptor subsites or vicinal opioid receptors. Moreover, the fact that the spacer lengths differed for maximum antagonism at μ and κ receptors suggested that these receptor types may have different recognition characteristics or may be organized differently from one another.

While such potency enhancements are in harmony with the bridging of vicinal opioid receptors (case a), it also is consistent with bridging between an opioid recognition site and a neighboring subsite that is part of the same receptor (case b). In an effort to distinguish between these two possibilities, a bivalent ligand containing a combination of (-) and (+)-enantiomeric pharmacophores has been synthesized (figure 2, meso isomer) (Portoghese et al. 1985). This ligand possesses the same spacer (n=2) that afforded peak antagonism at μ receptors [threo isomer, series *3* (n=2)]. If the

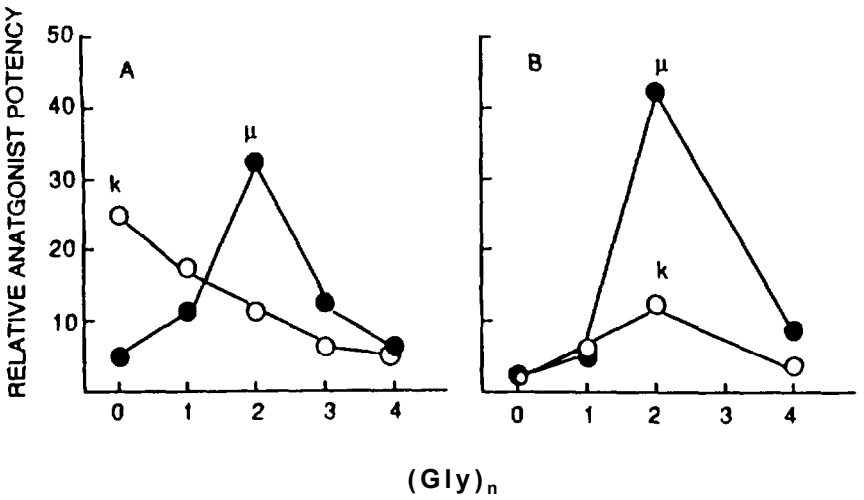


FIGURE 1. *Relationship between relative opioid antagonist potency and the number of glycol units, (Gly)n, in each half of the spacer*

NOTE: Panel A illustrates this for the succinyl series *3;* panel B is for the fumaryl series *4.* Note the different profiles for antagonism at kappa receptors in series *3* and *4.*

bivalent ligand containing two identical (-)-pharmacophores (threo isomer) bridges vicinal opioid receptors, then replacement of one of the pharmacophores with its inactive (+)-enantiomer (meso isomer) should afford a potency decrease. It was found that the meso isomer and the monovalent ligand *5* possessed nearly equal antagonist potencies, but had approximately one-thirtieth the threo isomer, thereby confirming that the neighboring site has an enantio-preference characteristic of an opioid receptor site.

The closely related series *4* in which the spacer contains a fumaryl moiety was synthesized and the μ and κ antagonist potencies were evaluated on the GPI preparation (figure 1, panel B). The fumaryl moiety was employed in order to determine the effect of conformational restriction of the spacer on opioid receptor selectivity. It can be seen that the fumaryl series *4* possesses an SAR profile at μ receptors that is very similar to that of the succinyl series *3.* However, the interaction of each of these two series at κ receptors differed substantially from the other. This difference was characterized by the significantly longer spacer requirement for peak κ-antagonist potency in the fumaryl series relative to the succinyl series. We have postulated (Portoghese et al. 1986b) that the conformational restriction imposed by the fumaryl group in a short spacer (n=0) prevents effective interaction of both pharmacophores with vicinal recognition sites of the

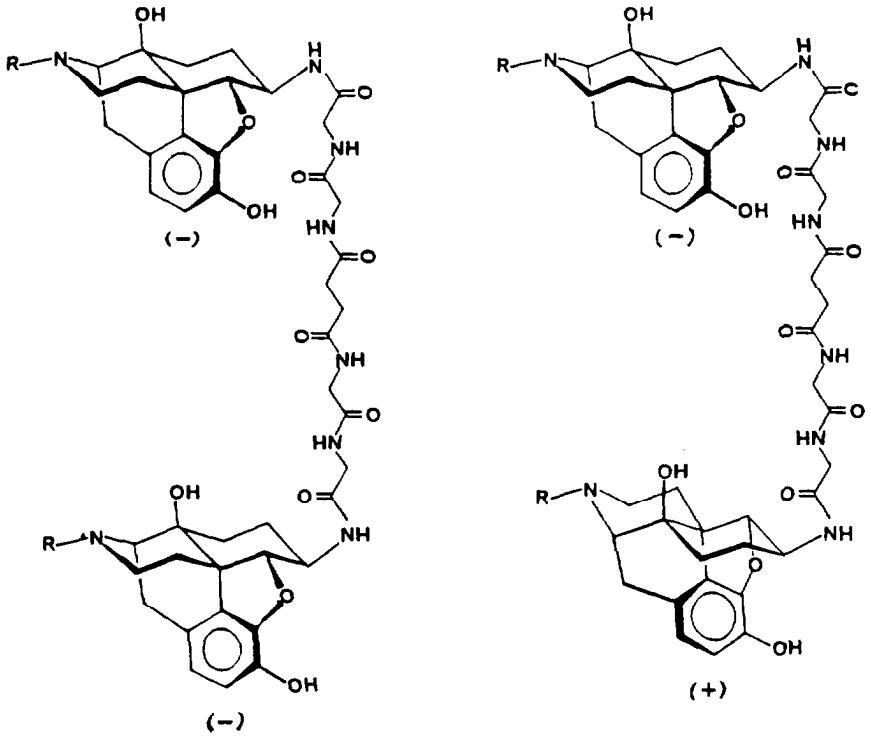


FIGURE 2. *Meso and threo [3, n=2, R=$CH_2CH(CH_2)2$] bivalent ligands with identical spacers*

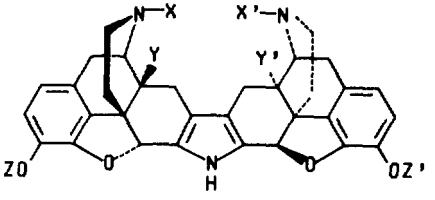
κ -receptor system. As the spacer is lengthened (n=2) and becomes more flexible, the simultaneous occupation of proximal sites can then occur with greater facility.

These results led to investigation of bivalent ligands whose pharmacophores are immobilized by a rigid spacer. This approach was based on the idea that immobilization of the antagonist pharmacophores in the proper orientation might facilitate simultaneous occupation of proximal recognition sites. Since their prior studies suggested that short spacers favored κ opioid receptor antagonist activity, we chose pyrrole as a spacer based upon its synthetic accessibility via the Piloty synthesis (Lipkowski et al. 1986a). This heterocyclic ring should restrict conformational mobility of both pharmacophores by virtue of fusion at positions C-6 and C-7 of the morphinan structure.

Some of the members of this series *7* arc listed in table 1 (Portoghese et al. 1987; Portoghese et al. 1988b). The parent member of the series,

nor-binaltorphimine *7a* (nor-BNI), possessed exceptionally high κ-opioid receptor antagonist potency and unprecedented K-antagonist selectivity. This high *in vitro* antagonist selectivity of nor-BNI is paralleled by its high binding selectivity for κ opioid receptors (K_i ratios: μ/κ =169, δ/κ =153). It is noteworthy that this selectivity arises from an increase in affinity at κ receptors and an affinity decrease at μ and δ receptors.

TABLE 1. *Opioid antagonist activities of series 7 in the GPI and MVD*



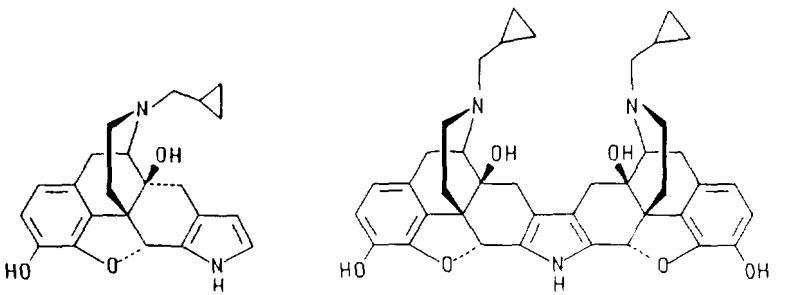
7

Compound No.	X	X'	Y	Y'	Z	Z'	Ke(nM)[a] κ[b]	μ[c]	δ[d]	Ke Ratio μ/κ	δ/κ
7a (norBMI)	$CH_2CH(CH_2)_2$	$CH_2CH(CH_2)_2$	OH	OH	H	H	0.41	13	20	32	49
7b	$CH_2CH(CH_2)_2$	$CH_2CH(CH_2)_2$	OAc	OAc	Ac	Ac	e	e	e	—	—
7c	$CH_2CH(CH_2)_2$	$CH_2CH(CH_2)_2$	OAc	OAc	H	H	0.38	42	12	111	32
7d	$CH_2CH(CH_2)_2$	$CH_2CH(CH_2)_2$	OH	OH	CH_3	CH_3	e	e	e	—	—
7e	$CH_2CH(CH_2)_2$	$CH_2CH(CH_2)_2$	OH	OH	H	CH_3	1.3	38	45	29	35
7f	CH_3	CH_3	OH	OH	H	H	e	e	e	—	—
7g	$CH_2CH(CH_2)_3$	CH_3	OH	H	H	H	1.9	21	41	11	22
8	Monovalent naltrexone-pyrrole derivative						2.1	0.65	3.2	0.3	1.5
	MR 2266						2.3	2.9	e	1.3	—
	Naltrexone						5.5	1.0	24.4	0.2	4.4

[a]Ke=(antagonist]/(IC_{50} ratio-1).
[b]Antagonism of ethylketazocine in the GPI.
[c]Antagonism of morphine in the GPI.
[d]Antagonism of [D-Ala^2,D-Leu^5]enkephalin in the MVD.
[e]Ke not calculated because IC_{50} ratio is not significantly greater than 1.

The minimum requirements for κ selectivity are (1) at least one free phenolic OH group and (2) at least one N-cyclopropylmethyl or N-allyl substituent. Several compounds (*7c, 7d*) possessed κ selectivity that was as good or better than nor-BNI *7a*. The fact that the ligand *8*, which contains only one morphinan with a fused pyrrole, is not κ selective, suggests that the pyrrole moiety indeed functions as a spacer. Moreover, the data (table 1) suggested that only one antagonist pharmacophore may be required for κ selectivity and that the other morphinan portion of the molecule confers selectivity by interacting with a unique proximal subsite on the same receptor.

This question was addressed further by the synthesis of the meso isomer *9* of nor-BNI (Portoghese et al. 1988c). The logic for the synthesis of *9* was based on the fact that it contains a combination of the antagonist pharmacophore derived from (-)-naltrexone and its inactive (+)-enantiomer. The perspective formulas (figure 2) corresponding to *7a* and *9* illustrate the different geometry of these molecules. Thus, if κ opioid antagonist selectivity is retained, it could not be ascribed to bridging two vicinal opioid receptors. In smooth muscle preparations, *9* was approximately five times more potent than nor-BNI (*7a*) but possessed reduced κ selectivity, presumably as a consequence of more effective interaction with μ and δ opioid receptors. This confirmed the idea that only one of the two antagonist pharmacophores of nor-BNI is required for κ opioid antagonist selectivity. Accordingly, one of the antagonist pharmacophores of nor-BNI was postulated to serve as the "message" and the second pharmacophore to function as the "address" at the same opioid receptor. It can be noted that although the portions of the molecules *7a* and *9* that contain the postulated address component are enantiomeric, the basic nitrogen function is in approximately the same location (figure 3). Is it possible that this nitrogen mimics the Arg^6 or Arg^7 residues in the address sequence of dynorphin?



8 and *9*

Nor-BNI has become widely used in opioid research. In addition to its use *in vitro,* it also has been effective *in vivo* (Takemori et al. 1988). The fact that the same ligand can be employed both *in vivo* and *in vitro* is a distinct advantage in its use as a research tool.

Message-Address Approach

The message-address concept was developed by Schwyzer (1977). In essence, this concept proposes that the message comprises those amino acid residues of a peptide hormone that are responsible for triggering a specific

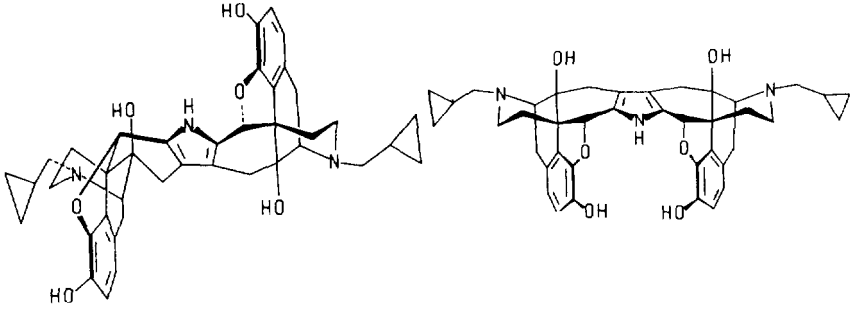


FIGURE 3. *Perspective formulas of nor-BNI (left) and its meso isomer (right)*

biological effect. The address component is composed of amino acid residues that contribute to the specific binding force between the ligand and receptor without being able to trigger the biological effect. In some cases, the message and address components appear to be confluent (e.g., insulin), while, in other cases, the message and address peptide sequences are clearly separated (e.g., ACTH). The latter case is schematically illustrated in figure 4.

The endogenous opioid peptides appear to conform to the latter case (figure 4) in that they contain an N-terminal tetrapeptide sequence Tyr-Gly-Gly-Phe that is an important requirement for opioid activity (Morley 1980). It has been proposed that this N-terminal tetrapcptide sequence carries the "message" responsible for mediating the opioid effect and that segments of these peptides that differ in amino acid sequence play an "address" role in conferring selectivity (Chavkin and Goldstein 1981). That is, its function is to bind to a unique recognition locus that is complementary to a specific receptor type.

Lipkowski and colleagues have reported that attachment of a peptide "address" segment to an opiate alkaloid pharmacophore (figure 5) changes selectivity by increasing the affinity for the target receptor type (Lipkowski et al. 1986b; Lipkowski et al. 1988). Thus, a C-terminal dynorphin-related peptide fragment attached to an opiate increased κ selectivity, while a C-terminal enkephalin dipeptide enhanced δ selectivity. The Phe^4 residue was considered to be part of the address sequence in that study because in opiate alkaloids, such as morphine. clearly only one aromatic ring is part of the message. The results of these studies suggested that the address subsite is proximal to the message-recognition locus and that it might be possible to modulate selectivity by attachment of a suitable molecular "appendage" other than a peptide to the opiate pharmacophorc.

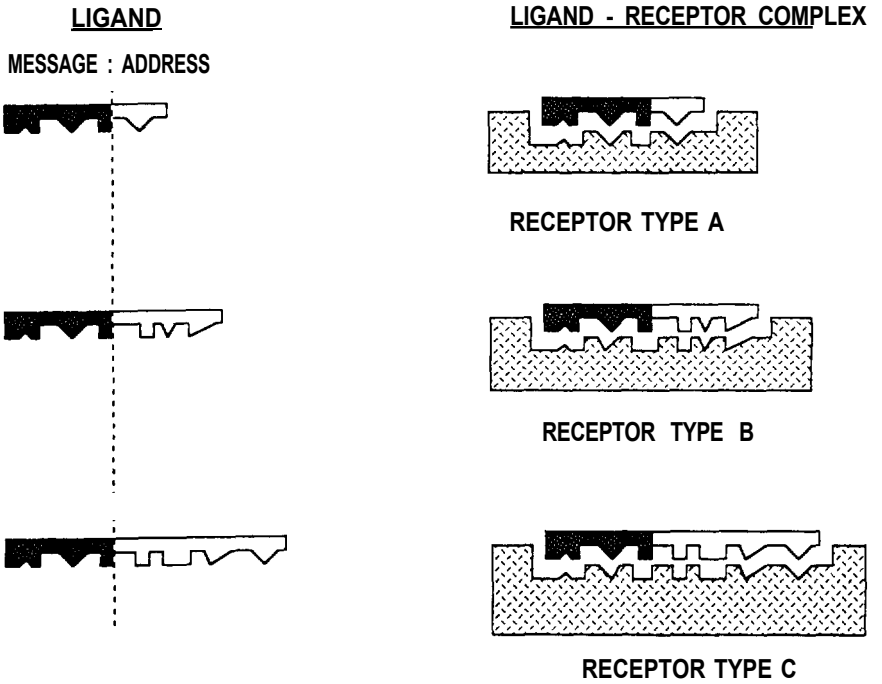


FIGURE 4. *A schematic illustration of the message-address concept*

NOTE: Note that the message is identical. while the address sequence differs. Selectivity at different receptor types is conferred by the address sequence.

Subsequent investigation of the nor-BNI series (table 1 and figure 2) discussed earlier in this chapter indicated that the κ selectivity of these ligands may involve a message-address mechanism. The possibility of designing nonpeptide δ opioid receptor antagonists was investigated using a naltrexone-derived pharmacophore joined to a compact "address" moiety. An important consideration in this design was the conformational restriction of this moiety. The desire to build conformationally restricted ligands, particularly with respect to the address component, was based upon the idea that a rigid address moiety might confer greater δ selectivity because this would preclude possible conformational adaptation in the binding to other opioid receptor types.

A logical choice of the address component in the target compounds was a flat aromatic system whose conformation is restricted by virtue of fusion at positions 6 and 7 of the morphinan structure. This aromatic ring was

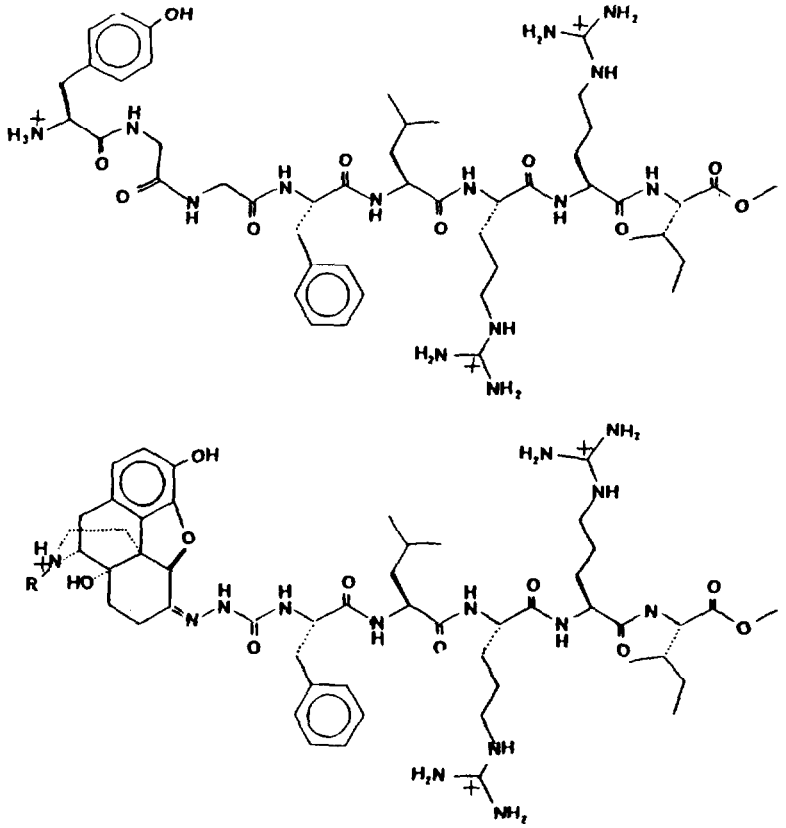


FIGURE 5. *Structural comparison of dynorphin(1-8)OMe (top) and alkaloid-dynorphin address hybrid (bottom)*

envisaged to mimic the phenyl group of the Phe[4] residue of enkephalin (figure 6). Several aromatic heterocyclic systems were employed for this purpose and were synthesized from naltrexone 2. These compounds include the indole, benzofuran, quinoline, and quinoxaline rings (Portoghese et al. 1988a). In this series, the pyrrole or other hetero-substituted monocyclic components in these bicyclic aromatic systems represents the rigid spacer that connects the benzene ring (the address) to the morphinan nucleus (the message).

Early on, the indoles *10* (table 2) were considered reasonable candidates because they fulfilled the criteria discussed above and were readily accessible from naltrexone 2 via the Fischer indole synthesis (Portoghese et al.

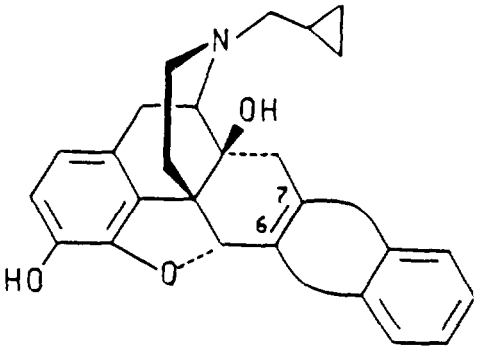
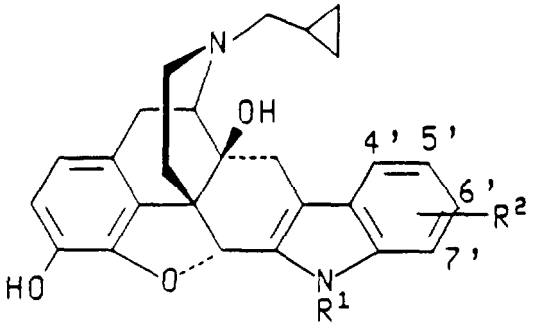


FIGURE 6. *Naltrexone-derived pharmacophore fused through a spacer to a benzene ring δ-address component*

1988d; Portoghese et al. 1988e). Testing of the indoles on the electrically stimulated GPI longitudinal muscle and mouse vas deferens (MVD) preparations revealed that they were devoid of opioid agonist activity and possessed a unique opioid antagonist profile. All of the compounds were very poor antagonists of morphine and ethylketazocine (EK), which act selectively in the GPI at μ and κ opioid receptors, respectively. Two members in the series potently antagonized the effect of the δ agonist [D-Ala^2,D-Leu^5]enkephalin (DADLE) in the MVD. These were naltrindole *10a* (NTI) and the 7'-fluoro derivative *10d,* with the κ_e values in the 0.2 to 0.4 nM range. The receptor binding of NTI was consistent with these results. NTI *10a* was greater than 100 times more effective in reversing the agonist effect of DADLE relative to morphine or EK. Substitution of the indole nucleus in the 5'-position with electron-donated or electron-withdrawing substituents (*10c, e, g, h*) reduced potency and selectivity at δ receptors. In this regard, the data suggest that size is the most important factor that contributes to this reduction of antagonist potency. Substitution at the 4'-position (*10b*) similarly reduced antagonist activity at δ receptors. The 7'-position appears to be the least affected by substitution in view of the higher potency of the fluoro and methyl congeners (*10d* and *10f*) relative to the other positional isomers. Methylation of the indole nitrogen (*10i*) afforded a reduction of δ--antagonist potency (K_e=1 nM) and reduced selectivity ratios (δ/μ ~20, δ/κ ~35) relative to NTI *10a.* Thus it appears that aromatic substitution may sterically interfere with the interaction of the address component with a unique subsite that is proximal to the opiate recognition locus at the δ opioid receptor.

The question of the necessity of the indole nucleus for δ antagonist selectivity was addressed through the preparation of *11,* since the benzofuran ring system is isostcric with that of indole. The benzofuran *11 is* a

TABLE 2. *Opioid antagonist activities of naltrindole 10a and its analogs*



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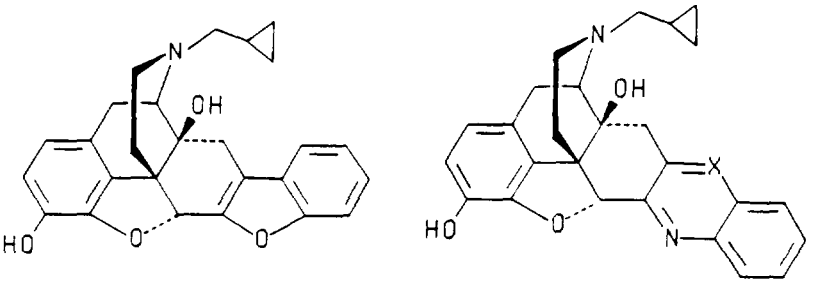
Compound	R^1	R^2	Ke(nM[a]) DADLE	Morphine	EK
10a (NTI)	H	H	0.21	32	58
10b	H	4'-F	4.2	125	160
10c	H	5'-F	2.0	61	46
10d	H	7'-F	0.35	23	>300
10e	H	5'-Me	4.2	160	250
10f	H	7'-Me	1.5	19	>300
10g	H	5'-OMe	5.7	63	13
10h	H	5'-NO_2	168	>300	>300
10i	Me	H	1.5	35	22

NOTE: κ_e =[antagonist]/(DR-1) where DR=the agonist IC_{50} in presence of antagonist divided by control agonist IC_{50}.

δ-selective antagonist but with reduced potency (K_e=1.5 nM) and selectivity (δ/μ=21, δ/κ=35) relative to NTI. It is noteworthy that this decreased antagonist selectivity is a consequence of the decreased efficiency with which *11* blocks the effect of DADLE. These results suggested that the isosteric nature of the indole and benzofuran ring systems confers δ selectivity.

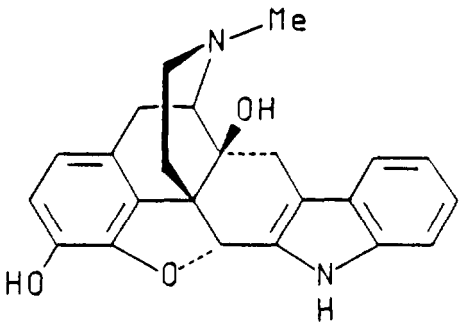
The data support the possibility that pyrrole or furan ring in *10* or *11* functions simply as a spacer in holding the benzene moiety in a specific conformation relative to the naltrexone-derived pharmacophore. This is consistent with the observation that the naltrexone-pyrrole derivative *8* is not selective. Presumably, this rigid spacer permits the aromatic ring to associate with a subsite on the "address" recognition locus of the δ receptor while reducing the affinity for μ and κ receptors. Further evidence for this was obtained with the quinoline *12* and quinoxaline *13* analogs, as these



11; 12, X=CH; *13, X=N*

ligands are not isosteric with *10* and *11* but yet have the benzene moiety fixed in a similar coplanar orientation with respect to the C-ring. Both *12* and *13* are δ antagonists but with potencies and selectivities lower than that of benzofuran *11,* a finding that is in harmony with the aforementioned model. Also, the fact that *12* and *13* were not as potent as NTI or the benzofuran *11* is consistent with the fact that the benzene ring is oriented somewhat differently because the spacer is a six-membered ring.

Interestingly, the NTI analog *14,* derived from the μ agonist oxymorphone, possessed no opioid agonist or antagonist activity in the GPI and was a partial agonist (70 percent maximum) in the MVD preparation. This suggested that its agonist activity may be mediated primarily through δ receptors. The apparent qualitatively similar recognition characteristics for NTI and *14* may mean tha δ opioid receptor antagonism and agonism are mediated through identical or similar recognition sites. Thus, it is conceivable the Try' and Phe[4] aromatic rings of enkephalin bound to the δ receptor may approximate the orientation of those in NTI or *14* (figure 7).



The most potent δ antagonist (NTI) in the series was evaluated in mice (Portoghese et al. 1988e) for its effectiveness in antagonizing the antinociceptive effect of δ-, μ-, and κ- -selective agonists using the writhing assay. When administered SC, it effectively blocked the δ- -selective agonist Tyr-D-Ser-Gly-Phe-Leu-Thr (Gacel et al. 1981) without blocking the effects of μ- or δ--selective agonists. Thus, NTI exhibited an *in vivo* pharmacologic profile that was qualitatively similar to that found in smooth muscle and receptor-binding studies.

NTI is the first example of a highly selective and potent δ opioid antagonist that is not a peptide. Pentapeptides related to the enkephalins (e.g., ICI 174864) have been reported (Cotton et al. 1984). However, the affinity and potency of ICI 174864 are approximately two orders of magnitude lower than NTI, and it has the disadvantage of low *in vivo* potency when administered peripherally due to poor penetration into the CNS.

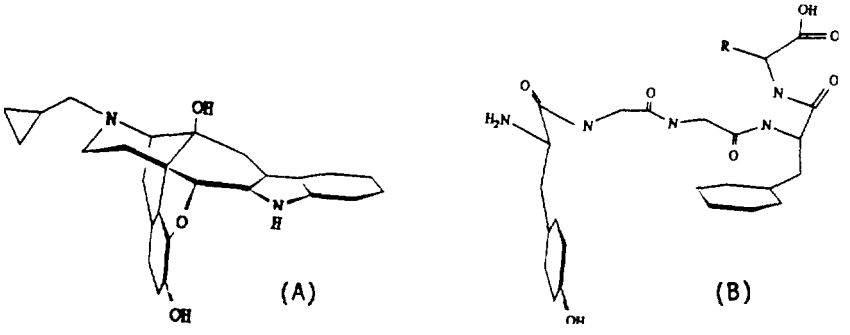


FIGURE 7. *Possible similarity between NTI (A) and the δ opioid receptor-bound conformation of enkephalin (B)*

CONCLUSION

The bivalent ligand approach has led to the design and synthesis of highly selective opioid receptor antagonists. One version of this approach, which involved the synthesis of ligands that contained two pharmacophores connected by a rigid spacer, resulted in the κ-selective antagonist nor-BNI. Further studies revealed that only one pharmacophore is required for κ selectivity. This suggested that part of the second pharmacophore confers selectivity by simulating a portion of the "address" sequence in dynorphin, a κ- -selective opioid peptide. In this context, peptides that conform to the message-address concept can be viewed as bivalent ligands because they contain two recognition units, only one of which functions as a pharmacophore; the second recognition unit, the "address," provides selectivity through interaction with an address subsite on the receptor. This principle

was employed to design the δ-selective antagonist NTI and related ligands. In this case, the δ address is a benzene ring attached to the pharmacophore (the message) through a rigid spacer. As a consequence of its conformational rigidity, this address component has been postulated to bind to the δ-address subsite and is incapable of conformationally adapting to address subsites associated with other opioid receptor types. The success of these studies suggests that it may be possible to alter antagonist selectivity in a predictable way by simulating a portion of the address peptide component with a rigid nonpeptide moiety.

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Opium Alkaloids and Affinity Labels

Sydney Archer, Jean Bidlack, and G. Keith Mulhollund

INTRODUCTION

The first direct evidence that heroin addiction may influence the immune system came from the observation that lymphocyte mitogenesis was depressed in heroin addicts (Brown et al. 1974). Street opiate addiction produced a significant depression in the absolute number of total T lymphocytes in peripheral blood (McDonough et al. 1980). Binding sites for both classical opioids, i.e., naloxone-reversible agonist binding agents, and nonclassical opioids insensitive to naloxone reversibility have been detected on lymphocytes (Lopker et al. 1980; Hazum et al. 1979; Schweigerer et al. 1985; Mehrishi and Mills 1983; Dave et al. 1985). However, the functions of these multiple receptors are unknown. To the best of the authors' knowledge, affinity labels derived from the opium alkaloids have not been applied to such studies.

METHODS

Affinity labels are generally derived from reversibly binding ligands that are so modified that they can be made to bind covalently to the receptor itself or to an insoluble matrix such as omega-aminohexyl Sepharose to form a material that can be used to purify a receptor by means of affinity chromatography. There are affinity ligands that can perform one of these functions well but can be unsuitable for the other. For example, the affinity ligand FIT was prepared from a μ ligand, yet it selectively acylated δ receptors prepared from NG 108-15 membranes (Klee et al. 1982). Yet, under reversible conditions FIT was a nonselective μ and δ agonist and for this reason was not suitable for use in affinity chromatography. On the other hand, 14β-bromoacetamidomorphine (BAM I), served well as a ligand for affinity chromatography but did not bind irreversibly to the μ receptor at 25 °C unless dithiothreotol (DTT) was present.

BAM *1* and its companion 14 β -bromoacetamidomorphine (BAMO) 2 were prepared as shown in figure 1 (Archer et al. 1983). Thebaine 3 was treated with chloronitrosocyclohexane to give an adduct that, after reduction with

zinc dust in the presence of methanol, gave a mixture of the 14β-aminocodeinone *4* and the corresponding ketal *5*. The latter was treated with bromoacetyl bromide to furnish the ketal *6,* which upon treatment with boron bromide followed by mild acid hydrolysis gave BAMO *2*. The codeinone *4* gave dihydrocodeinone *9* by catalytic hydrogenation. In order to reduce the ketone to 14β-aminocodeine *7,* sodium borohydride was used as the reducing agent. Treatment with boron bromide gave 14β-aminomorphine *8,* which, when allowed to react with bromoacetyl bromide, gave a mixture of amide esters, which on mild acid hydrolysis gave the desired BAM *1* (figure 1).

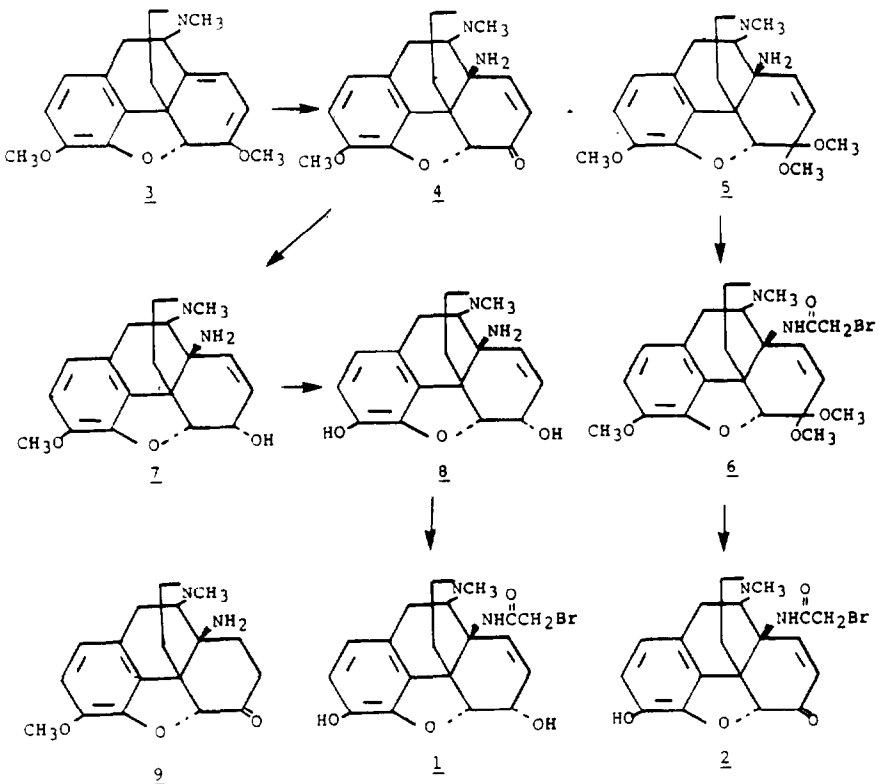


FIGURE 1. *Preparation of BAM and BAMO*

The ability of BAM and BAMO to inhibit the binding of [^{3}H]-dihydromorphine in rat neural membranes was determined using seven different concentrations of the ligands in three different experiments. The rat neural

membranes were prepared as described by Bidlack and Abood (1980), and the receptor binding assays were carried out according to the procedure described by Pert and Snyder (1973). Under these conditions, the IC_{50} value of morphine was 4 nM, whereas that for BAM and BAMO were 15 nM and 10 nM, respectively.

The ability of the two ligands to bind covalently to the opioid receptor was carried out by first incubating rat neural membranes with 20 nM concentrations of *1* and *2* at 37 °C for 30 minutes. An equal concentration of morphine served as a control. The membranes were centrifuged at 10^5Xg for 15 minutes. The pellet was resuspended in 50-mM Tris buffer, pH 7.5, and the washing procedure was repeated four times to remove any unbound ligands. [^{3}H]-Dihydromorphine and [^{3}H]-naloxone were used at a final concentration of 4 nM to determine the inhibition of binding. The results are summarized in table 1.

TABLE 1. *Inhibition of binding by BAM and BAMO*

Compound	Percent Inhibition[a] [^{3}H] Dihydromorphine	[^{3}H] Naloxone
BAM	19±4	37±3
BAMO	35±3	36±2
Morphine	1±3	2±2

[a]The ability of the neural membranes to bind 4 nM [^{3}H]-dihydromorphine and [^{3}H]-naloxone was measured by the glass-fiber filter technique in the presence of either 10^{-6} M dextrophan or 10^{-6} M levorphanol. The data are reported as the mean percent inhibition plus or minus standard error for five experiments.

Both BAM and BAMO partially inhibited the binding of the two radiolabeled ligands, with BAMO being slightly more effective against the agonist than BAM. Morphine had no inhibitory effect. It is noteworthy that even to achieve this relatively modest inhibition of ligand binding, the neural membranes had to be incubated with BAM and BAMO at 37 °C for 30 minutes.

To demonstrate that BAM was a selective reversible blocker of the μ receptor, a series of experiments on the inhibition of opioid binding were performed. DAGO was used as the highly selective μ agonist; DDDPE was used as the specific δ ;agonist; and bremazocine, in the presence of DAGO and DADLE, μ and δ blockers, respectively, was used as the κ agonist. The procedures used were those reported earlier (Bidlack and Abood 1980; Pert and Snyder 1973). The results are summarized in table 2. The selectivity of BAM for the μ site was at least seventyfold greater than for the δ site and almost two hundredfold greater than for the κ site. The

insertion of a bromoacetamido group at C-14 of morphine did not have an effect on receptor selectivity and only decreased slightly the affinity of this ligand for the opioid receptors.

TABLE 2. *Comparison of the inhibition of opioid binding to rat brain membranes by morphine and BAM*[a]

Radiolabeled Ligand	IC_{50} (nM) Morphine	BAM
0.25 nM [^{3}H]-DAGO	1.1±0.2	5.0±0.7
3.0 nM [^{3}H]-DDDPE	137±7	345±33
0.2 nM [^{3}H-Bremazocine] plus μ and δ blockers	324±33	950±116
2 nM [^{3}H]-naloxone	11±2	37±10
1 nM [^{125}I]-β-endorphin	6±4	46±5

[a]Rat membranes were incubated with 12 different concentrations of morphine or BAM in the presence of the radiolabeled opioid in 50 mM Tris HCI. pH 7.5 at 2.5 °C. IC_{50} values were calculated by least squares fit to a log-probit analysis. The results are expressed as the mean IC_{50} value ± SEM from three experiments performed in triplicate.

Under the above conditions, BAM behaved as a selective reversible μ ligand. However, when similar experiments were carried out in the presence of dithiothreitol (DTT), a different picture emerged. Rat membranes were incubated with either 20 nM of DTT or Tris HCI buffer (pH 7.5) for 20 minutes at 25 °C. After this preincubation period, 10 μM of BAM or a buffer (to act as a control) was added, and incubation at 25 °C was continued for an additional 30 minutes. The membranes were diluted to 40 ml with Tris buffer and washed six times by centrifugation at 48,000Xg for 15 minutes to remove all noncovalently bound BAM. The specific binding of 0.25 nM of [^{3}H] DAGO was measured. The results are summarized in table 3.

In contrast to the results reported in table 1, in which only modest irreversible inhibition of binding by BAM was achieved by incubation at 37 °C in the absence of DTT, in the presence of the latter almost complete irrevetsible binding was observed at 25 °C. Apparently there is a disulfide bond present near the active site of the μ receptor that must be reduced to sulfhydryl groups, which are then alkylated easily by BAM. Even the presence of a large excess of DTT, which itself may be susceptible to alkylation, does not interfere with the alkylation of the receptor by RAM. Apparently the ligand binds too rapidly to the active site, so that alkylation of DTT does not occur.

TABLE 3. *The effect of BAM and DTT on [^{3}H] DAGO binding to rat neural membranes*

Conditions	Percent Control Binding
10 μM BAM	93±6
20 mm DTT	106±4
20 mm DTT + 10 μM BAM	10±3

Very Little alkylation of δ and κ receptors occurs under conditions wherein the binding of [^{3}H] DAGO is prevented by BAM as shown in figure 2. N-Ethyl maleimide (NEM), a reagent that binds avidly to sulfhydryl groups, has been shown to decrease [^{3}H]-dihydromorphine binding to rat neural membranes and to suppress the effect of GTP on opioid binding (Childers 1984). Studies such as these suggest that there are free sulfhydryl groups in the vicinity of opioid binding sites in neural membranes of the rat brain. However, at 25 °C, BAM does not appear to alkylate these group, since in the absence of DTT no irreversible inhibition of opioid binding occurs.

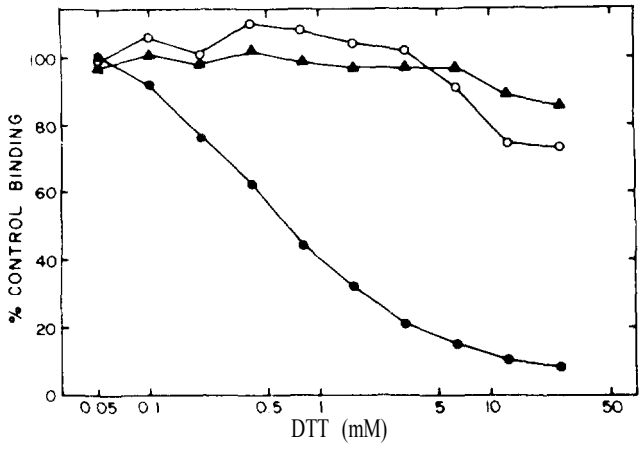


FIGURE 2. *Comparison of the inhibition of μ, δ,, and κ binding to rat brain membranes as a result of incubating membranes with DTT and BAM*

Source: Ridlack et at. 1989. coyright 1989, American Chemical Society.

BAM was also used as a ligand for affinity chromatography, and the affinity column prepared by coupling BAM with omega aminohexyl Sepharose 4B was used to partially purify the μ receptor (Bidlack et al. 1981). Rat brains minus the cerebellum were homogenized, and the opioid receptors present were solubilized with the aid of the detergent Triton X-100. After centrifugation the supematant was treated with Biobeads SM-2. The supernatant was removed, concentrated until the protein concentration was 30 to 50 mg/ml, and then was applied to the affinity column. The opiate receptor was present in the fractions eluted with either 1 μM of levorphanol or 1 μM of etorphine. These fractions were combined and dialyzed against Tris-HC1 buffer to remove the opiate ligands (i.e., levorphanol or etorphine), and the viability of the receptor fractions was tested by equilibrium dialysis using [^{3}H]-dihydromorphine as the opiate ligand. $NaDodSO_4$/polyacrylamide gels prepared from the active fractions showed the presence of three bands of apparent molecular weights 43,000, 35,000, and 23,000 daltons.

This procedure was repeated in the presence of protease inhibitors, because it was suspected that material of higher molecular weight may have escaped detection owing to degradation by proteases in the initial crude isolates. Again, fractions were obtained that bound [^{3}H]-dihydromorphine, but $NaDodSO_4$/polyacrylamide gels revealed the presence of a new band with an apparent molecular weight of 58,000 daltons.

Another thebaine-derived opiate ligand Hybromet, *13,* was used by Maneckjee et al. (1985) to isolate and purify an opiate receptor. The synthesis of this ligand is shown in figure 3.

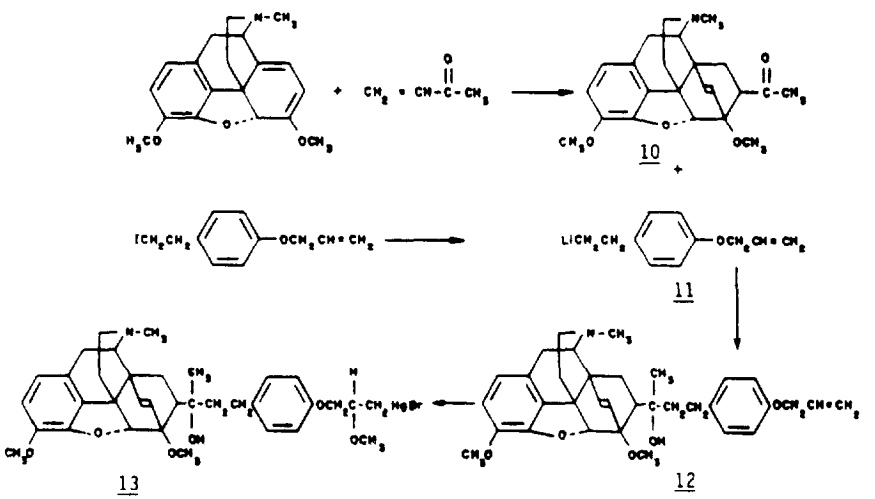


FIGURE 3. *Synthesis of hybromet*

The ketone *10,* a Diels-Alder condensation product of the reaction of thebaine and methyl vinyl ketone, was allowed to react with the lithium reagent *11* to give the tertiary carbinol *12,* which on methoxymercuration gave Hybromet *13.* An affinity column was prepared from *13* and Affigel 401, a sulfhydryl-containing matrix.

The homogenate derived from Sprague-Dawley rat brains minus the cerebellum was solubilized with the detergent CHAPS. This suspension was centrifuged at 105,000Xg for 60 minutes at 4 °C, and the supematant was applied to the affinity column. After elution first with Tris-HCI buffer containing CHAPS, the opiate receptor was eluted with 1 μM normorphine in 10-mM Tris-HCl buffer. Fractions exhibiting [^{3}H]-labeled opiate-binding activity were pooled and dialyzed against 10-mM Tris-HCI buffer.

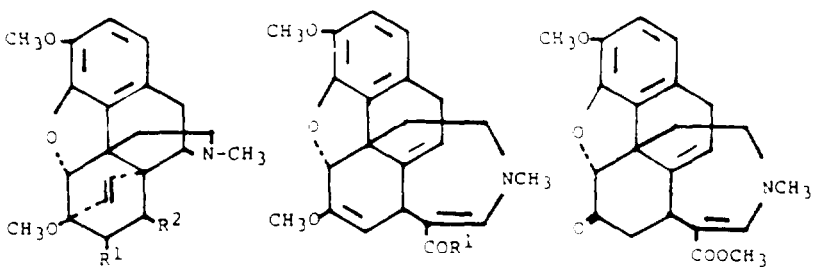
[^{3}H]-etorphine binding to the partially purified receptor was studied as a function of radioligand concentration. Half-maximal binding was observed at about 1 nM radioligand concentration. Scatchard analysis revealed a class of high-affinity sites with an apparent Kd=1.3 nM and β_{max} =2800 fmol/mg protein. Studies using [^{3}H]-ethylketocyclazocine as a selective κ agonist and DADLE, a δ agonist, showed that the partially purified receptor was primarily of the μ type. Electrophoresis under denaturation conditions revealed the presence of three bands of apparent molecular weights 94,000, 49,000, and 35,000 daltons. Two of these bands corresponded in approximate molecular weight to those reported by Bidlack et al. (1981).

While Hybromet appears to be a useful ligand for affinity chromatography, care must be exercised when attempting to use the ligand to bind to crude preparations of the opiate receptor because of the avidity of this ligand for sulfhydryl groups. For example, the presence of DTT or other exogenous sulfhydryl-containing compounds must be avoided because Hybromet, in contrast to BAM, will react with them instantly before binding to the receptor.

Rapoport and Sheldrick (1963) reported that when thebaine and dimethylacetylene dicarboxylate (DMAD) were heated in benzene for 1 hour at 50 °C, the normal Diels-Alder adduct *14* was obtained in 90 percent yield, but under comparable conditions, ethyl propiolate (EP) furnished the adduct *15* in only 6 percent yield. Despite the low yield, *15* appeared to be an attractive candidate for conversion to an affinity ligand because (1) the carboethoxy group could easily be transformed to a functionality suitable for use in irreversible binding and (2) the newly generated double bond could be reduced with tritium to yield a radioligand with high specific activity. With these considerations in mind, the authors decided to reinvestigate this reaction. Surprisingly, the interaction of thebaine with acetylenic dienophiles turned out to be far more complex than originally reported (Singh et al. 1986).

When thebaine was allowed to react with methyl propiolate (MP) in tetrahydrofuran for 24 hours at 35 °C, none of the expected Diels-Alder product *16* was obtained. Instead the enol ether *17* was obtained in 30 percent yield accompanied by some unreacted thebaine. The infrared (IR) and nuclear magnetic resonance (NMR) spectra are in agreement with the assigned structure. The outstanding features of the NMR spectrum of *17* were the signals for the H-9 proton, which appeared as a doublet of doublets at δ 5.92, and for the H-18 proton, which was a singlet at δ 7.35, which is characteristic of the β proton in β-aminoacrylic esters. When the acetylenic dienophile 3-butyne-2-one was used instead of MP, the analogous compound *18* was obtained. Hydrolysis of *17* using 6N HCI resulted in the formation of the ketone *19.* In the NMR spectrum of *19* the vinyl signal for the H-7 proton disappeared, but the δ 7.35 signal was still present. The structure of 19 was secured by means of a single crystal x-ray determination.

When the same reaction was carried out in methanol at room temperature, the adduct *17* was obtained in 32 percent yield accompanied by an ether-insoluble ketal 20 isolated in 62 percent yield. The NMR spectrum showed signals for four methoxy groups: the coupled H-7 and H-8 vinyl protons (J=10Hz), the H-9 proton at 8 5.94, and the H-18 proton at δ 7.35 coupled to the H-19 proton at δ 4.40 (J=13 Hz). This coupling constant suggested that the H-18 and H-19 protons were trans to each other. When DMAD and thebaine were allowed to react in tetrahydrofuran, the normal Diels-Alder adduct *14* was obtained as previously reported (Rapoport and Sheldrick 1963). When the reaction was carried out in methanol, the ketal 21 was obtained in 35 percent yield. Whether the two carbomethoxy groups are trans to each other as shown is not certain. When 3-butyne-2-one reacted with thebaine in methanol, the open-chain ketone *22* was obtained along with the enol ether *18,* which was isolated as an oil (figure 4).

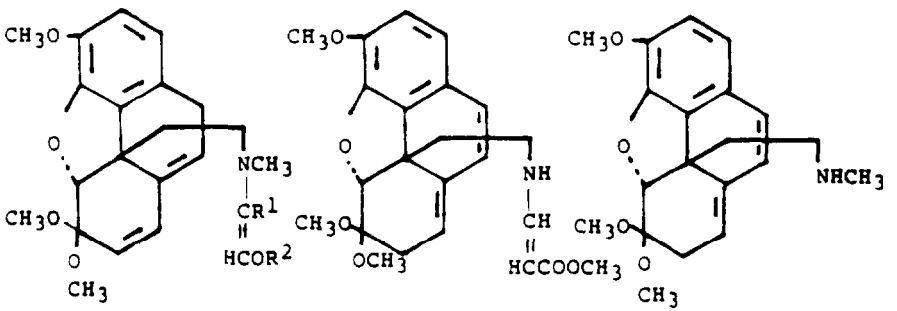


14, $R^1 = R^1 = COOCH_3$
15, $R^1 = COOC_2H_5$, $R^2 = H$
16, $R^1 = COOCH_3$, $R^2 = H$
17, $R^1 = OCH_3$
18, $R^1 = CH_3$
19

FIGURE 4. *Reaction of thebaine with acetylenic dienophiles*

When the ketal 20 was treated briefly with a slight excess of a strong acid, such as hydrochloric or p-toluenesulfonic, it cyclized almost immediately to give the enol ether *17*. Longer exposure to strong acid furnished the ketone 19. Heating the ketal under reflux with a dilute solution of aqueous methanolic sodium hydroxide resulted in the formation of a yellow isomeric ketal whose ultraviolet (UV) and NMR spectra were in agreement with the assigned structure 23. The UV spectrum of *17* showed one maximum at λ 280 nm and a shoulder at 225 nm, whereas that of 23 showed two maxima at 282 nm and 326 nm. The H-10 proton in the spectrum of 23 appeared as a doublet at δ 6.30 coupled to the H-9 proton.

Prolonged basic hydrolysis of 23 gave the amine 24 as an oil that furnished a crystalline fumarate. Treatment of *24* with MP converted it back to 23 (figure 5).



23 24

20, R^1 = H, R^2 = OCH_3
21, R^1 = $COOCH_3$, R^2 = OCH_3
22, R^1 = H, R^2 = CH_3

FIGURE 5. *Ketals derived from thebaine and acetylenic dienophiles*

Sodium borohydride reduction of the ketone *19* gave the alcohol 25 characterized as the acetate 26. Catalytic reduction of 26 resulted in the reduction of the double bond to give the dihydro ester 27.

If the catalytic reduction of *19* is carried out in acetic acid over a period of 25 hours, followed by treatment with acetic anhydride in pyridine, the ester 27 is obtained in modest yield. However, if the reduction is carried out for only 10 minutes and the base isolated, then dissolved in methanol, and the solution allowed to stand exposed to air for a period of about 2 days, a new substance separates from solution in 40 percent yield. The NMR spectrum showed a signal at δ 6.06, which appeared as a doublet and was assigned to H-9 as well as two other singlets at δ 5.82 and δ 6.38, which were assigned to the C-18 protons. The UV spectrum showed a maximum

at 284 nm, and the mass spectrum indicated that the molecular weight was 381. Structure *31* was assigned to this unusual product by means of single-crystal x-ray analysis (Singh et al. 1986). A possible mechanism to account for the formation of *31* is shown in figure 6.

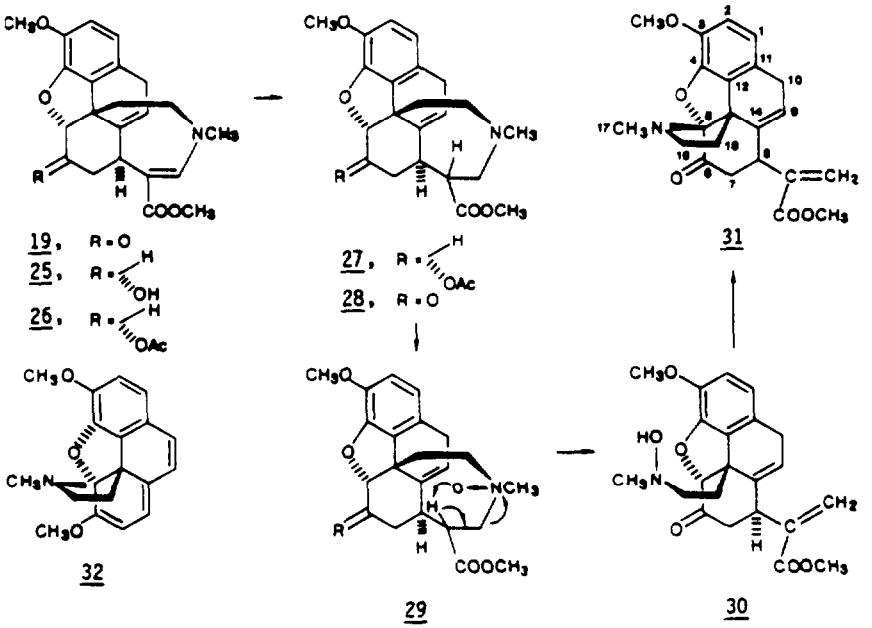


FIGURE 6. *A possible mechanism for the conversion of 19 to 31*

Brief hydrogenation of 19 gives the dihydroketo ester 28. When this ester is allowed to stand in air for 2 days, the N-oxide 29 is formed, which then undergoes a ring opening, as shown in 29, to give the hydroxylamine 30, which undergoes a dehydrative cyclization to afford the observed product *31*. Theuns et al. (1984) isolated the base 32 from *Papaver bracteatum* and commented that it may be an artifact. They postulate that it resulted from rearrangement of one of the thebaine N-oxides present in the plant extracts by a process analogous to that shown in figure 6.

Over the past several years, the authors' group has shown how thebaine, a companion of morphine and codeine in *Papaver somniferum,* can be converted to affinity chromatography and also as irreversibly binding ligands for the μ receptor. An unusual feature of BAM is that it binds irreversibly to the μ receptor under mild conditions only under reducing conditions (i.e., in the presence of dithiothreitol). We have succeeded in preparing a radiolabeled analog of BAM with high specific activity, in order to couple such

a ligand with the undenatured, partially purified μ receptor, to facilitate isolation of the pure receptor.

Lopker et al. (1980) have shown that human granulocytes and monocytes show stereospecific high-affinity binding for dihydromorphine, a highly selective μ agonist, and also for 3-quinuclidinyl benzilate (QNB) (figure 7) a specific ligand for muscarinic cholinergic receptors. Muscarinic binding occurred in viable cells as welt as in cell fragments.

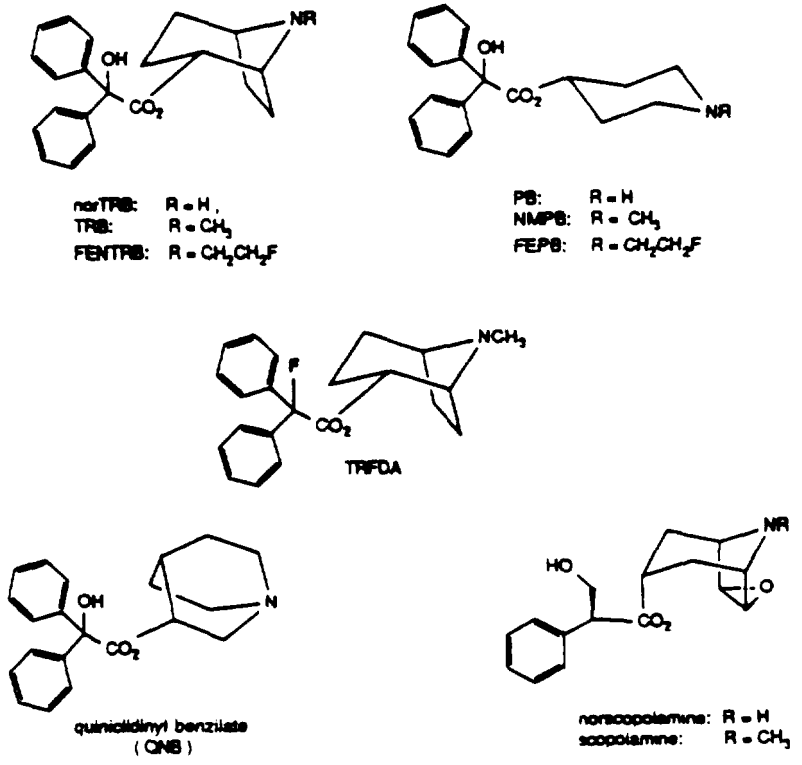


FIGURE 7. *Muscarinic agents*

Several years ago, the authors prepared 2-tropanyl benzilate (TRB) (Atkinson et al. 1977) and compared its central and peripheral antimuscarinic activity with QNB. These ligands were equipotent on peripheral muscarinic receptors, but TRB was about five times more potent than QNB on central muscarinic receptors (antitremorine assay).

More recently they have had occasion to compare TRB with QNB and a variety of other muscarinic acetylcholine receptor ligands (m AChR), using [^{3}H]-QNB in competitive binding assays in mouse-brain homogenates (table 4).

TABLE 4. *IC_{50} values for various m-AChR ligands obtained from competitive binding assays using [^{3}H] QNB in mouse-brain homogenates*

Drug	IC_{50} (nM)
QNB	0.8±0.2
Scopolamine	1.3±0.3
nor-Scopolamine	6.9±0.9
PB	17.3±2.7
NMPB	1.8±2.7
FEPB	6.4±0.4
TRB	0.7±0.3
nor-TRB	0.7±0.3
TRFDA	8.4±0.1
FENTRB	2.5±0.1

It is interesting to note that nor-scopolamine and PB are significantly less potent than their N-methylated counterparts, whereas TRB and nor-TRB are equipotent, and both have the same potency as QNB in this assay. The major advantage of TRB over QNB is that the former can easily be labeled with a positron-emitting isotope. [^{11}C]-TRB was easily prepared from nor-TRB. Excellent visualization of muscarinic receptors in the cerebellum and striatum of monkeys was achieved. [^{11}C]-TRB is an excellent candidate for studying muscarinic receptors in man, including heroin addicts using noninvasive techniques. The short half-life of the ^{11}C isotope makes the chemical synthetic procedure more difficult, but the advantage of ^{11}C- labeled drugs is that the very short half-life of this carbon isotope makes repeated administrations possible.

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Novel Thebainelike Morphinandienes and Their Diels-Alder Adducts

Leendert Maat

INTRODUCTION

Since the first isolation of morphine from opium, much chemistry has been done, and numerous derivatives and analogs have been prepared, primarily to obtain more specifically active central nervous system (CNS) analgesics. Nevertheless, new compounds with the morphinan skeleton can still be discovered. Their chemistry is interesting, and they may contribute to the knowledge of structure-activity relationships. Several of the new compounds have been developed on the basis of a Diels-Alder reaction with the opium alkaloid thebaine. Fifty years ago the first publications on this subject appeared (Schöpf et al. 1938), predicting the pharmacological importance of these cycloaddition compounds (Sandermann 1938). Some classes of new Diels-Alder adducts have been studied, and developments regarding them are reviewed in this chapter.

PEPTIDE-MORPHINANS BASED ON DIELS-ALDER ADDUCTS WITH ENKEPHALIN MOIETIES

Since the identification of the enkephalins, a new lead for molecules with opiate activity has been explored. The enkephalins should be able to assume conformations in aqueous solution that, at least partly, resemble morphine, the "classical" CNS analgesic (Aubry et al. 1988). Furthermore, there is some similarity between the C-terminal tail of the enkephalins and the lipophilic part of the highly potent Diels-Alder modified morphinans, such as etorphine, studied by Bentley (1971). Compounds have been synthesized that combine such a morphinan structure with a C-terminal residue of leucine-enkephalin (figure 1) (Beyennan et al. 1982). Work began with the cycloadduct of thebaine with ethyl actylate. Coupling of the acid group, obtained by hydrolysis of the ester substituent at position 7α, with a residue of leucine-enkephalin, resulted in a molecule with the peptide part attached

to the rigid C-ring system. The couplings were carried out by reaction of the freshly prepared acid chloride with, respectively, the ethyl ester of L-leucine, L-phenylalanyl-L-leucine, and glycyl-L-phenylalanyl-Lleucine. *O*-Demethylation at position 3 yielded the compounds that resemble the tyrosine moiety of both enkephalin and the A-ring of morphine.

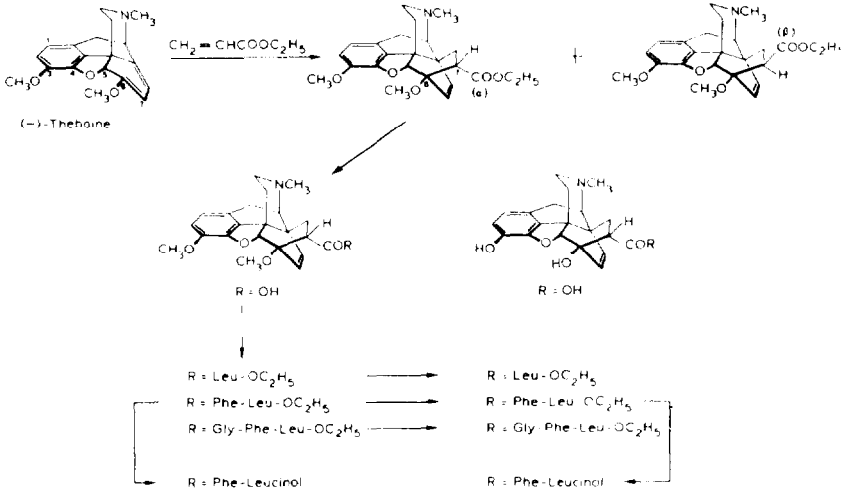


FIGURE 1. *Thebaine adducts coupled with enkephalin moieties*

Efforts to demethylate the 3-methoxy group selectively while maintaining the other one in position 6 were unsuccessful. Therefore, only the 3,6-dihydroxy compounds were prepared by a procedure using hydrogen bromide in glacial acid. In order to obtain the final products, the *C*-terminal carboxylic acid, formed by partial hydrolysis of the ester, was reesterified. In two cases, the leucinol derivatives were also prepared (figure 1), because the corresponding enkephalin reduction products showed a considerable increase in potency. The synthesis of more peptide-morphinans based on novel Diels-Alder adducts is in progress.

Some of the compounds were potent antinticeptives in the mouse hotplate assay (subcutaneous (SC) injection). The ethenoisomorphinan-3,6-diol with R=Leu-OC_2H_5 proved to be quite potent in the opiate receptor assay in rat brain membranes, in electrically stimulated guinea pig ileum, and in mouse vas deferens preparations. Recent investigations showed that the diols with R=Phe-Leu-OEt and R=Phe-leucinol are delta receptor agonists and that the diol with R=Gly-Phe-Leu-OEt is a potent antagonist of mu agonists. All three peptide morphinans were potent displacers of ^{3}H-etorphine in rat

cerebral membranes. The latter compound was less potent than naltrexone as a displacer. The first two diols were extremely potent displacers of ^{3}H-etorphine, with EC 50's in the range of etorphine (Smith et al. 1986).

DIELS-ALDER ADDUCTS WITH A NOVEL PATTERN OF OXYGEN-CONTAINING SUBSTITUENTS

Bentley (1971) studied the cycloaddition products starting from thebaine, which contains three oxygen functionalities on the morphinan skeleton. Extensive chemical and biological studies in the aromatic oxygenated morphinan-6-one series by Schmidhammer et al. (1983) revealed that the pharmacological activity of morphinans is strongly dependent on the number and position of oxygen-containing substituents in the aromatic nucleus as well as at C-6. The author has started to combine these two modifications of the morphine molecule. There are in principle two routes starting from thebaine: the synthesis of the cycloadduct followed by deoxygenation reactions or conversely beginning with a deoxygenated thebaine derivative followed by a Diels-Alder reaction.

For the latter route, the author developed two convenient syntheses of 6-demethoxythcbaine (Beyerman et al. 1984), one starting from the minor opium alkaloid neopine and one from codeine (figure 2). All simple dehydration reactions of neopine failed to give 6-demethoxythebaine. Methanesulfonyl (mesyl) chloride in pyridine gave the 6-mesyl derivative of neopine, which could be converted into 6-demethoxythebaine by treatment with potassium *tert*-butoxide. Starting with the easily accessible codeine, the 6-mesyl derivative was obtained quantitatively in the presence of 0.75 equivalent of tricthylamine instead of pyridine. Treatment with lithium bromide in boiling toluenc gave the 8 β-bromo morphinan that was converted into 6-demethoxythebaine with the aid of potassium *tert*-butoxide. Treatment of 6-*O*-mesylcodeine with sodium hydride gave directly 6-demethoxythebaine, but the overall yield was lower and the workup procedure was more laborious than the synthesis via the 8β-bromo morphinan.

6-Demethoxythebaine was the first morphinan-6,8-diene, which has been converted into Diels-Alder adducts with fewer oxygen-containing substituents (Hutchins et al. 1981; Crabbendam et al. 1981). With ethyl acrylate or methyl vinyl ketone, it gave predominantly the 7α-substituted 6,14-ethenoisomorphinan in analogy to the reaction with thebaine. Conversion of the 7α-acetyl group with propyl magnesium bromide into a tertiary alcohol substituent yielded compounds closely related to etorphine (figure 3).

Selective conversion of the 7α-ethoxycarbonyl substituent into a methyl propyl carbinol substituent, which was feasible with the thebaine derivative, failed with this 6-demethoxythebaine compound. Treatment with methyl magnesium bromide gave the dimethyl carbinol (figure 3, R^1=R^2=Me), a diprenorphine analog, which possesses the advantage of not having the extra chiral center of the carbinol group.

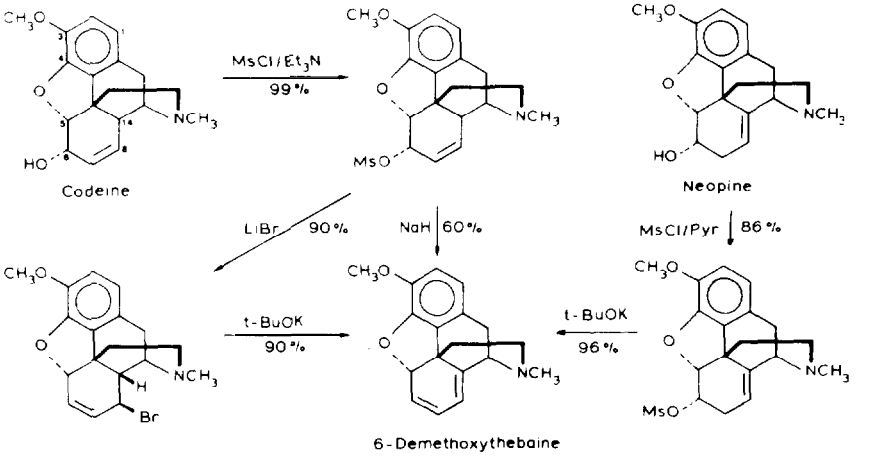


FIGURE 2. *Syntheses* of *6-demethoxythebaine*

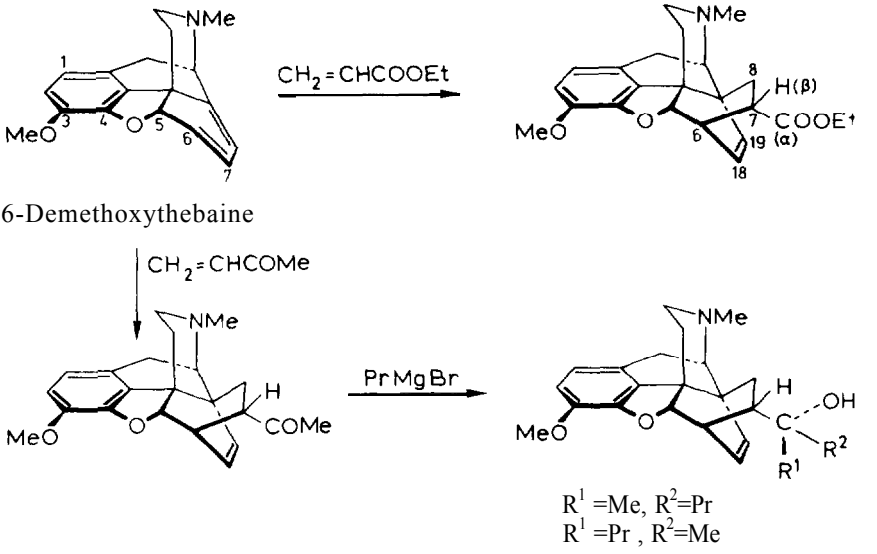


FIGURE 3. *Diels-Alder adducts of 6-demethoxyrhebaine*

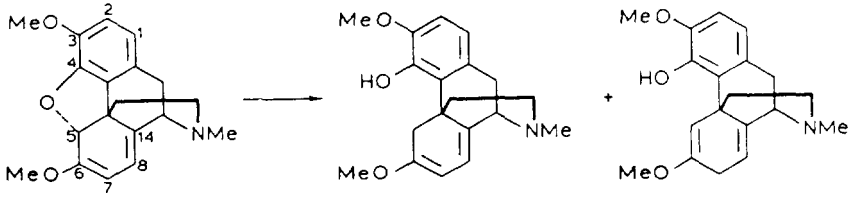
The pharmacological activity of 6-demethoxyetorphine-3-*O*-methyl ether (R^1=Me, R^2=Pr) was shown to be approximately 40 times that of morphine

in the mouse hotplate assay (SC injection). The 6-demethoxyetorphine epimer (R^1=Pr, R^2=Me) with the (S)-configuration of the tertiary alcohol function at C-7 'α was only 2 times more potent (Crabbendam et al. 1984). Further deoxygenation at position 3 of the 7 α· -dimethylcarbinol (R^1=R^2=Me) derivative was accomplished by catalytic hydrogenation (Pd-C) of the 3-(1-phenyltetrazol-5-yl) ether. It yielded a compound that proved to be a strong agonist (approximately 200 times that of morphine) in the mouse tail-flick assay (Linders et al. 1988b).

SCISSION OF THE EPOXY RING IN 4,5 α·-EPOXYMORPIIINANS

In order to obtain the series of 4-hydroxyethenomorphinans, several epoxy ring opening procedures were tried on 4,5 α·-epoxyethenomorphinans. A selective ring opening was difficult. Therefore, it was necessary to start from a morphinandiene. The reductive opening of the epoxy ring in thebaine with retention of the conjugated 6,8-diene function had been studied extensively (Razdan et al. 1978). The results were poor, mainly because the ring opening is accompanied by double-bond isomerization, giving the isomeric 5,8-diene (table 1).

TABLE 1. *Reductive scission of the* 4,5α-epoxy *bridge of thebaine*



	Thebaine	β–Dihydrothebaine (Percent)	Dihydrothebsine-ø (Percent)
1899	Freund[a] Na/EtOH		50
1939	Small[a] Na/EtOH		50
1951	Karrer[a] $LiA1H_4$/benzene	42	
1952	Bentley[a] Na/NH_3		95
1978	Portlock[a] K/NH_3	34	
1984	Kanematsu[b] $Fe(CO)_5$ / Zn/HOAc	47 (4-MeO-)	
1984	Delft[C] Zn/NH_4C1/EtOH,aq	70 (6-H-)	30
1986	Delft[d] Zn/2% KOH	98	1

[a]Razdan et al. 1978; [b]Fujii et al. 1984; [C]Crabbendam et al. 1984; [d]Linders et al. 1986

When 6-demethoxythebaine was treated with lithium aluminum hydride, according to one of the methods described for the epoxy ring opening of thebaine, a mixture of three major compounds was obtained, the third compound being the 5,7-diene, desoxycodeine-A. To prove the structure of the latter compound, it was prepared via bromocodide, starting from codeine in a way somewhat more convenient than the older procedure via chlorocodide.

It is remarkable to note that the 5,7-diene system in desoxycodeine-A failed to give Diels-Alder adducts. The reaction was studied with desoxycodeine-A itself and some 4-phenolic ethers and esters under both atmospheric and elevated pressures. Although the torsion angle around the C6-C7 bond, according to both x-ray analysis and molecular modeling calculations, does not differ much from that around the C7-C8 bond in morphinan-6,8-dienes, no cycloaddition product could be detected.

Finally, it was found that a simple treatment of thebaine in boiling 2-percent aqueous potassium hydroxide with zinc powder afforded almost exclusively and quantitatively β-dihydrothebaine (Linders et al. 1986). This reaction was then applied successfully to 6-demethoxythebaine, yielding 6-demethoxy-β-dihydrothebaine. However, the scission of the epoxy ring in 6-demethoxynorthebaine according to this procedure is still accompanied by approximately 15 percent of 5,8-diene formation.

DIELS-ALDER ADDUCTS OF 6-DEMETHOXY-β-DIHIYDROTHEBAINE

Diels-Alder reaction of thebaine with monosubstituted ethenes may afford, in principle, eight isomeric adducts. This was first recognized by Schöpf et al. (1938), who proposed on the basis of methylation reactions that the cycloadditions he studied took place from the P-side and that the oxygen-containing substituents were directed to the newly formed (C7-C8) double bond, in agreement with the so-called endo-rule according to Diels. Indeed, all further cycloaddition reactions described until recently yield predominantly one isomer with the dienophile approaching from the p-face of thebaine and with the substituent at position 7α (figures 1 and 3). These compounds were initially named 7α-substituted 6,14-*endo*-ethenomorphinans.

Heating 6-demethoxy-β-dihydrothebaine in an excess of methyl vinyl ketone for 60 hours yielded two new adducts in a ratio of 3:2, according to high performance liquid chromatograph (HPLC). The reaction when performed under conventional conditions caused extensive polymerization, which made the workup and the isolation of the adducts cumbersome. A dramatic improvement was achieved when the reaction was carried out using a modified microwave oven (Lindens et al. 1988a). Recently, the use of microwave heating in organic synthesis has been reported (Emsley 1988). The reactions described were carried out in closed vessels, which resulted in high reaction pressure and temperature. In the author's setup, under atmospheric conditions, the reaction, surprisingly, was complete within 24 hours with

substantially less formation of polymeric material, although the reaction temperature must have been similar to that in his earlier experiments. The usual workup procedure involving acid-base extraction gave the pure adducts after fractional crystallization. In contrast to all previous Diets-Alder reactions, the cycloaddition to 6-demethoxy-β--dihydrothebaine, lacking the epoxy bridge, occurred exclusively from the a-face of the diene function (figure 4).

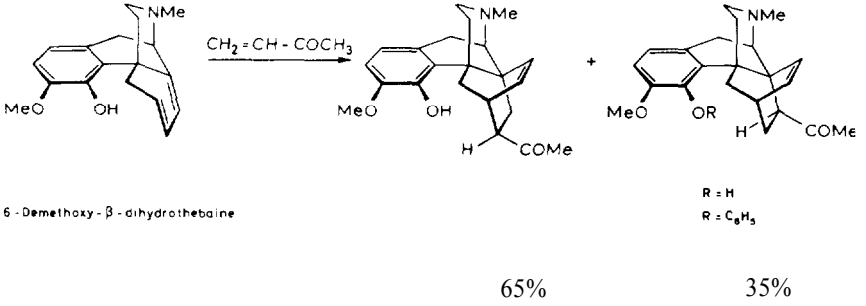


FIGURE 4. *Diels-Aider reaction of Memethoxy-β--dihydrothebaine with methyl vinyl ketone*

At first, structure elucidation of the new addition products met with difficulties. From the shifts of the vinylic protons H-18 and H-19, it was concluded that the etheno bridge is at the p-face of the molecule. The vinylic protons of the α–face etheno-bridged compounds show a downfield shift that can be attributed to the deshielding effect of the aromatic nucleus. The signals of the protons at C-7 and C-8 coincide with those of the acetyl and N-methyl groups. Mass spectrometry showed the expected molecular ions at *m/z 353*. The presence of a fragment *m/z* 57 (C_3H_5O) in the second compound can be explained by a McLaffetty rearrangement of the acetyl group. Molecular models show that this rearrangement can only occur in two 6β,14β-ethenomorphinans, viz, 7α- and 8β-acetyl, and in two 6α,14α--ethenomorphinans. However, the latter two can be ruled out on the basis of the nuclear magnetic resonance (NMR) data. From these spectral data, again with the application of the Diets endo-rule, the author tentatively assigned the 8β-acetyl-6β,14β -ethenomorphinan structure to the amorphous second compound. For x-ray analysis, it was converted into the crystalline 4-*O*-phenyl ether (R=C_6H_5). Detailed NMR as well as mass spectrometty did not result in an unequivocal proof of the two structures. Therefore, single-crystal x-ray analyses were used for conclusive evidence. Simultaneously with the author's findings, Ghosh et al. (1983) reported on the Diels-Alder reaction of the *O*-phenyl ether of β--dihydrothebaine with methyl vinyl ketone also giving rise to α--face cycloaddition.

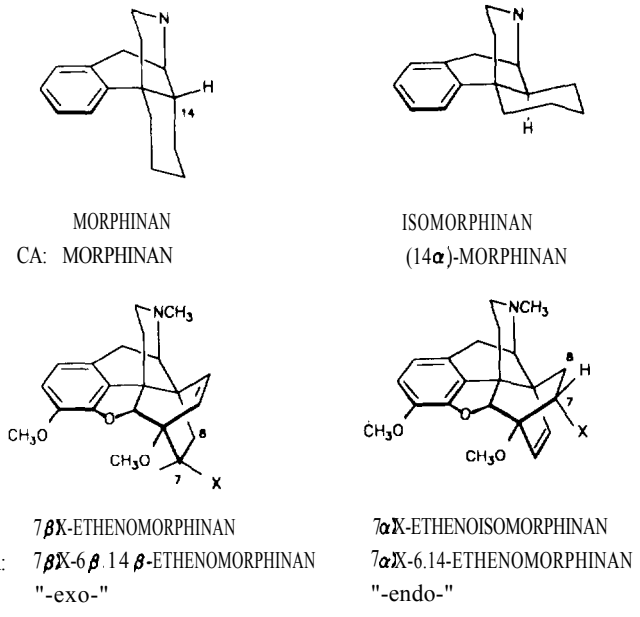
It is obvious that a new class of etheno-bridged morphinans is now accessible starting from the 4-hydroxymorphinandienes. As target molecule, reminiscent both of diprenorphine and of compounds previously prepared by the author, 3-hydroxy-α,α,17-trimethyl-6β,14β-ethenomorphinan-7β-methanol was chosen for pharmacological testing, starting from the first cycloadduct (figure 4). Reaction of the 4-hydroxyl group with bromobenzene in boiling pyridine in the presence of potassium carbonate and copper powder gave the 4-*O*-phenyl ether. The 7β-acetyl group was converted into the dimethyl carbinol substituent with methyl magnesium bromide. The phenoxyl group was then easily removed by means of reduction with sodium in liquid ammonia. The compound appears to show activity in only one of the biological assays (SC injection in mice). In the paraphcnylquinone (PPQ) stretching assay, it is morphinelike. In the tail-flick test at 1.0, 10.0, and 30.0 mg/kg, the compound is inactive as it is in the mouse hotplate assay. Since it has been previously noted that the PPQ assay is less discriminating with respect to the opioidlike activity, the compound will be examined further at the receptor level (Linders et al. 1988a).

The naming and numbering of the novel morphinans is confusing because different conventions are in use. The author suggests naming the "Bentley-type of so-called *endo*-etheno adducts" 6,14-ethen*oiso*morphinans and the new class of compounds 6,14-ethenomorphinans. In both cases, an etheno bridge is added to a distinguishable molecule, namely, isomorphinan and morphinan respectively. This leaves the usual numbering of the ring system unaltered at the positions 7 and/or 8, together with the statement of a and β with respect to the "phenanthrene projection" (figure 5).

N-FORMYLMORPHINANDIENES IN REACTION WITH NITROETHIENE

The Diels-Alder reaction of thebaine has been studied with a great variety of dienophiles. However, nitroethene has not received any attention. Although nitro compounds are not biologically important, the nitro group can be converted into the versatile amino substituent, interesting, for example, for opioid receptor studies (Lessor et al. 1984).

Direct reaction between thebaine and nitroethene did not lead to cycloaddition. Under alkaline conditions nitroethene undergoes polymerization. Therefore, an indirect route was chosen via the neutral *N*-formylnorthebaine (Maat et al. 1985). Now the addition proceeded quantitatively, and the product was, as expected, almost exclusively the 7α-nitro-6,14-ethenoisomorphinan (figure 6). The *N*-formyl substituent can be easily replaced by the N-methyl substituent via the northebaine derivative, which has the advantage that here any other useful alkyl group can also be introduced. Reduction of the nitro group with reagents such as lithium aluminum hydride and formamidinesulfinic acid stopped at the oxime stage. However,



MORPHINAN

CA: MORPHINAN

ISOMORPHINAN

(14α)-MORPHINAN

7βX-ETHENOMORPHINAN

CA: 7βX-6β.14β-ETHENOMORPHINAN

"-exo-"

7αX-ETHENOISOMORPHINAN

7αX-6.14-ETHENOMORPHINAN

"-endo-"

FIGURE 5. *Naming the different etheno-bridged morphinans*

treatment with aluminum amalgam in an aqueous mixture of ether and methanol gave the desired 7α--amine-6,14-ethenoisomorphinan (figure 6).

The cycloaddition of nitrcethene to 6-demethoxy-*N*-formylnorthebaine seems to be more peculiar. Preliminary experiments show that at least three cycloaddition products can be isolated (figure 7). Separation of the isomers was achieved by column chromatography. The major product (~ 50 percent) is the 8α-nitro-6,14-ethenoisomorphinan. Also, the usual 7α-nitro-6,14-ethenoisomorphinan is present (~ 10 percent). However, for the first time, an α--face addition to a 4,5α-epoxymorphinan-6,8-diene occurs in a fairly high yield (Prazeres et al. 1986). The structure was based on the ^{1}H NMR data of the N-H compound, obtained after acid hydrolysis. The signals in the 200-MHz ^{1}H NMR spectrum were assigned with the use of a homonuclear 2D correlation (COSY). From the magnitude of the vicinal couplings between H-7α and H-7β and H-8 ($J_{7\alpha,8}$ 8.5 Hz and $J_{7\beta,8}$ 4.0 Hz), it can be concluded that the nitro group is at the 8β-position. The 6β,14β orientation of the etheno bridge with respect to the isomorphinan skeleton was established by the long-range coupling between H-5 and H-7β (1.3 Hz), which is in agreement with the W-arrangement of the respective protons in that structure. Moreover, long-range couplings between H-5 and H-18 and between H-7β and H-18, as present in the case of 6α,,14α-etheno-bridged adducts, were not observed in this compound. In addition, it

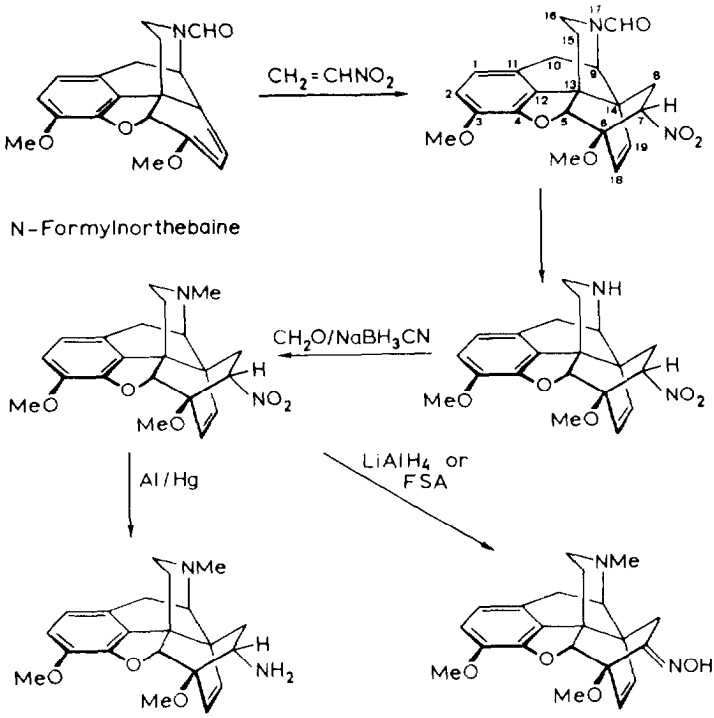


FIGURE 6. *Synthesis of 7α-nitro-6,14-ethenoisomorphinans and their reduction products*

is to be noted that proton H-8α is shielded (1.2 ppm) with respect to the corresponding H-7β in the 7α-nitro-6α,14α-ethenoisomorphinan, which might be explained by the shielding effect as a result of the proximity of the benzene ring in the 6β,14β-etheno adduct. A downfield shift in the resonance of the vinyl protons H-18 and H-19 of 0.4 and 0.8 ppm respectively was also observed. In conclusion, the structure of this compound is 4,5α-epoxy-3-methoxy-8β-nitro-6β,14β-ethenomophinan (~ 30 percent).

In order to see if this phenomenon also occurs with the usual dienophiles, the Diels-Alder reaction of 6-demethoxy-N-formylnorthebaine was carried out with methyl vinyl ketone. Chromatographic analysis showed only one major product, and the expected 7α-acetyl-6,14-ethenoisomorphinan could be isolated in 80-percent yield (figure 8).

The regio- and site-selectivities of the Diels-Alder reaction of morphinan 6,8-dienes have been explained as a combination of steric and electronic effect. Apparently, the strongly regio-directing properties of the small and

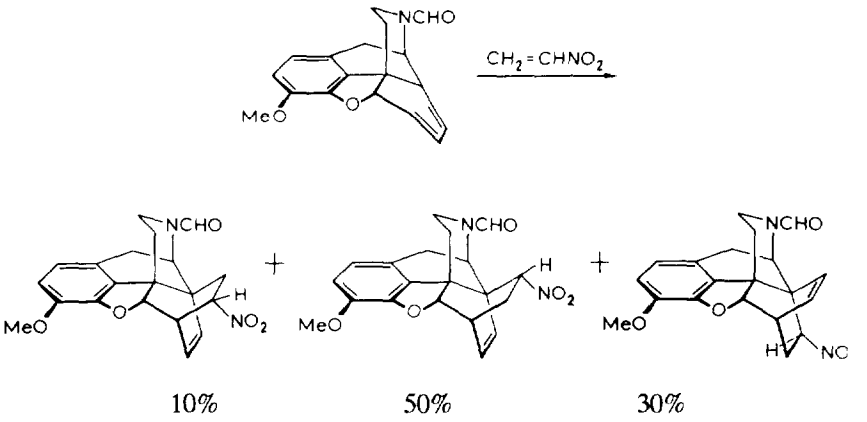


FIGURE 7. *Diels-Alder reaction of 6-demethoxy-*N*-formylnorthebaine with nitroethene*

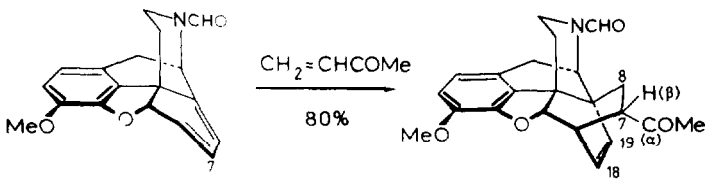


FIGURE 8. *Diels-Alder reaction of 6-demethoxy-*N*-formylnorthebaine with methyl vinyl ketone*

planar nitroethene govern the site selectivity of the Diels-Alder reaction when the 6-methoxy substituent is absent, giving rise to 8-substituted cycloadducts. Flattening at the nitrogen by replacing the *N*-methyl group by an *N*-formyl group, together with the removal of the 6-methoxyl group, also makes a-face approaching of nitroethene possible.

Until now the author synthesized the *N*-formylmorphinan-6,8-dienes by demethylation of the corresponding *N*-methyl derivatives followed by formylation. However, the demethylation with diethyl azodicarboxylate proceeds sometimes in low to moderate yields. Therefore, an alternative route was studied. The author started from *N*-formylnorcodeine, which was prepared by treatment of codeine with 2,2,2-trichloroethyl chloroformate in the presence of potassium hydrogen carbonate, a modification of a known procedure, which effected complete conversion into the carbamate. The latter

compound was reduced using zinc dust and, subsequently, formylated with ethyl formate. Along the lines of the pathway via the 6-*O*-mesylcodeine analog, depicted in figure 2, it was found that treatment of the 8β-bromo intermediate with an excess of potassium *tert*-butoxide gave at 0 °C, after 1 minute, the desired *N*-formylmorphinan-6,8-diene. Due to the instability in strong alkaline medium, careful control of the reaction conditions is necessary. When 6-*O*-mesyl-*N*-formylnorcodeine was treated with lithium bromide for a prolonged period, the initially formed 8β-bromo intermediate rearranged to (+)-(Z)-7-formyl-8,9-dihydro-l-hydroxy-2-methoxy-7*H*-dibenz[*d,* f]azonine (figure 9), the structure of which was proven by means of a single-crystal x-ray analysis.

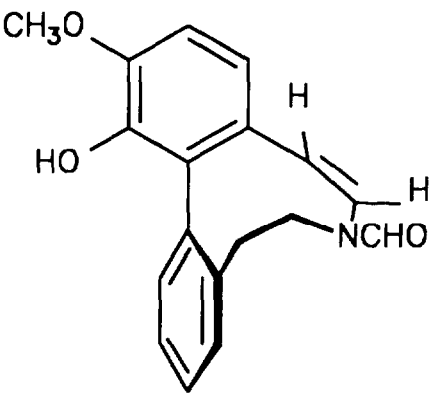


FIGURE 9. *(R)(Z)-7-Formyl-8,9-dihydro-l-hydroxy-2-methoxy-7H-dibenz[*d,f*]azonine*

CONCLUSION

Although many Diels-Alder reactions of thebaine and thebainelike morphinandienes have been studied, it is expected that still more reactions can be discovered, all affording new compounds that may add new data on structure-activity relationships. The *N*-formylmorphinans, especially, form an interesting group of compounds. They are not only useful in synthetic pathways, but they also can be easily converted into *N*-substituted morphinans.

From the data available it is clear that the cycloaddition to 4,5α-epoxymorphinan-6,8-dienes takes place from the β-face. Only in the case of the reaction with nitroethene does a substantial α-face addition of approximately

30 percent co-occur. Morphinan-6,8-dienes that lack the 4,5 α-epoxy ring give rise to α -face addition, as the few examples demonstrate (figure 10). The latter reaction needs further investigations and the reaction products, eventually converted into further modified compounds, will constitute new classes of morphinans.

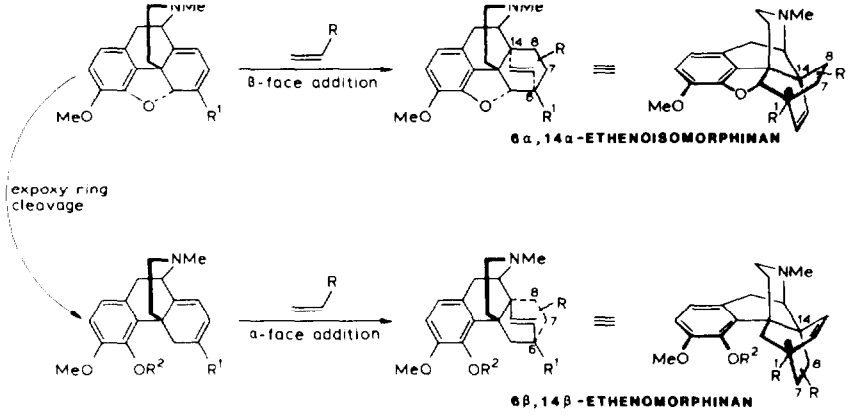


FIGURE 10. *Cycloadditions to morphinan-6,8-dienes*

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Opioid Antagonists: Structure Activity Relationships

Dennis M. Zimmerman and J. David Leander

INTRODUCTION

Considerable progress has been made toward understanding the functions of the various opioid receptors. Three opioid receptors have been well characterized (the mu, kappa, and delta), and substantial evidence has indicated the existence of other opioid receptor subtypes. Endogenous ligands for these receptors have been identified, and this has resulted in the realization that the endogenous opioids and their receptors have important physiological functions beyond the modulation of pain. One such function may be immune system modulation.

Much of what is now known concerning the opioid receptor system can be attributed to extensive structure activity relationship (SAR) studies where rank correlations between pharmacological potencies and binding affinities have been carefully compared. In such studies, opioid antagonists have played important roles. and it is likely that similar pharmacological comparisons will be equally important for the delineation of any endogenous opioid regulation of the immune system. Therefore, an understanding of the SARs of the opioid antagonists may be useful in the characterization of any opioid involvement in immune system regulation.

BACKGROUND

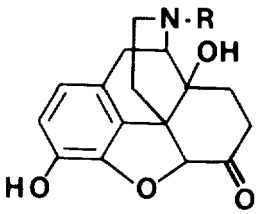
Opioid antagonist activity was first discovered with N-allyl derivatives of rigid opiate agonists. Nalorphine, the N-allyl derivative of morphine, figure 1, was the first antagonist approved to treat narcotic overdose (Robinson 1974). It was soon discovered that nalorphine was not a "pure" antagonist but had partial agonist activities, which caused nalorphine to be referred to as having mixed agonist-antagonist properties (Martin et al. 1976). In addition, following the characterizations of the different opioid receptor subtypes, it was discovered that nalorphine had relatively high affinities for all three classes of opioid receptors (Magnan et al. 1982).

FIGURE 1. *Comparison of the molecular structures of morphine, nalorphine, and naloxone*

The first opioid antagonist discovered to have no opioid agonist effects (zero intrinsic activity) was naloxone (Blumberg and Dayton 1974). Like nalorphine, naloxone has significant affinity for the mu, kappa, and delta receptors; however, it has highest affinity for the mu receptor (Magnan et al. 1982). Compared to other neurotransmitter receptors, it has been difficult to discover opioid antagonists devoid of agonist properties. In opioid pharmacology, the ability to measure agonist activity of a partial agonist is test dependent. The most sensitive means of detecting opioid agonist activity is to determine a compound's ability to block the electronically stimulated contractions of smooth muscles, particularly the isolated guinea pig ileum and mouse vas deferens tissues (Kosterlitz et al. 1974; Leslie 1987). Compared to the number of opioid agonists and partial agonists that have been identified, relatively few opioid antagonists with zero intrinsic activity are known. It is the SARs of these pure antagonists that are the focus of this review.

IMPACT OF STRUCTURE ON ACTIVITY

Naloxone is the N-allyl derivative of oxymorphone (figure 2). The N-cyclopropylmethyl derivative naltrexone is also an opioid antagonist but is more potent and longer acting than naloxone (Blumberg and Dayton 1974). The SAR shown in figure 2 for oxymorphone and derivatives is typical of those observed with other morphine-based structures. There is generally a loss of intrinsic activity, in an approximate stepwise manner, through replacement of the N-methyl with dimethylallyl and the other groups as shown. The allyl and cyclopropylmethyl derivatives generally have the lowest relative intrinsic activities, and this relationship is usually found for all three opioid receptors. With the oxymorphone molecule, these derivatives have no opioid agonist properties (Blumberg et al. 1967). However, with other rigid opioid molecules, the N-allyl and cyclopropylmethyl derivatives often have partial agonist activities. For example, cyclazocine (a benzomorphan derivative) (Eddy and May 1966) cyclorphan (a morphinan



R		
CH_3	Oxymorphone	Agonist
$CH_2\ CH = C(CH_3)_2$		Partial agonist
$CH_2\ C{\equiv}C{-}H$		Partial agonist
CH_2–cyclopropyl		Partial agonist
$CH_2\ CH = CH_2$	Naloxone	Antagonist
CH_2–cyclopropyl	Naltrexone	Antagonist

FIGURE 2. *Antagonist effects of N-substituted oxymorphone analogs*

derivative) (Hellerbach et al. 1966), and LY 113878 (a phenylisoquinoline derivative) (Zimmerman et al. 1988a) (figure 3) are potent, high-affinity, partial agonists. Much is now known concerning the impact of the various structural features of naloxone or naltrexone on intrinsic activity. The C-14 hydroxyl substituent of the oxymorphone molecule is essential for pure antagonist activity. For example, Compound 1, which is the naltrexone analog without a C-14 hydroxyl (figure 3) is a potent opioid partial agonist (Kosterlitz et al. 1973). Similarly, addition of a hydroxyl substituent to the same relative position of cyclorphan (figure 3) giving oxilorphan (originally referred to as BC2605), results in a dramatic loss of opioid intrinsic activities without having a significant effect on opioid receptor affinites. Oxilorphan, however, still has significant partial agonist activities (Kosterlitz et al. 1973; Pachter 1974).

Other structural modifications of rigid opiate structures have led to the discovery of new opioid antagonists (figure 3). MR2266 (figure 2) a N-furylmethylbenzomorphan derivative, is a well-characterized opioid antagonist. It has, at times, mistakenly been referred to as a selective kappa antagonist. However, MR2266 has approximately equal affinity for mu and

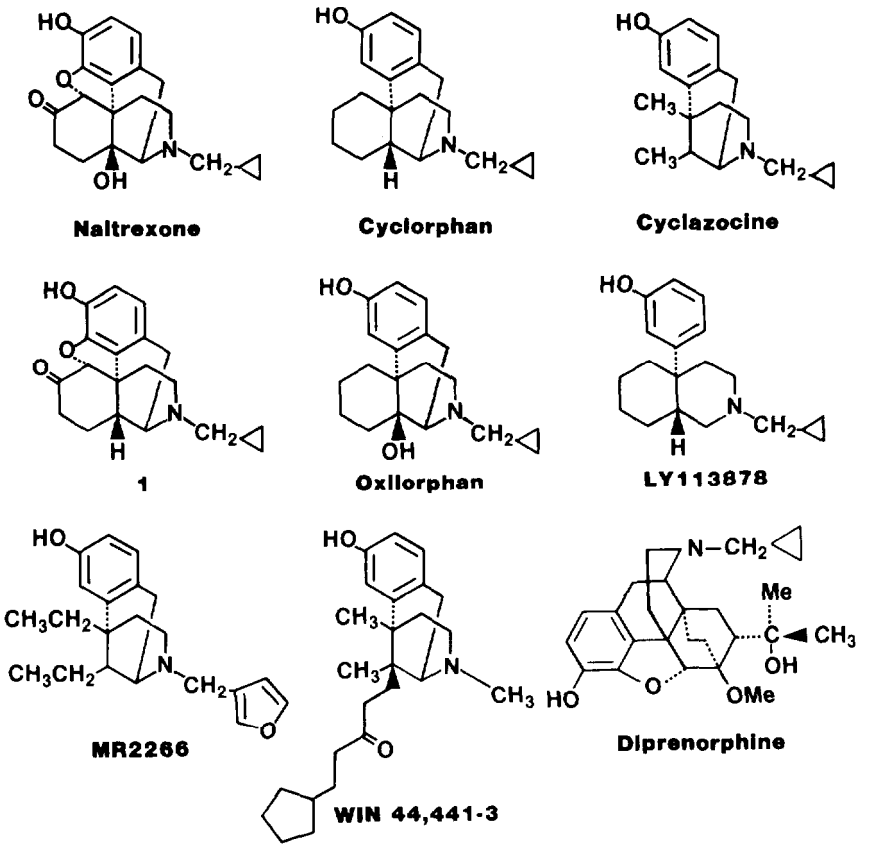


FIGURE 3. *Structural comparisons of various opioid antagonists*

kappa receptors. It also has weak opioid partial agonist properties in smooth muscle bioassays (Magnan 1982). Diprenorphine, an oripavine derivative, has highly potent antagonist activities. It has at times been referred to as a universal antagonist, with the implication that it is a pharmacologically pure antagonist; however, it is known to have significant kappa agonist activity (Traynor et al. 1987). WIN 44,441-3 is a potent, nonselective, opioid antagonist devoid of agonist activities (Michne et al. 1978; Wood 1984). Remarkably, it has a methyl-substituted nitrogen, and replacement of the cyclopentane group at the terminus of the 3-alkanone substituent with other phenyl or other alkyl groups imparts partial agonist activities to the molecule.

Naltrexone and naloxone have served as precursors for the synthesis of several novel pure opioid antagonists through structural modification of the

C-6 keto group (figure 4). These would include the opioid antagonists nalmefene (Hahn et al. 1975), naloxonazine (Pastemak and Wood 1986), naltrindole (NTI) (Portoghese et al. 1988), and norbinaltorphimine (nor-BNI) (Portoghese et al. 1987). NTI and nor-BNI are recently discovered selective antagonists of delta and kappa receptors, respectively. Their discovery represents a significant advance in SARs of the opioid antagonists.

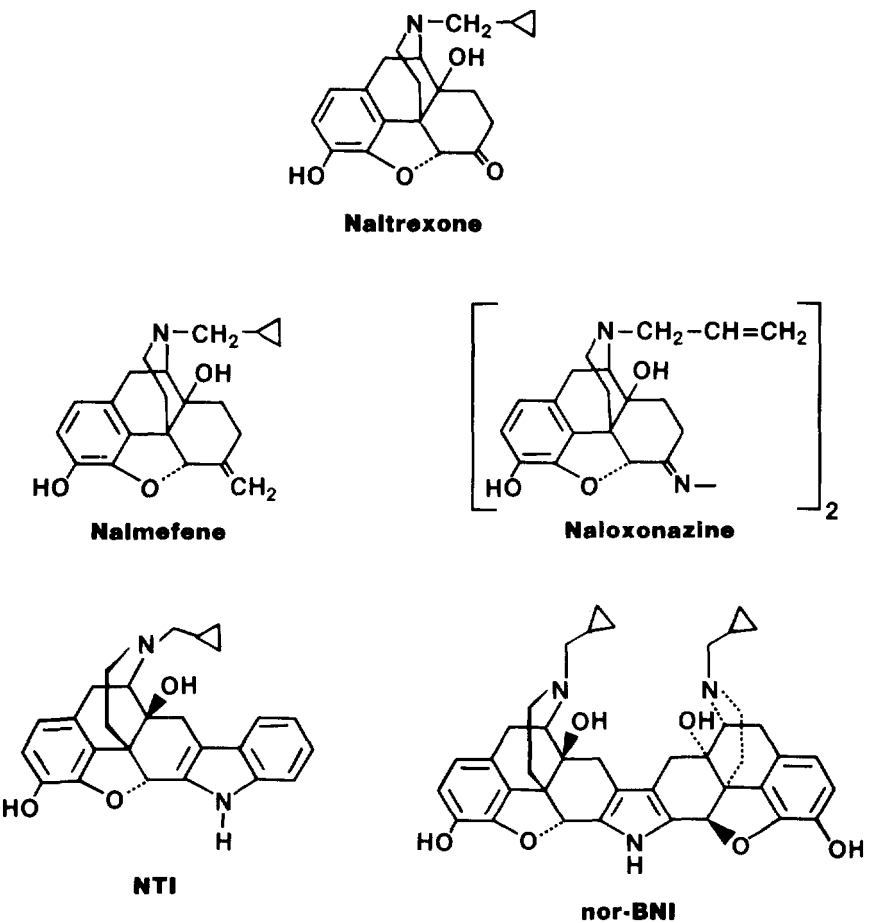


FIGURE 4. *C-6 antagonist analogs of naltrexone*

Structural alteration of the enkephalins has led to the discovery of the peptide antagonist ICI174864 (N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH; Aib=α-aminoisobutytic acid), which has high selectivity for the delta receptor

(Cotton et al. 1984). Although it does have weak agonist activity (Cohen et al. 1986), the selective antagonist properties of ICI174864 have proven highly useful for investigating delta receptor activities. Its utility *in vivo* is limited, however, because it crosses the blood-brain barrier poorly. Recently, the discovery of a somostation analog CTP (D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2), a selective mu receptor antagonist, was reported (Shook et al. 1987). Its selective reversible antagonist properties likely will make it a useful pharmacological tool.

The authors have reported the discovery of potent opioid antagonist activity in a series of trans-3,4-dimethyl-4-phenylpiperidines (figure 5) (Zimmerman et al. 1978). Quite surprisingly, in this series, the antagonist activity was shown to be a consequence of methyl substitution at the three position of the piperidine ring. LY99335 was a pure opioid antagonist, while its des-3-methyl analog, LY25506, was an opioid agonist. With the trans-3,4-dimethyl-4-phenylpiperidines, antagonist potency is not significantly affected by replacement of the N-methyl with an N-allyl or a cyclopropylmethyl substituent; however, antagonist activities were significantly increased with other substitutions at nitrogen. LY117413 is a pure opioid antagonist with affinities for the opioid receptors comparable to naloxone.

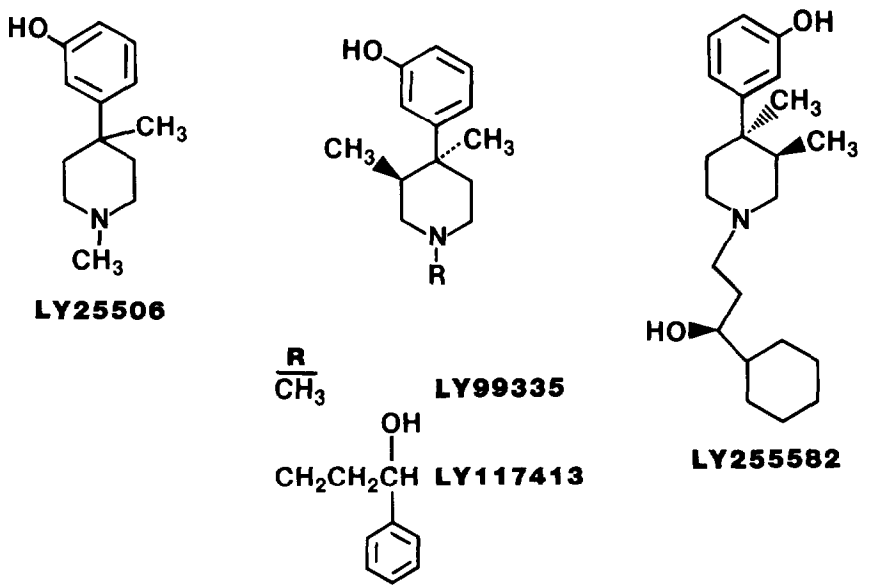


FIGURE 5. *Structures of the trans-3,4-dimethyl-4-phenylpiperidine antagonists*

Recently, at the Lilly Research Laboratories, the SAR of this 4-phenylpiperidine antagonist series has been further explored with the focus of this effort being directed to the possible use of an opioid antagonist as an appetite suppressant in the treatment of obesity. An extensive study was undertaken in an attempt to maximize the appetite-suppressant effect for possible clinical investigation and also to further characterize the opioid receptors involved in this effect (Zimmerman et al. 1988b). Opioid receptor affinities were compared to opioid antagonist potencies against mu (morphine) and kappa (U50,448) analgesia using the mouse writhing test and against bremazocine-induced diuresis (kappa effect) in rats. Selected compounds were evaluated for their effects on food and water consumption in meal-fed obese Zucker rats.

Some 75 different N-substituted analogs were synthesized, and many highly potent pure antagonists were discovered. All were devoid of opioid agonist effects, which further confirmed the antagonist pharmacophore nature of the trans-3,4-dimethyl-4-phenylpiperidine molecule. A few exhibited exceptional potency as appetite suppressants, but, surprisingly, the effects on food consumption were not well correlated with antagonist activities at either the mu, kappa, or delta receptors. Compound LY255582 (figure 5) emerged as having the best overall activity profile (Leander et al. 1988). As an appetite suppressant, it was at least 40 times more potent than naltrexone on an ED, basis (table 1). Furthermore, the maximum reduction achieved with LY255582 was significantly greater than that achieved with naltrexone (data not shown). Neither of these properties could be attributed to an increase in a particular opioid receptor antagonist activity.

CONCLUSION

In summary, until recently, only a small number of opioid pure antagonists were available for pharmacological studies. Most of these are nonselective for the three opioid receptors; however, they still serve as very useful probes to study possible opioid receptor-mediated effects. Recently, selective antagonists with affinities for the mu, kappa, and delta receptors have been discovered, and their use offers considerable promise for the further characterization of the functions of the endogenous opioids and their receptors. Pharmacological comparisons of the 4-phenylpiperidine antagonists as appetite suppressants indicate that other ways for the characterization of selective opioid receptor effects may lead to unexpected findings. It may be reasonable to expect other inconsistent findings in future pharmacological comparisons, and it is important to realize that we still have only a limited understanding of the opioid receptor system.

TABLE 1. *Opioid receptor affinities and antagonist effects of LY255582 compared to naloxone and naltrexone*

	Opioid Receptor Affinities Ki-Values (nM)			Antagonism of Opioid Analgesia Mouse Writhing Test AD_{50}[d]; (mg/kg, SC)		Antagonism of Kappa Diuresis in Rats	Effects on Food Consumption Obese Zucker Rats
	^{3}H-NAL[a] (mu)	^{3}H-EKC[b] (kappa)	^{3}H-DADL[c] (delta)	mu (morphine)	kappa (U50,488)	AD_{50}[e] (mg/kg, SC)	ED_{20}[f] (mg/kg, SC)
LY255582	0.41	2.0	5.2	0.015	0.05	0.38	0.05
Naloxone	3.7	66	32	0.08	1.1	3.5	1.4
Naltrexone	0.56	6.0	3.9	0.05	0.06	2.5	2.1

[a]Naloxone, using crude membranes from rat brain.

[b]Ethylketocycluocine, using crude membranes from guinea pig cortical tissue with fentanyl and DADL added to inhibit binding to mu and delta receptors

[c]D-Ala*-D-LeuJ-enkephalin, using crude membranes from rat brain.

[d]Dose required for SO-percent reduction in the analgesic response to either morphine (1.25 mg/kg, SC) or U50,488 (2.5 mg/kg, SC).

[e]Dose required to decrease the 5-hour bremazocine-induced (0.08 mg/kg, SC) urination by 50 percent.

[f]Dose required to reduce 4-hour food intake by 20 percent in rats trained to eat daily for 8 hours.

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Effect of Fluorine Substitution on the Anti-HIV Activity of Dideoxynucleosides

Victor E. Marquez

INTRODUCTION

Fluorine substitution has been extensively investigated in drug research and biochemistry as a means of enhancing biological activity and increasing chemical or metabolic stability (Goldman 1969; Ciba Foundation Symposium 1972; Filler 1979; Filler and Naqvi 1982; Bamette 1984). The most important factors considered for the synthesis of fluorine-containing compounds are: (1) the relative small size of fluorine, which is comparable to that of hydrogen; (2) the powerful electron-withdrawing properties of fluorine; (3) the increased stability of the carbon-fluorine bond relative to the carbon-hydrogen bond; and (4) the increased lipophilic character of fluorine relative to hydrogen.

In terms of size, fluorine has a small van der Waals radius (1.35 Å) that closely resembles that of hydrogen (1.20 Å) (Pauling 1960). Therefore, substitution of a hydrogen by fluorine in a molecule is expected to cause minimal steric perturbations with respect to the molecule's mode of binding to a receptor or an enzyme. In contrast, since fluorine is the most electronegative of the elements (Pauling 1960), its powerful electron-withdrawing properties can profoundly affect chemical reactivity. When attached to a reaction center, fluorine is a moderately good leaving group, and when placed near a reaction center, it can dramatically change chemical reactivity at that center via its strong inductive effect (Chambers 1973).

The combined effect of small size and powerful electron-withdrawing properties, for example, has been successfully exploited in the steroid field in optimizing the corticoid activity of 11-ß-hydroxy steroids by increasing the acidity of the 11-ß-hydroxyl group as in 9-α- -fluoro-ll-ß-hydroxyprogesterone (*1,* figure 1) (Wettstein 1972).

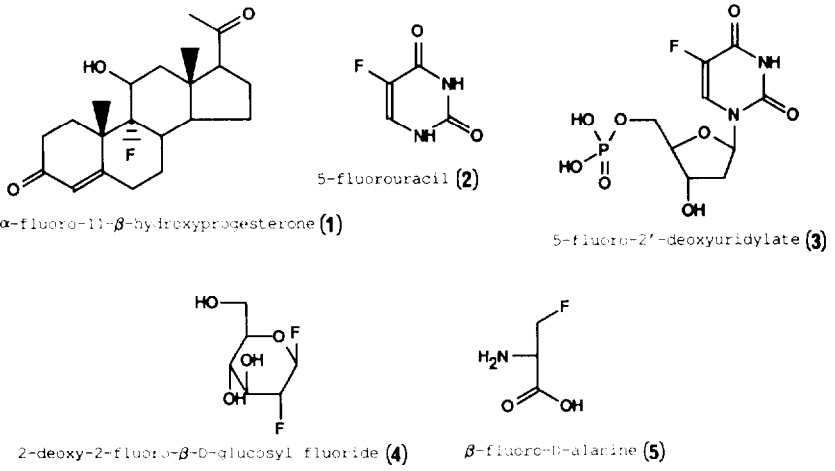


FIGURE 1. *Structures of some fluorinated bioactive molecules*

The higher energy of the C-F bond (108 kcal/mol) relative to that of C-H (104 kcal/mol) is another important property that is responsible for the increased metabolic inertness of some fluorine-containing compounds. A classical example in this category is the antitumor drug 5-fluorouracil (2, figure 1). The chemotherapeutic utility of 2 is due to its conversion to the active metabolite 5-fluoro-2'deoxyuridylate (*3,* figure 1), which behaves as a powerful inhibitor of thymidylate (dTMP) synthetase. This inhibition is caused by the unreactive fluorine at C-5, which blocks the ensuing addition of formate after a reversible complex between 3 and the enzyme is formed (Pogolotti and Santi 1977).

As mentioned previously, when the fluorine atom is attached to a reactive center, it undergoes facile elimination of fluoride, and the resulting electrophilic species can irreversibly inhibit enzymatic activity through the formation of a covalent bond to the enzyme (Johnston et al. 1979; Muehlbacher and Poulter 1985). For example, the glycosylase inhibitor 2-deoxy-2-fluoro-ß-D-glucosyl fluoride (4, figure 1) undergoes rapid elimination of the glycosylic fluorine to give an oxocarbonium ion capable of reacting very rapidly with the enzyme (Withers et al. 1988). The resulting 2-deoxy-2-fluoro-glycosyl-enzyme complex is then stabilized by the fluorine substituent at C-2. The combination of these two effects leads to enzyme inactivation through the generation of a more stable intermediate that traps the enzyme and prevents it from completing the final transformation (Withers et al. 1988). In other cases, the reactive center beating the fluorine atom can be unmasked at the active site by the normal enzymatic process, and the resulting inactivation of the enzyme is referred to as "suicide inhibition." The

well-known antimetabolite ß-fluoro-D-alanine (5, figure 1) is a good example of a "suicide inhibitor" that contains fluorine (Kollonitsch 1982). After the initial "normal," enzymatic-catalyzed reaction between ß-fluoro-D-alanine and pytidoxal phosphate, the resulting intermediate aldimine loses fluoride ion and generates an electrophilic species capable of alkylating the bacterial enzyme (alanine racemase) irreversibly (Kollonitsch 1982).

Another important property of fluorine, which is directly related to its high electronegativity, is that it can function effectively as a hydrogen bond acceptor (figure 2). This property of fluorine, allowing it to operate as a substitute for oxygen in a hydroxyl function, has not been extensively studied in drug design. In the area of carbohydrate chemistry, however, the systematic replacement of hydroxyl functions by fluorine has permitted the accurate mapping of important hydrogen-bonding interactions with the various receptors involved in the transport of sugars (Bamctt 1972). As seen in table 1 (Barnett 1972), the similar size and electronegativity of fluorine and oxygen suggest that fluorine may indeed be better compared as an isostere of oxygen rather than of hydrogen because of the similarity of the C-F and C-OH bonds in polarity and bond length. It is possible, therefore, that fluorinated analogs of hydroxyl-containing compounds will retain biological activity or behave as powerful inhibitors of the enzymes that process the nonfluorinated substrates. All of these properties are potentially exploitable in drug design.

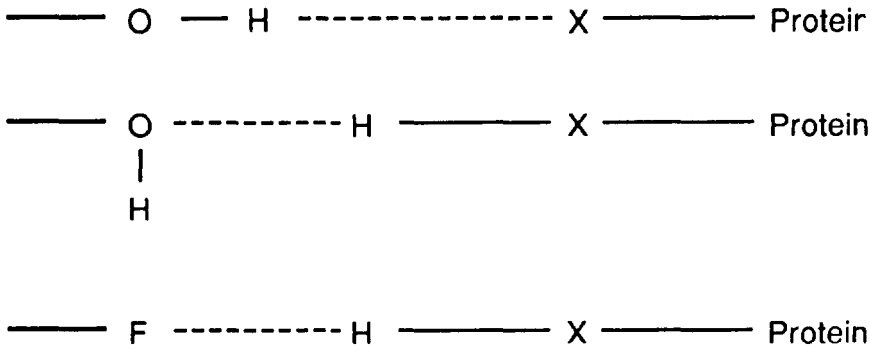


FIGURE 2. *Possible hydrogen bonds between protein and drugs*

The various properties of the C-F bond discussed above have provided the necessary incentive to investigate the effects of fluorine substitution on the antiretroviral activity of dideoxynucleosides.

TABLE 1. *Comparison of the size and electronegativity of some elements*

Element	Bond Length (CH_3-X) (Å)	Van der Waals radius (Å)	Total (Å)	Electronegativity
H	1.09	1.20	2.29	2.1
F	1.39	1.35	2.74	4.0
0 (in OH)	1.43	1.40	2.83	3.5
CL	1.77	1.80	3.57	3.0
S (in SH)	1.82	1.85	3.67	2.5

Source: Table based on data in Barnett 1972.

DIDEOXYNUCLEOSIDES AS ANTIRETROVIRAL AGENTS

The unique mode of replication of retroviruses, requiring virion-encoded reverse transcriptase (RT), provides a virus-specific target for effective chemotherapy. Dideoxynucleosides have been shown to inhibit the replication of human immunodeficiency virus (HIV) and other retroviruses by inhibiting RT (Mitsuya and Broder 1986; Dahlberg et al. 1987). The inhibition is believed to result from the incorporation and consequent chain termination effect of the dideoxynucleosides, which must be metabolically activated from the monophosphate to the triphosphate level by cellular rather than viral enzymes (Johnson et al. 1987; Johnson et al. 1988).

The anti-HIV activity of these compounds is normally determined *in vitro* by measuring the increase in cell viability after cells infected with HIV have been treated with various concentrations of the test drugs. In one experimental approach, the anti-HIV activity is measured in MT-4 cells, and the ED_{50}, defined as the concentration of the test agent capable of conferring 50-percent protection to HIV-infected cells, is reported along with the equivalent parameter for cytotoxicity (CD_{50}) (Pauwels et al. 1987; Baba et al. 1987). Another protocol uses an immortalized helper-inducer T-cell clone (ATH8). and the results are normally expressed in percent protection vs. percent cytotoxicity for a given dose (Mitsuya and Broder 1986; Kim et al. 1987). Aside from the inherent difficulties in comparing data from different laboratories that use different assay systems, the *in vitro* tests give an end-point result that encompasses a complex sequence that spans biochemical events from activation (three cellular enzymes) to interaction with viral RT. Consequently, drawing clear-cut structure-activity conclusions from these studies is not possible unless one looks at each step individually by preparing and testing each metabolite. Despite these shortcomings, the *in vitro* assay is the more practical and economical method for rapid screening.

EFFECT OF FLUORINE SUBSTITUTION IN DIDEOXYNUCLEOSIDES

This problem can be divided into two general areas dealing with the effects of fluorine substitution on either the sugar or the aglycon moieties of the dideoxynucleosides. Each of these areas, in turn, could be further divided into pyrimidines and purines.

Sugar-Modified Dideoxynucleosides

In looking at the activation process from the nucleoside level to the triphosphate form (figure 3), it is possible to envision that a fluorine substituent at C-3' on the sugar might be a hydrogen-bonding equivalent of a hydroxyl group (figure 2). In addition, one could anticipate that for this 3'-fluoro substituent to be an effective isostere of oxygen, it should have a-stereochemistry. In the case of a 2'-fluoro substitution, however, in which fluorine functions as a steric isostere of hydrogen, it is more difficult to predict which stereochemical orientation will preserve substrate recognition by the different enzymes involved in the activation process.

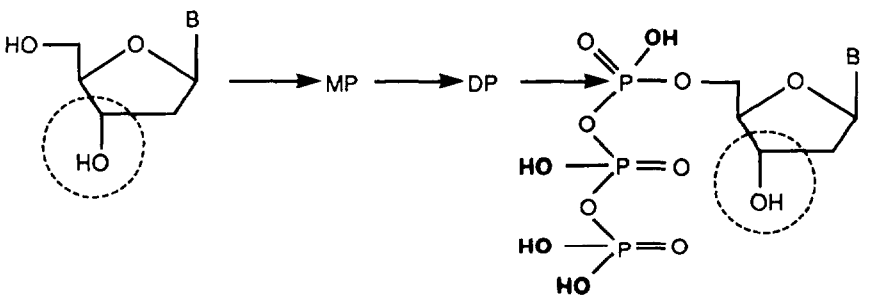
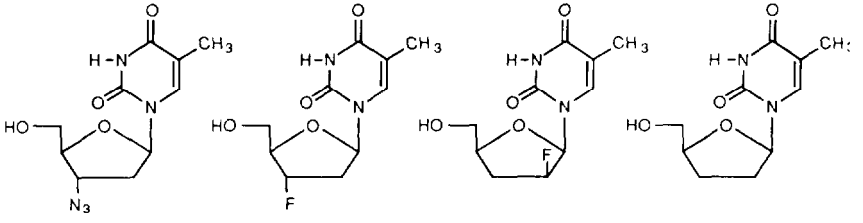


FIGURE 3. *Nucleoside activation steps*

Pyrimidines. The biological evaluation of some of the first fluorine-substituted pyrimidine dideoxynucleosides (Herdewijn et al. 1987; Balzarini et al. 1988) confirmed the anticipated results mentioned above. When the anti-HIV activities of 3'- α -fluoro-2',3'-dideoxythymidine *(7*, figure 4) and 2'-ß-fluoro-2',3'-dideoxythymidine (8, figure 4) were compared to those of 2',3'-dideoxythymidine (ddTh, *9,* figure 4) and the prototype drug used clinically against AIDS, AZT (3'-azido-2',3'-dideythymidine, *6,* figure 4), the results confirmed the importance of the 3'-α-hydroxyl group at some stage of the activation process or at the level of interaction with RT. In MT-4 cells, isosteric fluoro and azido groups both appeared to be equipotent, albeit the fluoro analog was more toxic. In the ATH8 system, however, the two compounds were nearly equipotent and equitoxic (Herdewijn et al.



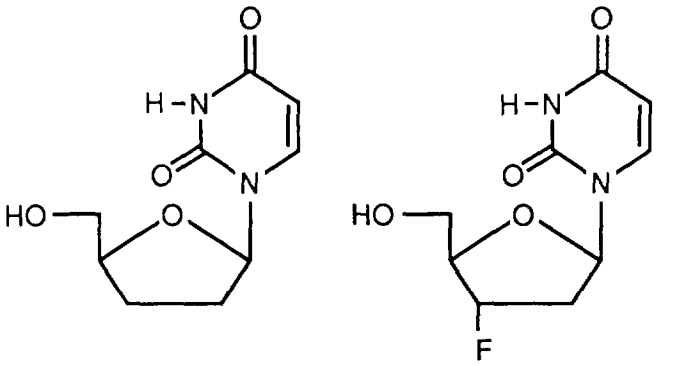
6 **7** **8** **9**

	ED_{50} (μM)	CD_{50} (μM)	SI
6	0.004	20	5000
7	0.001	0.197	197
8	>500	>500	
9	6	>625	104

FIGURE 4. *Anti-HIV activity (MT-4 ceils) of AZT vs. fluorinated and nonfluorinated thymine nucleosides*

1987). This near equivalence between fluoro and azido groups is not too surprising since the azido group is often considered to be a pseudohalogen (Treinin 1971). At the 2'-position, however, fluorine substitution above the plane of the sugar ring destroyed activity (figure 4). A more striking effect at 3' was observed for the dideoxyuridine case (Van Aerschot et al. 1988). As seen in figure 5, the compound with the 3'-fluoro substituent in the α--configuration (*11,* figure 5) displayed a 5,000-fold increase in anti-HIV potency over the parent unsubstituted compound (*10,* figure 5) (Van Aerschot et al. 1988).

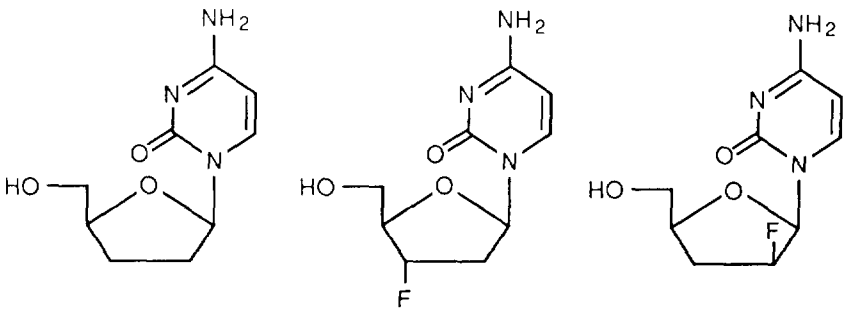
When the pyrimidine base was cytosine, both test systems (ATH8 and MT-4) revealed that the same pattern of fluorine substitution on dideoxycylidine (ddC, *12,* figure 6) was in general detrimental and with opposite effects to those observed in the dideoxyuridine and dideoxythymidine cases (Herdewijn 1987; Van Aerschot et al. 1988). This is probably a reflection of the fact that the two drugs are activated by different cellular kinases, deoxycytidine kinase and thymidine kinase, respectively (Baizarini et al. 1988). In this instance (figure 6), the 2'-ß-fluoro substituted analog (*14,* figure 6) was slightly superior to the 3'-α--fluoro compound (*13,* figure 6) (Van Aerschot et al. 1988). When two fluorine atoms in the α- -configuration were simultaneously introduced at the 2' and 3' positions (compounds *15* and *16,* figure 7). there was no difference between the dideoxyuridine and dideoxycytidine series, and both classes of compounds were completely devoid of anti-HIV activity (Van Aerschot et al. 1988). Compounds with the substitution pattern of *17* and *18* (figure 7) have not yet been reported.



10 **11**

	ED_{50} (μM)	CD_{50} (μM)	SI
10	210	>625	>3
11	0.04	16	400

FIGURE 5. *Anti-HIV activity (MT-4 cells) of fluorinated vs. nonfluorinated uracil nucleosides*



12 **13** **14**

	ED_{50} (μM)	CD_{50} (μM)	SI
12	0.3	40	120
13	16	26	1.6
14	9.8	117	12

FIGURE 6. *Anti-HIV activity (MT-4 cells) of fluorinated vs. nonfluorinated cytosine nucleosides*

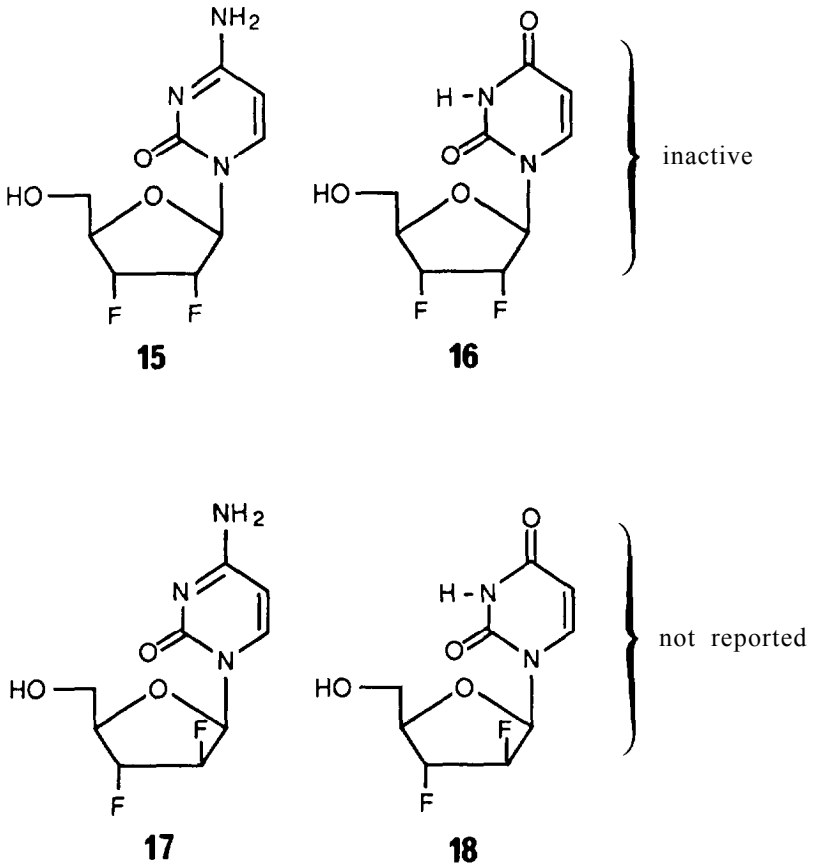
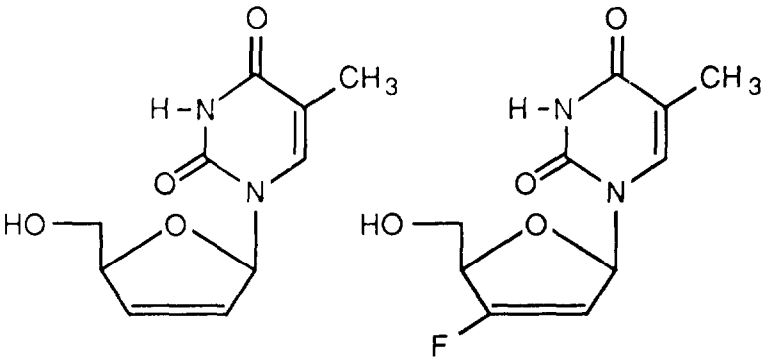


FIGURE 7. *Structures* of *polyfluorinated nucleoside analogs*

It is worth noting that, contrary to the pronounced increase in potency brought about by fluorine substitution on an sp^3-hybridized carbon, as observed with compounds 7 and II, substitution at the same relative position on an sp^2-hybridized carbon was detrimental to the anti-HIV activity. Fluorine substitution on the very potent 3'-deoxy-2',3'-didehydrothymidine (19, D4T) caused a 1,000-fold reduction in potency (compound 20, figure 8) (Van Aerschot et al. 1988).

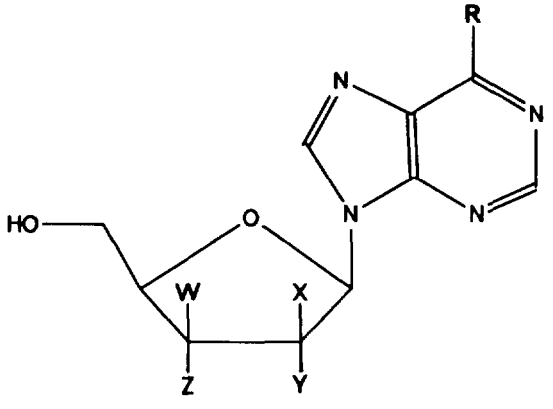


19 **20**

	ED_{50} (μM)	CD_{50} (μM)	SI
19	0.01	1.2	120
20	10-50	232	4.5-2.3

FIGURE 8. *Anti-HIV activity (MT-4 cells) of fluorinated vs. nonfluorinated D4T*

Purines. Among the dideoxypurine nucleosides, 2',3'-dideoxyadenosine (*21,* figure 9) is a compound in clinical trial that shows potent activity and better selectivity than AZT in the lymphocyte ATH8 *in vitro* test system (Johnson et al. 1988). In addition to the changes that fluorine substitution would be expected to have on the activation of this substance by its two operational kinases (adenosine kinase and deoxycytidine kinase), the possibility of altering the chemical properties of this drug through fluorine substitution became very attractive. Although all dideoxynucleosides are hydrolytically less stable than their patent nucleosides, this difference reaches staggering proportions in the case of dideoxyadenosine, which undergoes acid-catalyzed cleavage of the glycosylic linkage at a rate 40,000 times faster than adenosine (York 1981). The half-life of this drug at pH 1 and 37 °C was determined to be 35 seconds (Marquez et al. 1987). Clearly, this precludes any intended use of this drug by the oral route.



21, W=X=Y=Z=H, R=NH_2
22, W=X=Y=Z=H, R=OH
23, W=X=Z=H, Y=F, R=NH_2
24, W=Y=Z=H, X=F, R=NH_2
25, W=Y=Z=H, X=F, R=OH
26, W=X=Y=H, Z=F, R=NH_2
27, X=Y=Z=H, W=F, R=NH_2

FIGURE 9. *Structures of fluorinated and nonfluorinated dideoxy purine nucleosides*

Since the acid-catalyzed hydrolysis of purine nucleosides is thought to proceed by an Al mechanism in which the protonated nucleoside dissociates in the rate-controlling step to a glycosyl carbonium ion and free purine, it was felt that introduction of an electronegative fluorine atom adjacent (C-2') to the reaction center should destabilize the resulting oxocarbonium ion and decrease the rate of hydrolysis (figure 10).

The syntheses of both α- and ß-fluoro dideoxyadenosine analogs (23 and 24, figure 9) permitted confirmation of this hypothesis by showing that both compounds were completely inert to acid hydrolysis under the same conditions that rapidly hydrolyzed dideoxyadenosine (Marquez et al. 1987). Although minimal steric changes were expected to occur after fluorine substitution in both isomers, it was impossible to predict beforehand the effect that the stereochemical orientation of the fluorine would have on biological activity. Testing of both drugs in the ATH8 system indicated that only the ß-fluoro analog 24 was an effective anti-HIV agent, virtually indistinguishable from its parent compound, dideoxyadenosine (Marquez et al. 1987). Another important consequence of fluorine substitution and the increased chemical stability of the glycosylic bond was manifested in the enzymatic

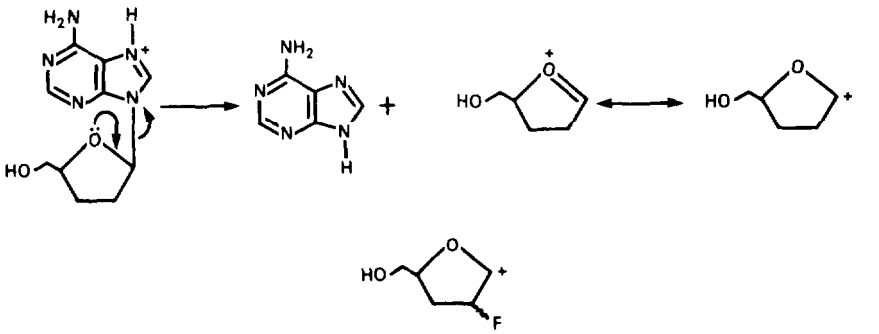
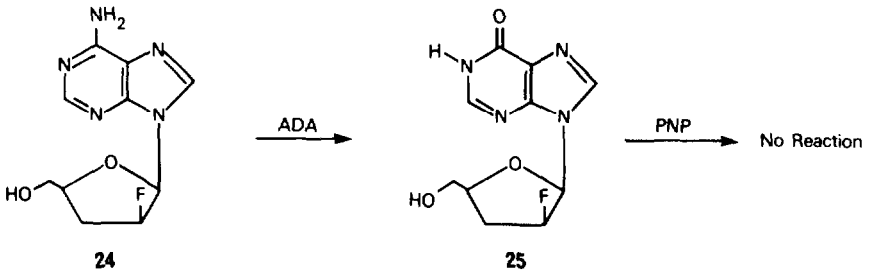


FIGURE 10. *Mechanism of acid hydrolysis of dideoxynucleosides and acid stabilization rationale*

inertness of the dideoxyinosine metabolite 25 towards cleavage by purine nucleoside phosphorylase (Marquez et al., unpublished) (figure 11). Dideoxyinosine (22), as well as its fluorinated analog 25, is formed from the parent adenosine nucleosides via adenosine deaminase. However, such metabolic transformation has little or no bearing on the resulting anti-HIV activity, since both dideoxyadenosine and dideoxyinosine, as well as the corresponding 2'-ß-fluoro analogs, are equally effective against HIV. On the other hand, resistance to purine nucleoside phosphorylase is a desirable



24 **25**

FIGURE 11. *Metabolism of 2'-β-fluoro-dideoxypurines*

feature, since this enzyme is capable of depleting useful levels of anti-HIV-active dideoxyinosine. Chemically synthesized 2'-ß-fluorodideoxyinosine (25) was shown to be completely resistant to purine nucleoside phosphorylase upon incubation with the enzyme (Marquez et al., unpublished). In addition, the anti-HIV activity of 2'-ß-fluorodideoxyinosine (25) was identical to that of its progenitor compound *24* and dideoxyadenosine (figure 12). In this system, a 10-μM concentration of the drug was able to provide com-

plete protection against HIV without significant cytotoxicity, as judged by the equal height of the bars representing infected vs. noninfected cells, respectively (figure 12).

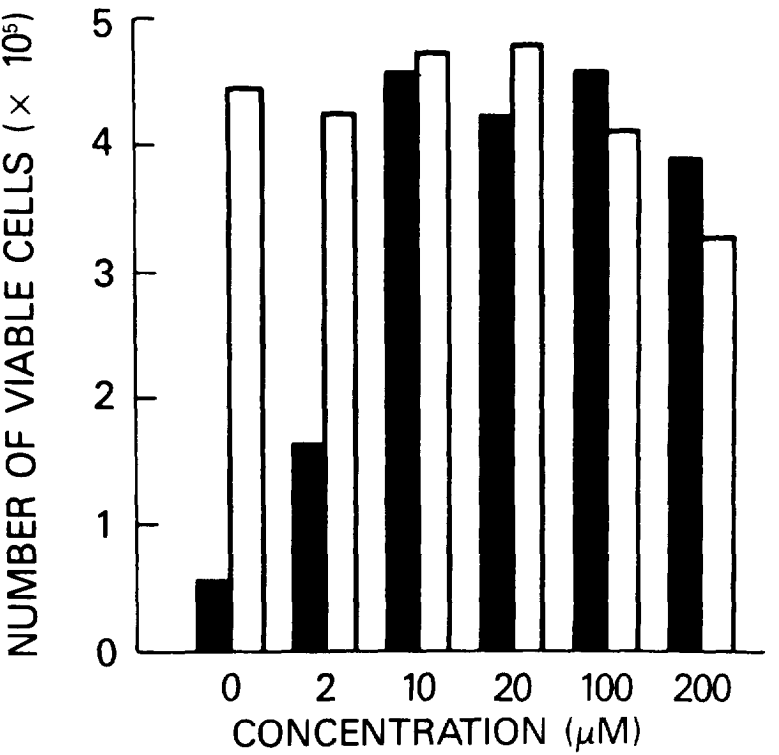


FIGURE 12. *Inhibition of the cytopathic effect of HIV by 2'-β-fluoro-dideoxyinosine (25) in ATH8 cells*

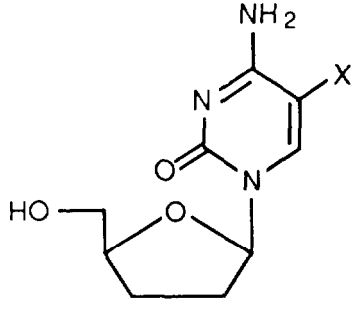
Compounds 23 and 24 (figure 9) were also independently synthesized and tested in the MT-4 cell system (Herdewijn et al. 1987). In this system, the same difference was observed: the 2'-ß-fluoro isomer 24 showed potent anti-HIV activity, whereas the 2'-α-fluoro isomer 23 was completely inert. The fact that in MT-4 cells dideoxyadenosine appeared to be slightly more potent than the active fluoro isomer 24 indicates that there are differences in sensitivity between the ATH8 and MT-4 test systems (Baba et al. 1987).

In the case of the two possible 3'-fluoro isomers, 26 and 27 (figure 9), the results in the MT-4 assay were consistent with the premise that the 3'-α-fluoro substituent appears to be a good isostere for the hydroxyl oxygen of 2'-deoxyadenosine (Herdewijn et al. 1987). This compound (26), although

approximately one-eighth as potent as the parent dideoxyadenosine, coofirmed the previously observed trend in the pyrimidine system for a preferred α-fluoro stereochemistry at the 3'-position. The 3'-ß-fluoro isomer 27 was totally devoid of activity (Herdewijn et al. 1987).

Aglycon-Modified Dideoxynucleosides

Pyrimidines. The most obvious and chemically accessible position for substitution on a pyrimidine base is at carbon 5. In the uracil series, however, fluorine substitution was considered unwise, since cleavage of the glycosylic bond in the resulting compound, 5-fluorodideoxyuridine, would have been a source of highly cytotoxic 5-fluorouracil. However, because dideoxycytidine, which is one of the most potent known anti-HIV agents, was not a substrate for cytidine deaminase (Kelley et al. 1987), it was decided to synthesize and investigate the anti-HIV activity of analogs in the cytidine series (Kim et al. 1987). In an effort to increase the lipophilic character of dideoxycytidine, the 5-fluorodideoxycytidine analog 28 (figure 13) was synthesized along with other members of a small series that included the more lipophilic analogs, methyl- and bromo-substituted derivatives (29 and 30, figure 13).



	Dose (μM)	% Protection	% Cytotoxicity
12, X = H	5	100	0
28, X = F	0.5	100	0
29, X = Br	>200	<10	10
30, X = Me	>100	<20	20

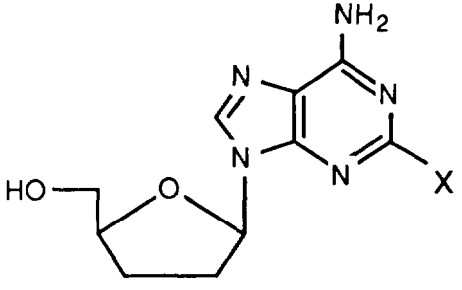
FIGURE 13. *Anti-HIV activity (ATH-8 ceils) of 5-substituted cylosine nucleosides*

Active compounds with increased lipophilic character appeared important enough, since it is known that HIV has a selective tropism for certain cells of the central nervous system (CNS), and a wide variety of CNS

complications, including dementia, can result from this CNS infection (Price et al. 1981; McArthur 1987). With the parent compound, dideoxycytidine, CSF:plasma ratios of only 3 to 5 percent are achieved in rhesus monkeys, and its poor CNS penetration is suspected to be related to its strong hydrophilic character, as indicated by its negative log P value (-1.26) in the octanol/water system (Kelley et al. 1987; Cheung 1988). Among the 13 AIDS-related nucleosides recently measured for partition coefficient values by the National Cancer Institute, AZT was the single compound that was lipophilic, but only barely so (log P=+0.04) (Cheung 1988). Unfortunately, the more lipophilic methyl- and bromo-substituted dideoxycytidines proved to be inactive against HIV in the ATH8 assay system (Kim et al. 1987). On the contrary, the 5-fluorodideoxycytidine analog was just as potent as the parent dideoxycytidine and afforded complete protection against HIV at a dose of 0.5 μM (figure 13) (Kim et al. 1987). However, the modest increase in lipophilicity afforded by this compound (log P=-1.05) did not change the CSF:plasma ratio, which was virtually the same as for dideoxycytidine (Kelley et al., unpublished data). In this instance, therefore, the fluorine atom in the pyrimidine ring behaves as a simple isostere of hydrogen, in which its stronger electronegativity appears not to have any effect, positive or negative, on the resulting anti-HIV activity of dideoxycytidine.

Purines. Only one published report addresses halogen substitution in the aglycon of a didcoxy purine (Haertle et al. 1988). The main thrust behind this project was provided by the finding that 2-chloro-2'-deoxyadenosine was not significantly deaminated in human cells, while at the same time being efficiently phosphorylated in T lymphocytes. As discussed previously, dideoxyadenosine is catabolized first by adenosine deaminase, and the resulting product dideoxyinosine is further cleaved and rendered inactive through the action of purine nucleoside phosphorylase. The 2-haloderivatives (compounds *31* through *33,* figure 14) were found to be extremely poor substrates for adenosine deaminase, and the corresponding 2-halodideoxyinosine analogs were generated only after prolonged incubation with the enzyme (Haertle et al. 1988). Prevention of the deamination reaction was critical in 2-halodideoxyadenosine series because the corresponding 2-halodideoxyinosine congeners had significantly less anti-HIV activity (Haertle et al. 1988). This is in sharp contrast with the 2-unsubstituted didcoxyadcnosine and dideoxyinosine nucleosides, which are virtually equal in their anti-HIV activity (Ahluwalia et al. 1987; Johnson et al. 1988). Inhibition of HIV infection, measured in cultured MT-2 lymphoblasts by the reduction in formation of syncytia, was achieved by 2-fluoro-, chloro- and bromodideoxyadenosine nucleosides at 10-μM concentration (figure 14) (Haertle et al. 1988). At the same concentration, these drugs also blocked HIV replication in CEM T lymphocytes, as measured by a p24 (*gag*) antigen capture assay. Cytotoxicity was observed generally at concentrations twofold higher than those required to inhibit HIV-induced syncytia formation (Haertle et al. 1988). However, the observed trend was that with increasing size of the halogen substituent, cytotoxicity was reduced without a concomitant

reduction of the anti-HIV activity. Another important difference, which was independent of the type of halogen substituent, was the failure of the 2-halodideoxyadenosine derivatives to inhibit HIV replication in deoxycytidine kinase-deficient CEM mutants (Haertle et al. 1988). By contrast, dideoxyadenosine exerted equivalent anti-HIV activity in wild-type CEM cells as well as in the deoxycytidine kinase-deficient variant. This would indicate that dideoxyadenosine is also a substrate for adenosine kinase as well as for deoxycytidine kinase (figure 14). In summary, introduction of a 2-halo substituent into the purine aglycon prevents catabolism by adenosine deaminase but limits the activation of the drugs by eliminating substrate recognition from adenosine kinase. The 2-halodideoxyadenosines are also more toxic than the parent dideoxyadenosine against T cells. The order of toxicity is F>Cl>Br (Haertle et al. 1988). These differences could be due in part to the release of toxic 2-halo-adenine bases from the very unstable dideoxynucleosides.



	HIV-induced Syncytia	Dose	dCK-	dCK+
21, X = H	0	10 µm	+	+
31, X = F	0	10 µM	-	+
32, X = Cl	0	10 µM	-	+
33, X = Br	0	10 µM	-	+

FIGURE 14. *Anti-HIV activity (MT-2T cells) of 2-substituted adenine nucleosides*

CONCLUSION

The most salient feature of fluorine as a substituent on the sugar moiety of active anti-HIV dideoxynucleosides is that it can effectively function as an isostere of oxygen or hydrogen, depending on its position and stereochemical orientation on the dideoxyribose moiety. When the aglycon base is

uracil or thymine, fluorine is an excellent isostere of the oxygen atom of the 3'-α--hydroxyl function. This hydroxyl group appears to be essential for enzymatic recognition at some critical stage towards the activation of these compounds by the various kinases. This effect of fluorine is significantly less, and in some cases detrimental to anti-HIV activity, when the aglycon bases are cytosine or adenine. In these instances, it appears that the 3'-α-hydroxyl function has a less critical role during the enzymatic activation that occurs by way of different kinases. When the fluorine is substituted at the 2'-position, only the ß configuration appears to be compatible with anti-HIV activity. This effect appears to be more pronounced in the dideoxypurine case, where the fluorine atom brings an additional stability to the glycosylic bond towards chemical or enzymatic cleavage. An a-fluorine at the 2'-position appears less desirable, since it will make these molecules look more like ribosides. As a result, all 2'-α-fluoro-substituted dideoxynuclecsides are inactive against HIV. Substitution by fluorine at various positions of the aglycon moiety of dideoxycytidine and didcoxyadenosine nucleusides does not appear to destroy activity, and only minimal changes are observed with respect to the parent compounds. Clearly, in these situations, fluorine appears to be functioning as an isostere of hydrogen.

In summary, the most important effect of fluorine in anti-HIV-active dideoxypurine occurs when the fluorine is substituted at the C-2'-ß position. Particularly for the purine case, it increases the stability of these drugs without compromising biological effectiveness. This increase in chemical and enzymatic stability offers many practical advantages for the development of effective long-lasting oral forms of this important class of antiviral agents.

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Opiate Antagonists and Anti-HIV Agents

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INTRODUCTION

It is unfortunate but true that most drugs of abuse were originally introduced as therapeutically useful agents that were intended to relieve and not to add to an individual's ills. In fact, clinical practice still regards many of these abused agents as valuable and essential tools. With the diversion of drugs to illicit use, it was recognized that opiates such as morphine impact on the immune system, since immunological deficits were commonly seen in opiate addicts.

The effects and some of the beneficial properties of the opium poppy were known to man long before medical records were established. Egyptian findings indicate that opium was used to calm children, and other discoveries described the early use of opium in some form as a source of relief from many types of aches, pains, and mental disorders. Aside from its medical applications, the poppy was also used (abused) in social settings, where it was either eaten or smoked.

Although the pharmacological properties of the poppy were recognized for centuries, an understanding of the nature of opium activity did not begin until the early 19th century, when the principal constituents of the resin of the poppy were isolated. These included the clinically useful compounds morphine and its 3-methyl ether, codeine. Another important component was identified as thebaine, which was shown to be medically useless because of its extreme toxicity. However, the value of thebaine resided in its transformation products, which include both opiate agonists, such as oxymorphone and oxycodone, and the pure opiate antagonists. The discovery of this latter type of agent, of which there are few, represents one of the most interesting developments in research associated with the opiates. By definition, opiate antagonists are compounds that are able to block or reverse the pharmacological effects of the opiate analgesics. The first observation of this type of drug was made in 1915. when N-allylnorcodeine

was shown to antagonize the respiratory depressant effects of morphine (Pohl 1915). It was 30 years later that N-allylnormorphine (nalorphine, Nalline) was made and its antagonistic action confirmed (Hart 1943). It was not until 1951 that this property was clinically utilized, when Eckenhoff demonstrated that nalorphine was an antidote for morphine poisoning in man (Eckenhoff et al. 1951). The discovery of these properties of nalorphine stimulated an intensive search for other such agents. The key to opiate antagonist activity appeared to reside with the substituent on the piperidine ring nitrogen (Winter 1957). Pharmacological testing showed that compounds containing a three-carbon chain, such as allyl or cyclopropylmethyl, displayed antagonist properties (Jacobson et al. 1970; Jacobson 1977). Naloxone was the first pure opiate antagonist to be synthesized (Lewenstein and Fishman 1966; Jasinski et al. 1967). More recently, naltrexone has become clinically available (Martin et al. 1973). The rest of the vast majority of other derivatives that were synthesized showed varying degrees of agonist activity.

Approximately 15 years ago, the author was involved in a research program to synthesize pure opiate antagonists that would exhibit greater potency and a longer duration of action than naloxone. He was also interested in a compound that would be orally active, since the possible use of pure opiate antagonists as therapeutic agents in the treatment of opiate addiction was being advocated. Initial studies (Linder and Fishman 1973) focused on the preparation of various esters at C-3 and C-6 of the molecule (table 1). Although an increase in potency was observed for a number of derivatives, no significant oral bioavailability was noted.

TABLE 1. *Biological activity* of *esters of naloxone*

Compound	Relative Intravenous (IV) Potency
Naloxone	1.0
3-Acetate	2.0
14-Acetate	1.5
3,14-Diacetate	0.66
3-Sulfate	0.22
14-Sulfate	0.10
3,14-Disulfate	0.09
3-Sulfate,l4-Acetate	0.25
3-Cinnimate	1.8
3-Nicotinate	1.3
3-Phosphate	0.53

The C-6 carbonyl group in the naloxone molecule lends itself readily to modification, and it appeared reasonable that structure-activity correlations derived at this position for opiate agonists might also translate to the antagonists. Replacement of the C-6 carbonyl by methylene resulted in morphine and codeine analogs up to 75 times more potent than the parent compound (Chadha and Rapoport 1957; Okun and Elliott 1958). Interestingly, this increase in potency was accompanied by a decrease in duration of action except in the 14-hydroxy series of compounds. Both naloxone and naltrexone underwent a Wittig reaction with triphenyl phosphonium methylide to yield the respective methylene analogs in greater than 90 percent yield (Hahn et al. 1975). Table 2 (Hahn et al. 1975) shows the relative potencies of a series of C-6 derivatives of naloxone and naltrexone as determined in the hot plate and tail clip tests. The methylene derivatives of both naloxone and naltrexone (nalmefene) exhibited significant increases in potency relative to the parent compounds.

TABLE 2. *Mouse hot plate and tail clip assay*

Substitution				Oral Antagonist Potency
C-3	C-6	C-14	N	
OH	=O	OH	$-CH_2CH=CH_2$	1.0
OH	=O	OH	$-CH_2-C_3H_5$	10.0
OAc	=O	OH	$-CH_2CH=CH_2$	2.0
OH	=O	OAc	$-CH_2CH=CH_2$	0.5
OAc	=O	OAc	$-CH_2CH-CH_2$	1.0
OH		OH	$-CH_2CH=CH_2$	1.0
OH	$\rightarrow CH_3\cdots OH$	OH	$-CH_2CH=CH_2$	5.0
OAc	$\cdots XH_3\rightarrow$	OH	$-CH_2CH=CH_2$	2.0
OH	$\rightarrow CH_3\cdots OH$	OH	$-CH_2-C_3H_5$	12.0
OH	$=CH_2$	OH	$-CH_2CH=CH_2$	1.5
OAc	$=CH_2$	OH	$-CH_2CH=CH_2$	3.0
OH	$=CH_2$	OH	$-CH_2-C_3H_5$	100
OAc	$=CH_2$	OH	$-CH_2-C_3H_5$	50

The effect of spatial orientation of the C-6 oxygen substituent as a determinant of agonist and antagonist potency was examined using the guinea pig ileum longitudinal muscle assay (Ronai et al. 1977). Table 3 indicates that, if the substituent is alpha to the plane of the molecule, an enhancement of agonistic properties is observed. In contrast, when the substituent at C-6 is beta, no agonist component is introduced. The 6-methylene derivatives that exhibit similar stereochemistry to that of the parent keto compound also are devoid of agonist activity and have increased potency (table 3).

TABLE 3. *Kinetic parameters of naloxone, naltrexone, and their 6-alpha- and 6-beta-hydroxy derivatives on the longitudinal muscle strip of guinea pig ileum*

Compound	ID50 (nM)	Ke (nM)
Naloxone	—	3.9±0.5 (n=7)
6-Beta-Hydroxy naloxone	— (n=7)	6.3±0.9 (n=3)
6-Alpha-Hydroxy naloxone	346.5±46.1 (n=4)	9.9±1.6 (n=3)
Naltrexone	71,433 (n=3)	0.8±0.1 (n=4)
6-Beta-Naltrexol	(n=4)	1.6±0.1 (n=4)
68-Alpha-Naltrexol	33.7±3.8 (n=4)	1.2±0.2 (n=4)

Further evaluation of nalmefene was carried out to establish its pure opiate antagonist character. Results from both *in vivo* and *in vitro* studies indicated that nalmcfene was without agonist properties. These results included the following:

(1) No signs characteristic of morphine were produced on injection into drug-naive animals (Heilman et al. 1976).

(2) It was inactive in the mouse acetic acid writhing test at doses of 100 mg/kg subcutaneously (SC) (Heilman et al. 1976).

(3) It did not depress the contraction of coaxially stimulated guinea pig ileum muscle (Nash et al. 1984).

(4) Nalmefcne had a sodium index of less than 1.0 at the opiate receptor in rat brain membranes (Michel et al. 1984).

The binding of nalmefene to opiate receptors in rat brain membranes was also tested (Michel et al. 1984). Nalmefene displaced the radiolabeled prototype ligands dihydromorphine (mu), D-Ala-D-Leu enkephalin (delta), and ethylketocyclazocine (kappa) with IC50s in the low nanomolar range. As can be seen in table 4, nalmefene was more effective at inhibiting agonist binding at central kappa sites than either naloxone or naltrexone.

TABLE 4. *Relative affinities to the mu, delta, and kappa*

	mu	delta	kappa
Naloxone	0.85	0.06	0.09
Naltrexone	0.77	0.13	0.10
Nalmefene	0.67	0.09	0.24

A comparison of the potency of nalmefene and naloxone after either parenteral or oral administration is shown in table 5 (Baker Cummins Pharmaceuticals' nalmefene information for investigators). Nalmcfene was more effective in reversing the effects of an ED80 dose of morphine after both SC and IV administration. Oral nalmefene (0.4 mg/kg) antagonized this dose of morphine 3 hours after administration. In contrast, oral naloxone (up to 5 mg/kg) was inactive.

TABLE 5. *Narcotic antagonist activity of nalmefene and naloxone*

Compound	Procedure	AD50* (mg/kg)	Time**
Nalmefene	Mouse tail flick	0.004 SC	20 min
Naloxone	Mouse tail flick	0.03 SC	20 min
Nalmefene	Mouse tail flick	0.2 PO	20 min
Nalmefene	Rat tail flick	0.008 IV	15 min
Naloxone	Rat tail flick	0.016 IV	15 min
Nalmefene	Rat tail flick	0.4 PO	3 hr
Naloxone	Rat tail flick	Inactive at 5.0 PO	3 hr

*Dose to get 50 percent antagonism of an ED80 dose of morphine
**Time from administration of narcotic antagonist.

In clinical studies, nalmefene was shown to prevent respiratory depression induced by fentanyl in a dose-dependent manner (figure 1) (Gal and Difazio 1986). In another study, nalmefene was compared to naloxone and placebo in reversing the effects of meperidine (figure 2) (Barsan et al., 1989). Nalmefene was shown to be effective through the course of the study (4 hours), while naloxone, after about 45 minutes, was no longer significantly different from placebo.

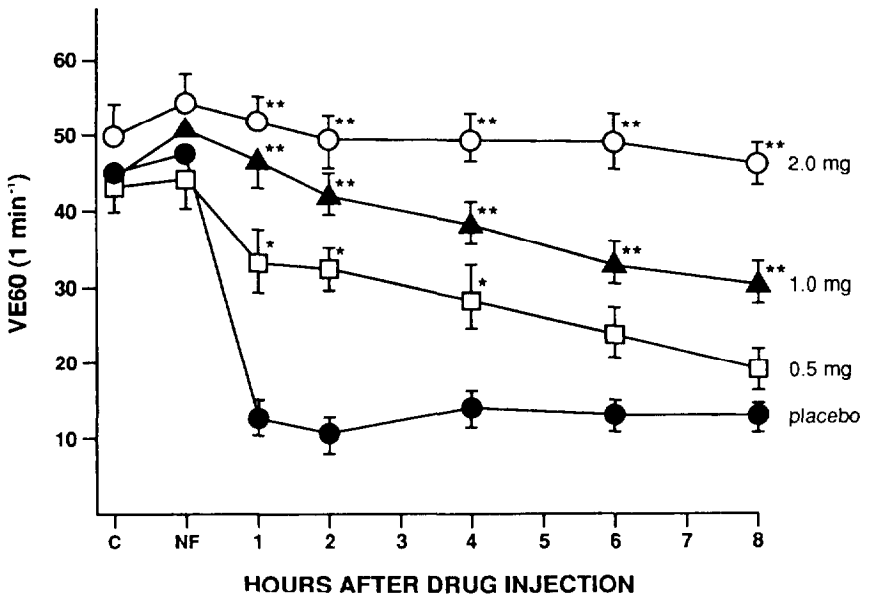

FIGURE 1. *Nalmefene prevents respiratory depression by fentanyl*

*p<.05, significant difference from placebo treatment.
**p<.01, significant difference from placebo treatment.

NOTE: Control (C) values for VEKI (Mean ± SEM) after placebo or nalmefene (NF) pretreatment and fentanyl challenge (2 µg/kg) 1, 2, 3, 6, and 8 hours later.

The bioavailability of nalmefene after oral administration was compared using tablets relative to an oral solution (figure 3) (Baker Cummins Pharmaceuticals' nalmefene information for investigators). Single 50-mg doses of nalmefene HCI were administered to six normal subjects as five 10-mg tablets or as a 50-mg oral solution with a l-week washout period between treatments. Blood samples were collected periodically for 48 hours, and the plasma concentrations of intact nalmefene were quantitated. Statistical analysis of biopharmaceutical parameters, such as maximum plasma

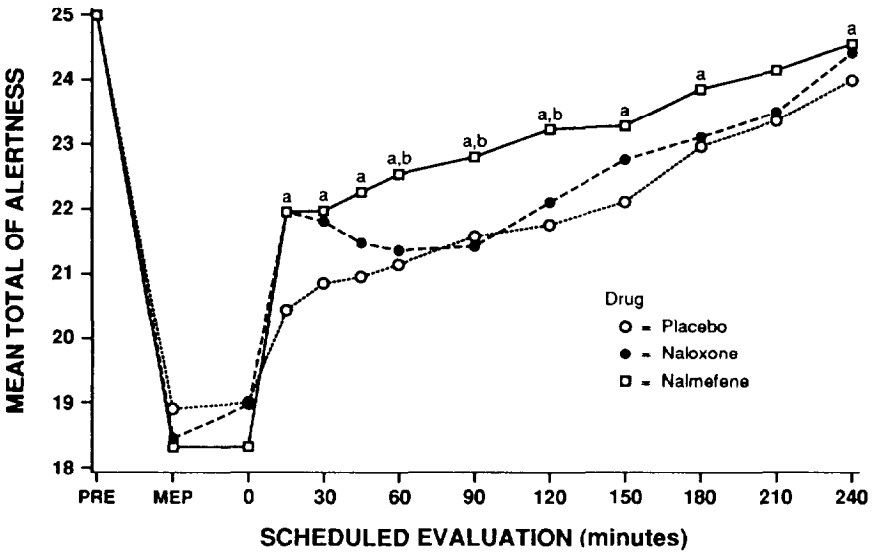

FIGURE 2. *Opiate antagonism of meperidine by naloxone and naltnefene*

[a]p<.05 nalmefene vs. placebo.
[b]p<.05 nalmefene vs. naloxone.

concentration (C_{max}), time to reach maximum plasma concentration (T_{max}), area under the plasma concentration-time curve (AUC), and elimination half-life (t 1/2), indicated bioequivalency of the tablets and solution.

The harmonic mean half-life of nalmefene following administration of the tablet was 8.3 hours; the corresponding value following the solution was 8.8 hours. These values are in close agreement with the terminal elimination half-life of nalmefene observed following IV administration. The absolute bioavailability of orally administered nalmefene has been estimated at about 40 to 50 percent by comparison of the AUCs following both IV and oral administration of the drug to four subjects.

With the discovery of endogenous opioids (Hughes 1975; Pastemak et al. 1975; Terenius and Wahlstrom 1975; Cox et al. 1975), the potential indications for the use of pure opiate antagonists has expanded dramatically. Listed below are some of the clinical uses that have been investigated and reported. A number of reviews describing these studies have been published (Olson et al. 1986; Zadina et al. 1986).

Parenteral:

(1) Anesthesia and opiate-overdose reversal
(2) Central nervous system (CNS) trauma
(3) Hypotension
(4) Cardiovascular

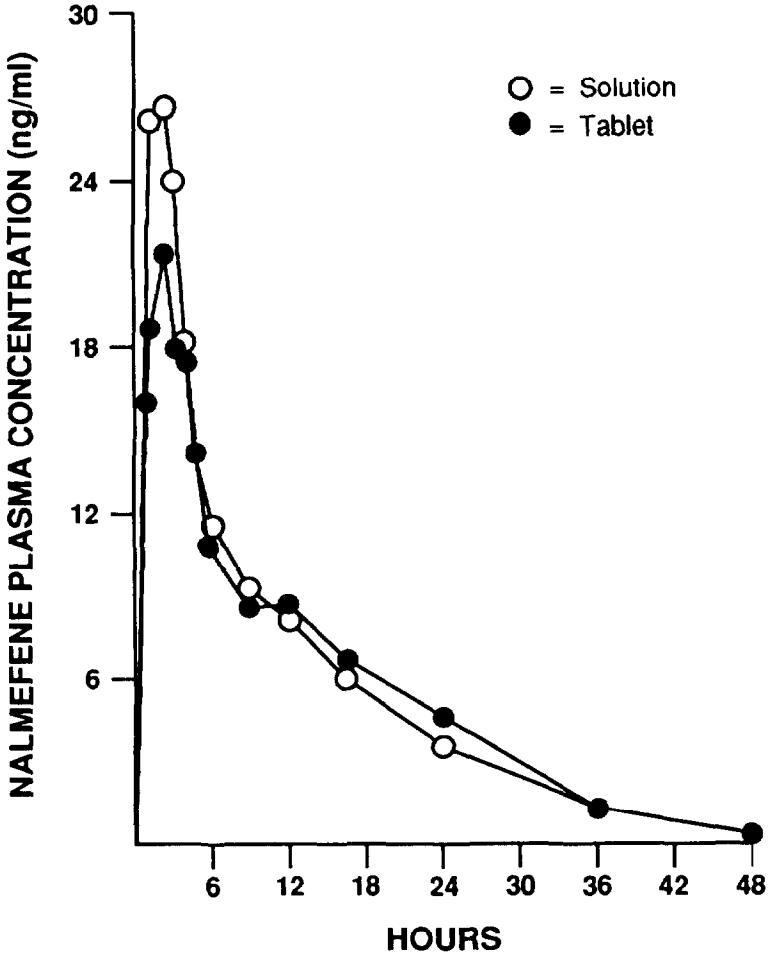

FIGURE 3. *Mean nalmefene plasma concentrations after administration of solution or tablet to healthy humans (n=6)*

Oral:

(1) Puritus
(2) Gastrointestinal—reversal of constipation
(3) Compulsive behaviors (anorexia nervosa, eating disorders)
(4) Premenstrual syndrome (PMS)
(5) Fatigue
(6) Antineoplastic activity
(7) Endocrine disorders
(8) AIDS—immune modulator

The interest in pure opiate antagonists as immune modulators stems from the numerous reports that show an impact of opioid peptides and exogenous opiates on the immune system. Table 6 lists some of the reports that support an interaction between the endogenous opioid and immune systems. Table 7 shows that at least some of these effects involve mediation by opioid receptors, since they are blocked or reversed by opioid antagonists. It should be noted that neither table 6 nor table 7 is meant to be all inclusive.

The recent interest in the use of opiate antagonists as agents for the treatment of AIDS stems from the report by Bihari et al. (1987) in which naltrexone (1.75 mg, four times a day) was administered to AIDS patients in a double-blind placebo-controlled study. After 3 months, the naltrexone group showed a significant drop in pathologically elevated serum alpha-interferon levels compared to controls, and this correlated with a lesser incidence of opportunistic infections. At this time, all patients including controls were given naltrexone and monitored for 12 months. Approximately 60 percent of the patients responded and showed decreases in serum alpha-interferon levels. Of the nonresponders, 87 percent died within 9 months, while only 17 percent of the responders died within 12 months, and 26 percent died within 18 months. No additional clinical studies using opiate antagonists as anti-HIV agents have been reported since the report by Bihari and coworkers (1987).

In conjunction with the National Cancer Institute Developmental Therapeutics Program, the author has evaluated the anti-HIV activity of nalmefene using *in vitro* assays. Nalmefene was inactive in both the MT-2 and CEM-V cell lines at concentrations up to $1.25X10^2$ μg/ml.

In studies carried out at Dupont Corporation (Dr. Ii. Tabor, personal communication), the effect of naltrexone on various immune responses was examined in both *in vitro* and *in vivo* assays. Nallrexone (0.012 to 100 μg/ml) had no effect *in vitro* on the proliferation of splenocytes in response to phytohemagglutin (PHA). Similarly, activation of tumoricidal

TABLE 6. *Evidence for an interaction between the endogenous opioid and immune systems*

(1) Normal human T lymphocytes possess receptors for met-enkephalin and beta-endorphin (Wybran et al. 1979; Hazum et al. 1979).

(2) Human phagocytic leukocytes contain opiate receptors (Lopker et al. 1980).

(3) Enkephalins prolong survival time of mice exposed to tumor cells (Plotnikoff 1982).

(4) Met-enkephalin increases active T-cell rosette-forming cells in peripheral blood lymphocytes (Miller et al. 1983).

(5) Enkephalins inhibit T-dependent antibody responses *In Vitro* (Johnson et al. 1982).

(6) Enkephalins or beta-endotphin enhance human peripheral blood lymphocyte (PBL) natural killer (NK) cell activity (Faith et al. 1984; Kay et al. 1984; Kay et al. 1987).

(7) Met-enkephalin enhances T cells in patients with Kaposi's sarcoma (Plotnikoff et al. 1986).

(8) Met-enkephalin temporarily enhances selected immune responses in HIV-infected patients (Zunich and Kirkpatrick 1988).

TABLE 7. *Impact of opioid antagonists on immune system functions*

(1) Naloxone reverses effect of enkephalins and beta-endorphin on PBL NK cell activity (Kay et al. 1984).

(2) Naloxone blocks beta-endorphin and met-enkephalin enhancement of cytotoxic T-cell generation (Carr and Klimpel 1986).

(3) Naloxone blocks alpha-interferon-induced changes in hypothalamic neuronal activity (Nakashima et al. 1987).

(4) Naltrexone has a dose-dependent stimulatory or inhibitory effect on neuroblastoma growth (Zagon and McLaughlin 1983).

(5) Naloxone binds to human T lymphocytes (Madden et al. 1987).

macrophages was unaffected by naltrexone (0.01 to 10 μg/ml). The generation of lymphokine-activated killer (LAK) cells in the presence of IL-2(1U/ml) was unchanged by naltrexone (0.01 to 10 μg/ml). *In vivo* studies in mice showed a lack of effect of naltrexone (25 and 100 mg/kg) on NKcell activity. In the tumor cell-clearance assay, naltrexone (0.01 to 10 mg/kg) was ineffective in changing the ratio in treated or control animals. Finally, no change in ear swelling was observed after naltrexone (0.01 to 10 mg/kg) in the assay that measures delayed-type hypersensitivity.

Taken together, the data are not suggestive of a beneficial effect resulting from the use of opiate antagonists in the treatment of AIDS. Although intriguing, the study by Bihari et al. (1987) needs to be repeated under more controlled conditions, since it is not clear that decreases in serum alpha-interferon levels are indeed beneficial to the patient. In fact, studies are currently ongoing in which alpha-interferon is administered in a regimen with AZT to reduce the toxic side effects of the latter drug.

Recently the author has prepared a series of compounds that are either homo- or heterodimers of specific dideoxynucleosides and elevated their activity against HIV. The phosphate-linked dimers of the dideoxynucleosides are of the general formula shown in figure 4, where R^1 and R^2 may be AZT, ddA, or ddI, and R^3 may be hydrogen, cyanoethyl, or either a metal anion or organic anion salt. The substituents R^1 and R^2 may either be the same or be derived from different dideoxynucleosidcs to yield phosphate dimers that are either homo or hetero analogs. The scheme for the synthesis of the dimers is illustrated in figure 5, which describes the preparation of AZT-P-ddA from AZT-5'-cyanoethylphosphate (Tener 1961) and ddA using various coupling agents generated *in situ.*

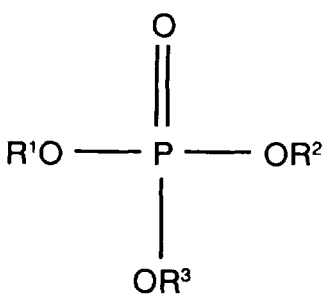

FIGURE 4. *Structure of dimers*

When the dideoxynucleosides are linked by a phosphate bridge, the dimerized compounds might be more effective agents because of the following:

(1) Two nucleosides are being delivered to the cell simultaneously.

(2) The compound contains a masked phosphate, with the masking unit also being active.

(3) The dimer could function as a prodrug.

(4) The intact dimer might also be active.

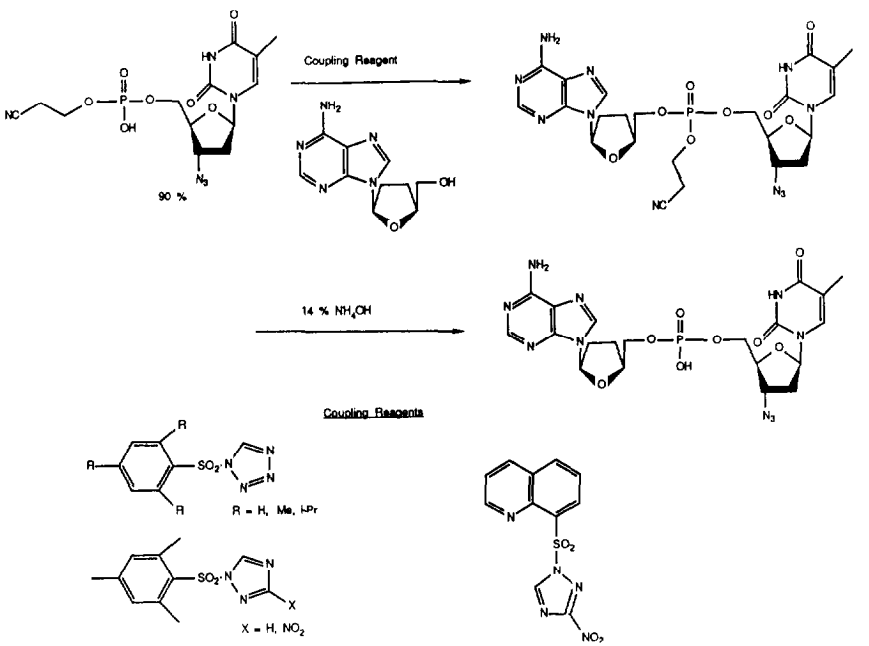

FIGURE 5. *Synthetic scheme for synthesis of dimers*

Table 8 lists the results of an assay in which the inhibition of syncytium formation is examined at various concentrations of drug. Over the concentration range examined, both AZT-P-ddA and AZT-P-ddI are at least as potent as AZT. Table 9 (Busso et al. 1988) shows the results of studies in which the cytotoxic effect (ID50) of specific compounds is measured so that a cytotherapeutic index (CTI=ID50/ED50) may be generated. Greater CTI values than that of AZT are indicative of drugs that are either less toxic to cells or more potent. The data (table 9) show that both AZT-P-ddI and

AZT-P-ddA have larger CTI values than AZT, a result that is due to their being considerably less toxic to the cells.

TABLE 8. *Primary screen results*

Drug	Percent Inhibition of Syncytia Formation						
	100 µg/ml	10 µg/ml	5 µg/ml	1 µg/ml	0.5 µg/ml	0.1 µg/ml	0.01 µg/ml
AZT-P-ddA	100	100	100	96	96	64	0
AZT	100	100	100	100	100	57	0
AZT-P(CyE)ddA	100	100	100	72	58	0	0
AZT-P-ddI	100	100	100	100	98	76	0
ddA	100	100	100	93	87	42	0

NOTE: MT-2 cells were incubated with HIV (TM) and then resuspended to a final concentration of $3X10^5$ cells/ml (96-well microtiter plate) with an MOI of 0.001. The drug was added after the cells were transferred to the wells. On day 4, the number of syncytia per well was compared to controls. Results are the mean of quadruplicate cultures (one experiment).

TABLE 9. *Comparison of the cytotherapeutic index of specific dimers*

Drug	ID50* (mg/ml)	ED50 (mg/ml)	CTI
AZT-P-ddA**	>100	0.08	>1,250
AZT	75	0.06	1,250
AZT-P(CyE)ddA**	>100	0.6	>150
AZT-P-ddI**	>100	0.05	>2,000
ddA	75	0.1	750

*MT-2 cells ($2X10^5$ cells/ml) were seeded with 100 µg/ml or 50 µg/ml of drug. Every 3 to 4 days, media changes were performed and fresh drug added. On day 14, cell counts were performed (trypan blue exclusion method). Results are the mean of two replicates per concentration.
**No evidence of cytotoxicity at 100 µg/ml.

Reverse transcription (RT) has been shown to be involved in the replication of HIV (Mitsuya et al. 1987). The inhibition of RT by various dimers is shown in table 10. AZT-P-ddI at a concentration of 1 µg/ml inhibited the enzyme through at least 11 days, whereas AZX at 1 µg/ml was effective at 7 days but not at 11 days. In this assay, the activity of AZT-P-ddA was comparable to that of AZT.

In other studies, the author examined the uptake of radiolabeled AZT-P(CyE)ddA into H-9 cells. After incubation for 24 hours at 37 °C, the cells were separated from the medium by the centrifugation. and the super-

natant was decanted. After washing and extraction with 70 percent methanol, the evaporated cellular extract was purified on silica gel, and bands that coeluted with authentic standards of AZT-P-(CyE)ddA and AZT-P-ddA were removed, extracted, and counted in a scintillation counter. The results showed that 7 percent of the incorporated radioactivity corresponded to AZT-P-(CyE)ddA, and 20 percent corresponded to AZT-P-ddA. The data suggest that at least some of the dimer enters the cell intact and that AZT-P(CyE)ddA is acting as a prodrug for AZT-P-ddA.

TABLE 10. *Inhibition of reverse transcriptase activity**

Drug	Evaluation Period (Days)		
	7	11	14
Control (No Drug)	11,000	38,000	34,000
ddA (10 μM)	500	3,000	4,500
ddA (1 μM)	5,500	45,000	39,500
AZT-P-ddA (5 μg/ml)	500	500	500
AZT-P-ddA (1 μg/ml)	1,500	12,000	26,500
AZT (1 μg/ml)	1,500	12,000	23,500
AZT (0.5 μg/ml)	2,000	16,500	28,000
AZT-P(CyE)-ddA (5 μg/ml)	1,000	4,500	12,000
AZT-P(CyE)-ddA (1 μg/ml)	6,000	43,000	36,000
AZT-P-ddI (5 μg/ml)	1,000	1,500	25,000
AZT-P-ddI (1 μg/ml)	1,000	3,000	10,000

*Target cells=H9 cells. MOI=1.0. Media changes with the addition of fresh drug were performed at 3- to 4-day intervals. Results are expressed as CPM-RT activity per ml in culture supernatant. CPM>5,000 signifies abnormal elevations in RT activity. The results represent the mean of duplicate studies in one experiment.

The stability of AZT-P-ddA as a function of temperature was examined in human plasma (figure 6) (Hahn et al. 1989). At 37 °C, approximately 10 percent of drug was metabolized per hour. This metabolism was found to be species dependent (figure 7) (Hahn et al. 1989). Analysis of the nature of the metabolites by HPLC showed that only AZT and ddI were formed.

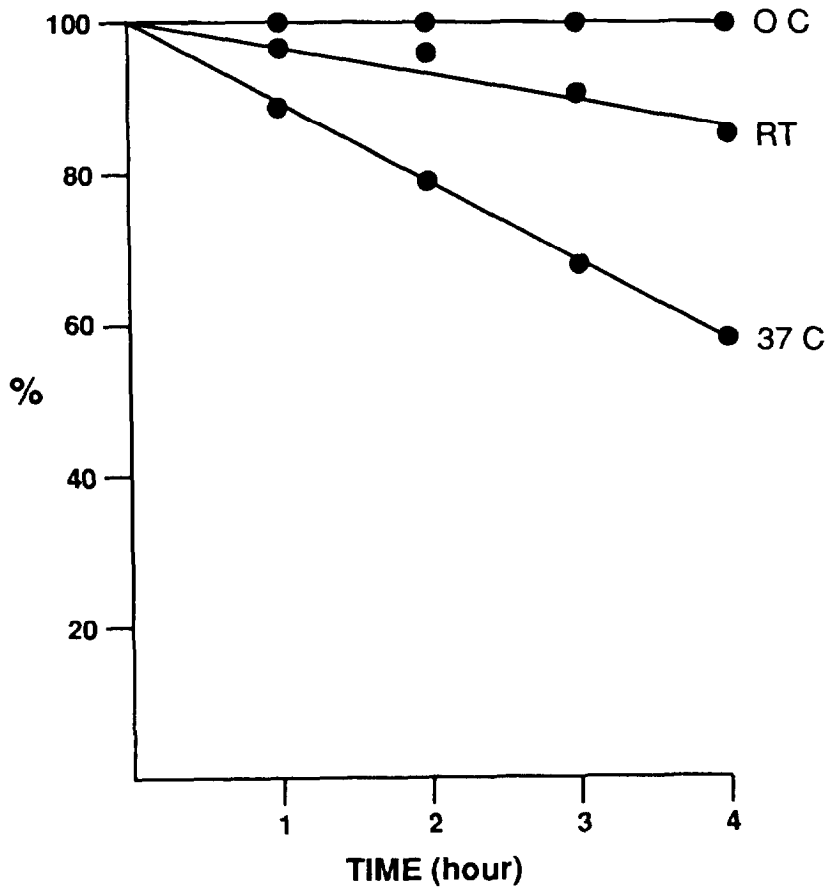

FIGURE 6. *Stability of AZT-ddA in human plasma (in vitro) at various temperatures (4 μg/ml)*

SOURCE: Hahn et al. 1989, copyright 1989, American Chemical Society

Figure 8 shows the distribution of radioactivity in serum and cells at various times following IV injection of [^{3}H]-AZT-P-ddA (5 mg/kg) into rats. After 1 hour, about 7.8 percent of the injected dose is found in red blood cells compared to 4.2 percent in the serum. This disproportionate uptake into cells is no longer seen at 2 and 3 hours after injection, at which time the serum contains approximately twice as much radiolabel when compared to cells. The nature of the radioactivity found in the kidney at 3 hours

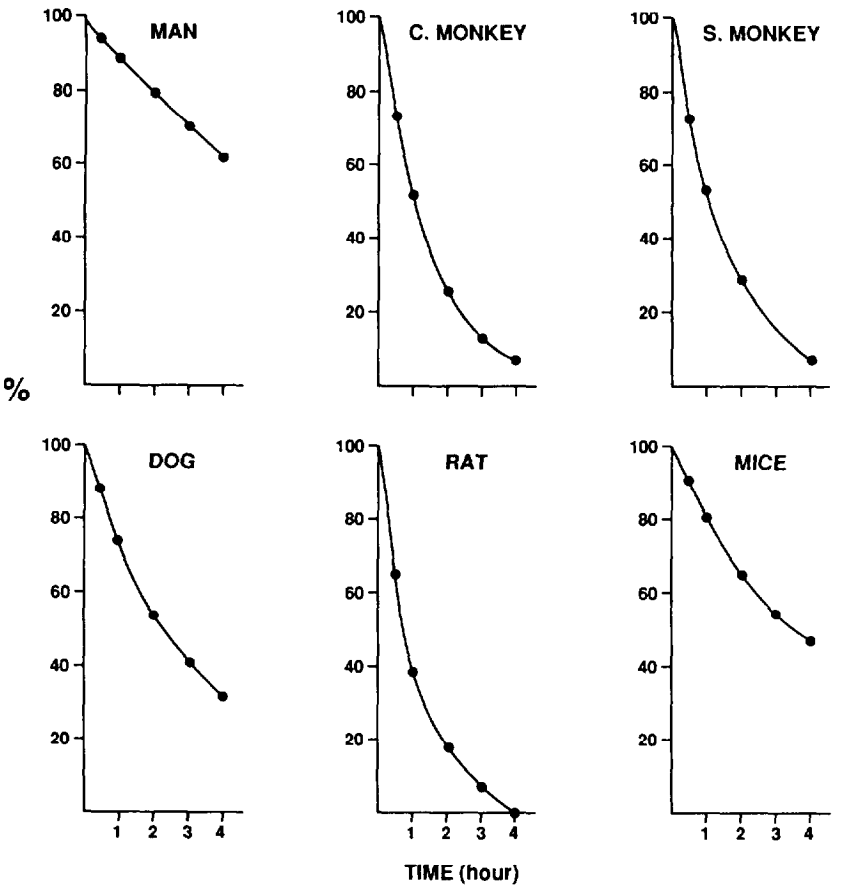

FIGURE 7. *Stability of AZT-ddA in plasma of various species at 37 °C (4 μg/ml)*

SOURCE: Hahn et al 1989, copyright 1989, American Chemical Society.

after administration was examined. After sacrifice, kidneys were removed, homogenized, and extracted. The residue after evaporation of the solvent was analyzed by radioscans of thin-layer chromatography plates developed in two different systems. The scans showed that the principal compound present was AZT-P-ddA, indicating that intact dimer was still found in tissue 3 hours after administration of the drug.

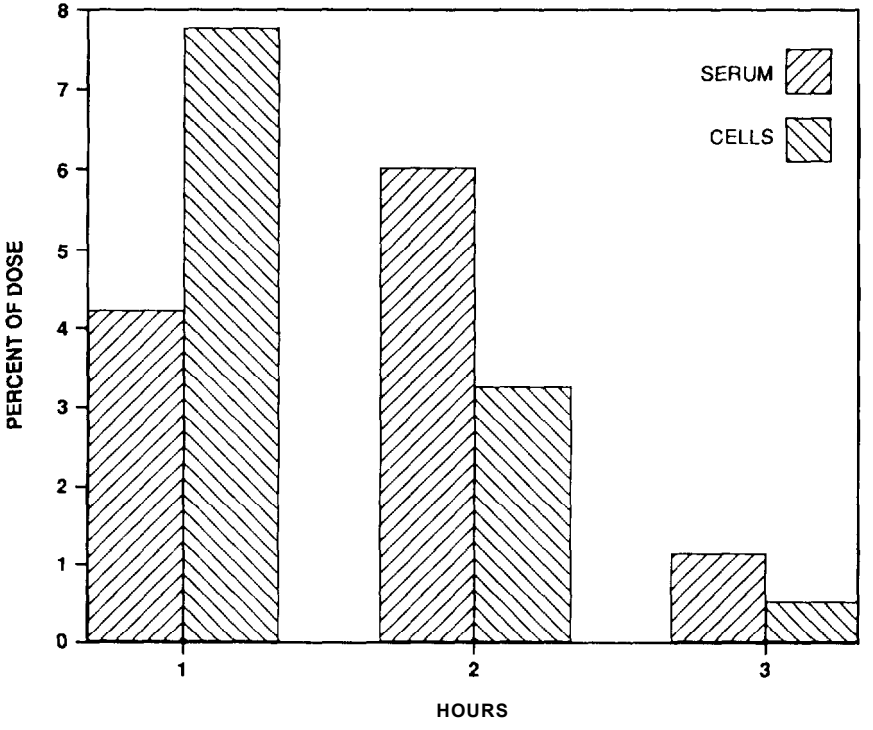

FIGURE 8. *[^{3}H]-Labeled material in serum and cells following IV injection of [^{3}H]-AZT-P-ddA (5 mg/kg) into rats*

In conclusion, a series of dimers of dideoxynucleosides linked by a phosphorous bridge has been synthesized. The results that have been obtained show the following:

(1) The cytotherapeutic index of some of the dimers is better than the corresponding value for AZT.

(2) The intact dimeric compounds exhibit cellular uptake.(3)The dimers are bioavailable after *in vivo* administration to animals.

(4) Since the dimers are not metabolized in a manner comparable to the individual nucleoside components, it is conceivable that additional advantages may be associated with their *in vivo* administration. Additionally, as an intact compound, both components are present in the cell simultaneously and may provide an added therapeutic effect.

(5) Since at least some of the administered drug enters the cell intact, a possible new mechanism of action is suggested.

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Classical and Nonclassical Cannabinoids: Mechanism of Action—Brain Binding

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INTRODUCTION

Following characterization and publication by Gaoni and Mechoulam (1964) of the structure of delta-9-tetrahydrocannabinol (delta-9-THC), researchers enthusiastically pursued new therapeutants for 17 years, based on this active principle from marijuana. These research efforts culminated with the marketing of delta-9-THC (Marinol) by Unimed and nabilone (Cesamet) by Lilly and clinical investigation of levonantradol from Pfizer and nabitan from SISA (figure 1). Delta-9-THC and nabilone are approved as an antiemetic for cancer patients being treated with chemotherapy. Levonantradol was studied both as an antiemetic and an analgetic, and nabitan was studied as an analgetic. This chapter will briefly review the research at Pfizer, Inc. related to discovery of the synthetic cannabinoid CP-55,940 as an analgetic in rodents and continuing research at St. Louis University Medical School responsible for the discovery of a mechanism of action and brain-binding site for this compound.

DISCUSSION

At the outset of these research efforts, two key points of information in the cannabinoid literature provided a basis for the discovery of a unique class of nonclassical cannabinoids. First, 9-nor-9-beta-hydroxyhexahydrocannabinol (HHC) was prepared (Wilson and May 1975) and found to exhibit analgetic activity in rodents, with equal potency to morphine (Wilson et al. 1976; Bloom et al. 1977). These results provided a novel chemical target from which potent. nonopiate analgetics could be discovered. Second, published and presented research supported a contention that significant biological activity was present in naturally occurring and synthetic cannabinoids only if they contained the rigid benzopyran ring system present in

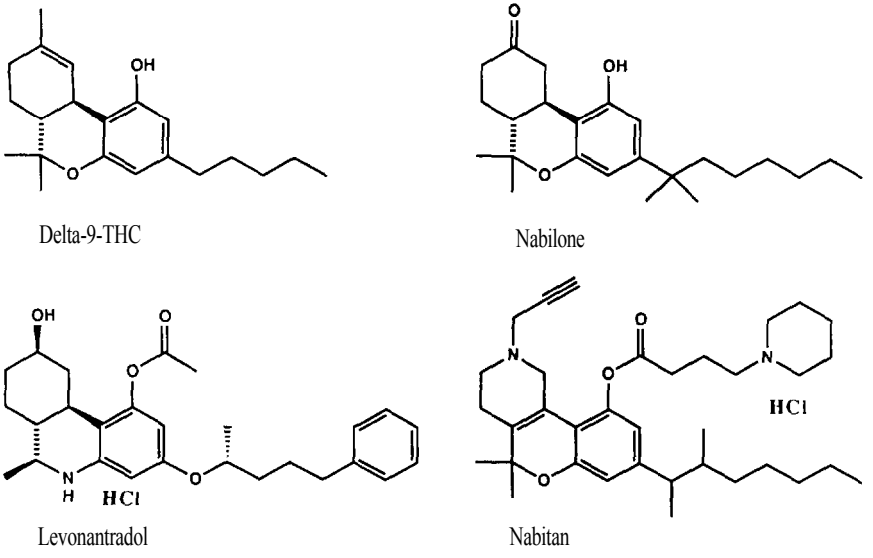


FIGURE 1. *Delta-9-tetrahydrocannabinol and derived drugs*

delta-9-THC (Mechoulam and Edery 1973; Razdan 1987; Mechoulam et al. 1987). This second point was inconsistent with several hypotheses that we had formulated to pursue a mechanism-based approach to the development of drugs based on HHC. Therefore, an early research objective was to directly challenge the need for incorporation of a benzopyran ring in cannabinoid-based drugs. An additional benefit from this structure-activity relationship (SAR) test was that it allowed synthesis of a greater diversity of structurally novel compounds than may have been anticipated from previous studies. This point was also necessarily important for the successful discovery and development of a drug based on HHC. Delta-9-THC and structurally related biologically active cannabinoids present a broad spectrum of activities in animals and, where tested, in man. Such a plethora of effects by a compound would usually contraindicate its use as a drug. The ultimate objective of this research was to structurally isolate the various pharmacophores present in delta-9-THC and HHC and then rebuild compounds with high specificity and potency of action. To achieve progress on this major objective, it was not fortuitous that we hypothesized that the cannabinoids must produce their biological effects on subtypes of an as-yet-unknown specific receptor system.

Initial research leading to the discovery of levonantradol provided substantial understanding of the SAR in the pyran ring portion of HHC (Johnson et al. 1981; Johnson and Melvin 1986). Using analgesia as an endpoint, it was shown that the pyran oxygen atom in HHC could be replaced by carbon,

nitrogen, or alkyl-substituted nitrogen. Equatorial substitution at C-6 was favored, but varied substitution was also found to be allowable and included just hydrogen. These results indicated the gemdimethyl pyran ring of HHC was not, in itself, a pharmacophore for analgetic activity in HHC. Nonetheless, it was still possible that the rigidity of the benzopyran ring was responsible for a favorable three-dimensional interaction of more distal functionality (alcohol, phenol, C-3 chain) with a receptor. This possibility was tested with the synthesis of structure *1,* lacking the pyran ring of HHC (Melvin et al. 1984a) (figure 2). Compound *1* also incorporated the 1,1-dimethylheptyl C-3 chain, previously used in nabilone (Archer et al. 1977; Archer et al. 1986) and developed in early work (Adams et al. 1949; Loev et al. 1973), which was expected to provide optimal potency. The phenylpentyloxy C-3 chain of levonantradol and similar side chains were equally suitable for use in structures such as *1*. Analgetically, *1* was more potent than HHC and equipotent with morphine. In fact, molecular modeling had predicted a low-energy conformation of *1* in which the spatial arrangement of the alcohol, phenol, and C-3 chain of HHC and *1* were superimposable. This initial result confirmed the original proposal that the pyran ring of HHC was not a necessary pharmacophore in cannabinoids and that any molecular rigidifying effects it provided were surmountable.

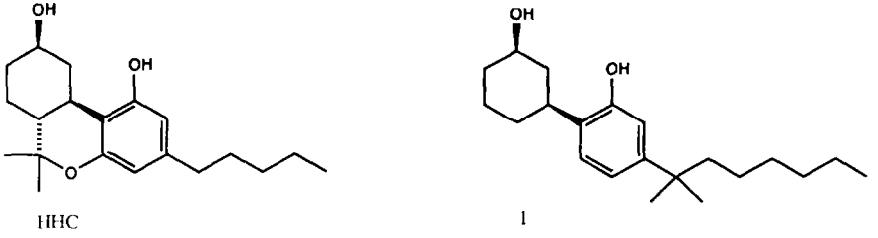


FIGURE 2. *Comparison of HHC and compound* 1

Broad SAR studies of *1* guided in part by an earlier and now less attractive proposal of a prostaglandin mechanism of action (Milne and Johnson 1981), led to the synthesis and extensive study of 2 (CP-55,940), a single enantiomer (Johnson and Melvin 1986) (figure 3). Structure 2 differs from *1* and HHC by having a hydroxypropyl residue in the space previously occupied by the pyran ring of HHC. Nuclear magnetic resonance (NMR) data confirmed that both the hydroxypropyl group and aryl-cyclohexyl bond are freely rotating. Compound 2 was shown to have significantly enhanced analgetic potency with respect to morphine (6 to 37 times as potent) and to be equipotent with levonantradol. Similar to levonantradol and desacctyllevonantradol, the analgetic activity of 2 was not blocked by naloxone

(McIlhenny et al. 1981; Yaksh 1981). The nonclassical cannabinoids, including 2, exhibit varying degrees of cannabimimetic activity, in addition to analgetic and antiemetic effects (Weissman et al. 1982; Koe et al. 1985; Little et al. 1988). The enhanced analgetic effect of 2 over *1* was attributed to the introduction of a potential new receptor binding element (hydroxypropyl), which interacted with a previously unutilized site at the proposed cannabinoid analgetic receptor. To gain support for the identification of a new binding element, compound 2 was modified in various ways to induce stable conformations of the hydroxypropyl unit. It was argued that such a conformationally restricted binding element would provide maximum biological potency when matched to its binding site at the receptor.

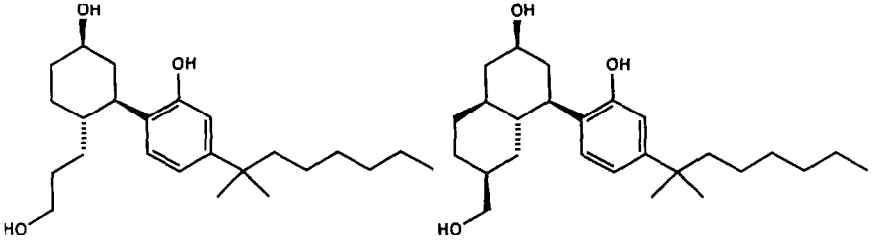


FIGURE 3. *Compound* 2 *(CP-55,940) and the rigid derivative* 3 *(CP-55,244)*

Ultimately, a conformationally restricted derivative of 2 was synthesized with a hydroxymethyl-containing decalin ring (Johnson and Melvin 1986). Compound 3 (CP-55,244), a single enantiomer, showed enhanced analgetic potency (2 to 13 times) over 2 and a strong stereochemical preference for an axial vs. equatorial hydroxymethyl group (31 to 53 times) (figure 3). The enantiospecificity of analgetic activity for 3 was dramatically increased to greater than 5000 times vs. 209 times for 2 and 3 times for *1,* implying a very specific receptor fit. These findings supported the conclusion that the hydroxypropyl functionality on 2 was a significant new contributor to receptor binding.

The less rigid bicyclic cannabinoid prototype illustrated by compound *1* was extended to two other interesting modifications. SP-1, the active form of the prodrug nabitan (Razdan et al 1976; Razdan and Howes 1983), possesses a unique N-propargyl group in place of the alcohol in 6a, 10a-dehydro HHC and utilizes a 1,2-dimethylheptyl C-3 chain. Removal of the pyran ring in SP-1 provided compound 4, which was still equipotent with HHC as an analgetic but lost potency on some endpoints vs. SP-1 (1 to 8 times) (figure 4). Optimum timing for drug administration was not taken into

consideration. A similar relationship also held true for the fully saturated piperidine ring derivative of 4 (Melvin et al. 1984b).

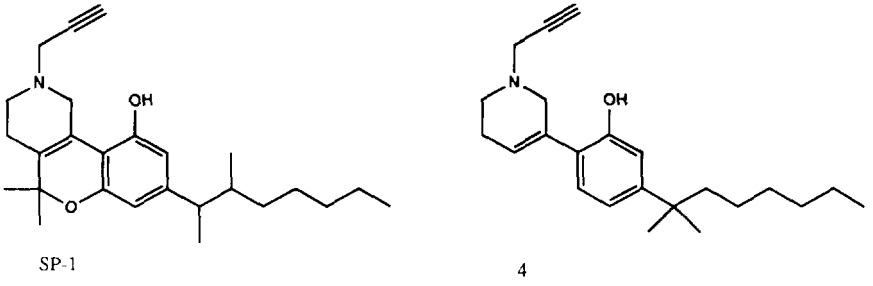
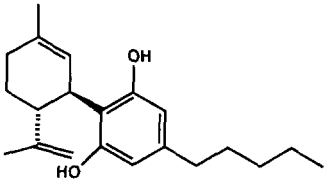


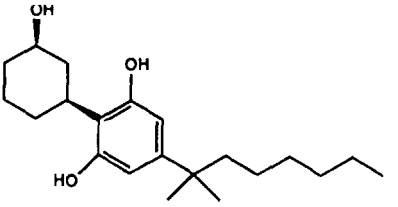
FIGURE 4. *Relationship of SP-1 and compound* 4

Cannabidiol (CBD) is the natural pyran-ring-opened derivative of delta-9-THC (Mechoulam and Shvo 1963). The pharmacology of CBD has been broadly studied, showing that it lacks analgetic activity and is a relatively weak cannabimimetic (Mechoulam et al. 1970; Dewey 1986; Razdan 1986; Consroe and Snider 1986). We speculated that the inactivity of cannabidiol was a result of an unfavorable steric interaction between the bulky isopropenyl group and a phenolic hydroxyl. Unlike *1, 2,* or *3.* cannabidiol cannot readily adopt a conformation that would overlap its vinyl methyl (possibly oxidized to hydroxymethyl), phenol, and C-3 chain with those of delta-9-THC. Since it was already shown that the pyran ring was not necessary for biological activity, it seemed unlikely that the simple presence of a second phenol in cannabidiol would be so deleterious. Syntheses of compound *5* would test this idea, since *5* contains both phenols of cannabidiol but lacks the isopropenyl group (figure 5). As predicted, compound *5* and its axial alcohol isomer exhibited analgetic activity comparable to that of *1* and its isomer (Melvin et al. 1984a).

With support and direction from accumulating research data, biological studies were initiated to investigate the mechanism of action for the analgetic synthetic cannabinoids. Earlier studies had demonstrated that active cannabinoids, in particular delta-9-THC and desacetyllevonantradol, were reversible inhibitors of adenylate cyclase activity in membranes from neuroblastoma cells (N18TG2) (Howlett 1984; Howlett 1985; Howlett 1987).



Cannabidiol



5

FIGURE 5. *Relationship of cannanidiol and diphenol 5*

Table 1 demonstrates the potent inhibition of adenylate cyclase and the high degree of enantiospecificity of the nonclassical cannabinoids and levonantradol (Howlett et al. 1988). The inhibition of neuroblastoma adenylate cyclase is exhibited by all analgetically active cannabinoids with a significant degree of correlation for potency, enantiospecificity and stereospecificity. Further work supports a conclusion that actual enzyme inhibition by cannabinoids occurs via interaction with a Gi protein complex (Howlett et al. 1986). The receptor mechanism whereby cannabinoids inhibit adenylate cyclase is distinct from that by which the opiates act (Devane et al. 1986). It is tentatively concluded that the mechanism whereby cannabinoids exhibit *in vivo* analgetic activity is identical to their *in vitro* inhibition of adenylate cyclase (Howlett et al. 1988).

TABLE 1. *Inhibition of adenylate cyclase activity* in vitro

Compound	K_{inh} (nM)* (-) Enantiomer**	(+) Enantiomer**
1	79	135
2	25	>5,000
3	5	>10,000
Levonantradol	100	>5,000
Desacetyllevonantradol	7	—
Delta-9-THC	430	—

*Inhibition of secretin-stimulated activity. Data is fmm a mean of at least three determinations.
**In all cases studied to date, the (-) rotation has been exhibited by the enantiomer with absolute configuration 6aR,10aR (using HHC as an example) and this is the absolute configuration in which all structures in this manuscript are drawn.

The original premise driving research to segregate the various biological activities of cannabinoids, i.e. that the effects of cannabinoids are the result of direct interaction with a discrete receptor(s), was initially pursued in rat brain (Devane et al. 1988). A potent, stable cannabinoid radioligand was needed for this research. Compound 2 (CP-55,940) was chosen and tritium labeled in the C-3 chain by catalytic reduction of the olefinic precursor 6 (figure 6) (Devane et al. 1987).

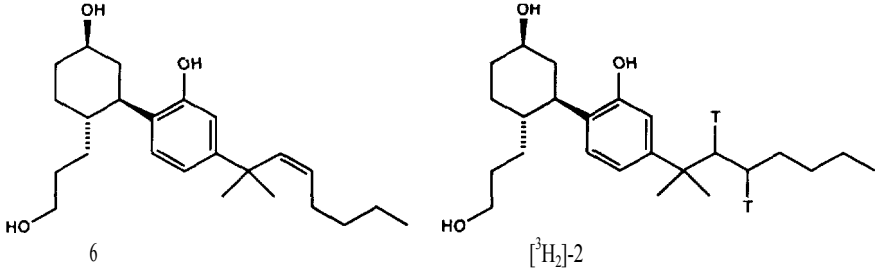


FIGURE 6. *Precursor 6 and tritium-labeled 2*

Specific binding in rat brain to cortical P2 membranes and synaptosomes from hippocampus plus prefrontal cortex was characterized with tritiated *2*. Compound 2 binds to the rate P2 membrane preparation with a Ki=133±11 pM and a Bmax of 1.85 pmol/mg of protein. Desacetyllevonantradol exhibits a binding Ki=123±34 pM, while delta-9-THC is approximately tenfold less potent as a ligand with Ki=1.6±0.56 nM. Conversely, the analgetically inactive cannabidiol gave less than 50 percent displacement of tritiated 2 at 1 μM.

The analgetically less active enantiomer of 2 showed a fiftyfold weaker affinity with Ki=3.4±1.9 nM. Together with unreported data and the continuing accumulation of new data, a significant correlation between analgetic potency *in vivo* and binding to the rat brain receptor *in vitro* defined by tritiated 2 has been shown. As was the case for inhibition of neuroblastoma adenylate cyclase, evidence has been obtained suggesting that the brain-receptor site is linked to a Gi protein complex (Devane et al. 1988). Table 2 summarizes the data that corroborate our conclusion that a specific brain receptor has been identified that is responsible for at least the analgetic activity of cannabinoids.

CANNABINOIDS AND THE IMMUNE SYSTEM

With respect to the possible effects of cannabinoids on the immune system, these lipophilic substances can now be evaluated more specifically for their cell receptor effects. This is important because the literature has not clearly

defined an effect of cannabinoids, particularly delta-9-THC, on the immune system of mammals. Several caveats should be considered when reviewing the reported immunological effects of marijuana or delta-9-THC. First, those effects ascribed to the inhalation route of delivery of delta-9-THC from marijuana are not usually controlled for effects resulting from the simple act of smoking a marijuana or generic cigarette. Second, when evaluating a user population of marijuana, it is difficult to control for the use of other potentially immuno-affecting substanas such as tobacco, alcohol, and other drugs of abuse. Finally *in vitro* effects on cells are difficult to assess because measurements are frequently conducted under nonphysiological conditions of drug and protein concentrations. Several recent reviews summarize research on the potential effects of marijuana on the immune system (Friedman et al. 1988; Yahya and Watson 1987; Kawakami et al. 1988; Murison et al. 1987; Mishkin and Cabral 1987; Press et al. 1987).

TABLE 2. *Fulfilled requirements for a cannabinoid drug-receptor site interaction*

Receptor Binding of 2 (CP-55,940) to Rat Brain*	
High Affinity	K=133 pM
Rapid Equilibrium	<45 min
Reversible/Saturable	100%/100%
Enantioselective	(-)/(+)=0.02
Functional Activity and Correlation with Analgesia	
High Potency	µg/kg
Enantioselective	(-)/(+)=0.004
SAR Correlatable With Binding	R=0.9

*Assay uses [^{3}H]-2 (CP-55,940) of specific activity ≥ 50Ci/mmole.

CONCLUSION

Utilizing a nonclassical cannabinoid analgetic as a radioligand, a specific receptor was identified in rat brain. *In vitro* binding of cannabinoids to this receptor and *in vivo* rodent analgetic potency correlate significantly. Additionally, analgetic cannabinoids inhibit neuroblastoma adenylate cyclase activity *in vitro* with a significant correlation to *in vivo* potency. Preliminary data support a further conclusion that inhibition of adenylate cyclase and brain receptor binding both occur via interaction with a single Gi protein complex.

Biological and chemical probes are now available to allow an indepth exploration of the pharmacology and mechanisms of action of the cannabi-

noids. Physiologically relevant receptor subtypes can be searched for, including perhaps peripheral receptors that are associated with pain. Antagonists and mixed agonists-antagonists for these receptors can be pursued, and endogenous binding substances can now be looked for. It is anticipated that worthwhile exploitation of these results will follow in the form of action-specific drugs based on cannabinoids.

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Cocaine Receptor-Design of Ligands

F.I. Carroll, Abraham Philip, and Anita H. Lewin

INTRODUCTION

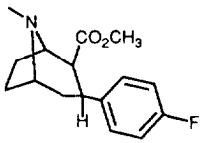
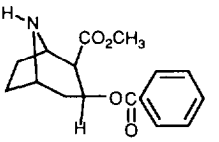
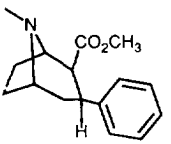
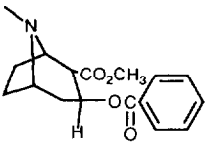
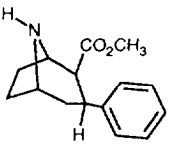
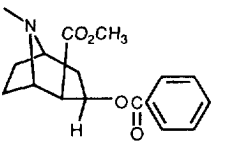
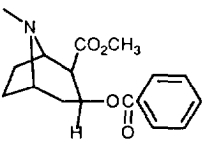
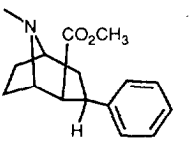
Abuse of cocaine, an alkaloid obtained from the leaves of *Erythroxylan coca,* has grown from a relatively minor problem a few years ago to a major U.S. health problem today (Grabowski 1984; Clouet et al. 1988). Concern is rising, especially over the much more potent effects obtained by the growing fashion of smoking the alkaloid itself as free base (crack). Since cocaine may affect immune system function (Watson et al. 1983; Van Dyke et al. 1986; Donahoe et al. 1986; Havas et al. 1987; Klein et al. 1988) and can be toxic to the point of being lethal (Clouet et al. 1988), studies directed toward gaining information about the mechanism of cocaine action and toxicity are of importance.

POSSIBLE BASIS FOR REINFORCING PROPERTIES OF COCAINE

Cocaine is a psychomotor stimulant with rewarding or reinforcing properties. Since cocaine has been shown to inhibit the transport of dopamine, norepinephrine, and serotonin (Coyle and Snyder 1969; Javitch et al. 1984; Ross and Renyi 1969; Blackburn et al. 1967; Horn et al. 1974), any one or a combination of these binding sites could presumably mediate the effects that are related to the abuse of cocaine. However, the pharmacologically relevant receptor must possess a significant correlation between potency of drugs in producing a response and in binding to the recognition site. Recently Kuhar et al. (1988) and Ritz et al. (1987) reported that ligand binding affinity to the ^{3}H-mazindol binding site on the dopamine transporter correlated with the reinforcing properties of cocaine and related drugs. In contrast. no correlation was found for binding sites on other monoamine transporters. In general, these workers found that compounds potent in self-administration studies were also potent inhibitors of binding at the transport site for dopamine. Conversely, compounds that were weak in self-administration studies were also weak inhibitors at the dopamine binding site. The study included compounds structurally similar to cocaine (table 1)

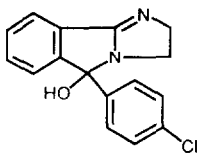
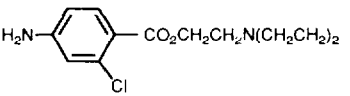
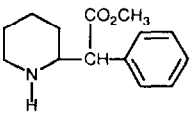
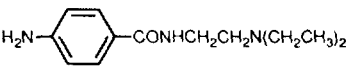
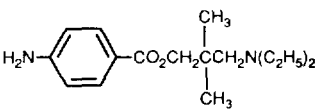
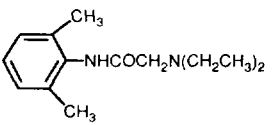
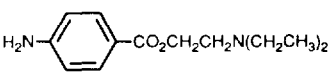
as well as other ester-linked local anesthetics and closely related psychostimulants (table 2). This information led Kuhar et al. to conclude that the dopamine transporter may be the cocaine receptor that is responsible for cocaine stimulant and reinforcing properties. Thus, cocaine euphoria would be explained on the basis of dopamine uptake blockade causing increased dopamine concentration in the synaptic cleft, leading to increased neurotransmission in the brain reward system.

TABLE 1. *Inhibition of binding at dompamine transporter: Cocaine and structurally similar compounds*

Compound	Ki (um)*	Compound	Ki (um)*
	0.17		1.21
WIN-35,428		(±)-Norcocaine	
	0.26		116
WIN-35,065-2		(+)-Pseudococaine	
	0.36		136
WIN-35,981		(+)-Cocaine	
	0.64		385
(-)-Cocaine		WIN-35,065-3	

SOURCE: Kuhar et al. 1988.

TABLE 2. *Inhibition of binding at the dopamine transporter: Local anesthetics and psychostimulants related to cocaine*

Compound	Ki (um)*	Compound	Ki (um)*
 Mazindol	0.023	 Chloroprocaine	65
 Methylphenidate	0.39	 Procainamide	1,943
 Dimethocaine	1.29	 Lidocaine	3,298
 Procaine	104		

SOURCE: Kuhar et al. 1988

The data in tables 1 and 2, while limited, do suggest some structural requirements for the receptor. An aromatic ring and an amino group are apparently required but are not sufficient for good affinity to the receptor. All the tropane structures as well as methylphenidate possess a carbomethoxy group. However, the fact that (+)-pseudococaine, (+)-cocaine, and WIN-35,065-3 show low affinity for the receptor reveals that the stereochemistry of the carbomethoxy group is important. Nevertheless, the high affinity of mazindol demonstrates that a carbomethoxy function is not a

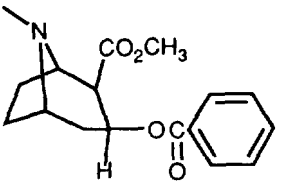
strict requirement for high affinity to the receptor. The high affinity of the 2-aryl substituted tropanes (WIN-35,428; WIN-35,065-2; and WIN-35,981) and methylphenidate shows that the receptor can accommodate aryl groups located in different positions relative to the amino group. The high affinity of methylphenidate and the comparable affinities of not-cocaine to cocaine and WIN-35,981 to WIN-35,065-2 demonstrate that a tertiary amine is not necessary for high affinity.

CONSTKUCTION OF COCAINE ANALOGS

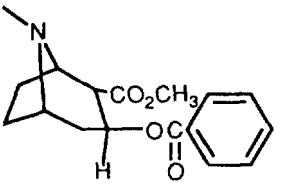
In collaboration with the Addiction Research Center (ADR) of the National Institute on Drug Abuse, the authors are conducting an SAR study to learn more about the cocaine receptor. The goal of this research is to gain information about the structural and stereochemical features required for good binding to the cocaine receptor. The scope of the study includes the design, synthesis, and receptor binding evaluation of cocaine analogs. The design and synthesis of several cocaine analogs are presented in this chapter. The receptor binding data are not available at this date and will be published later.

Structurally, natural cocaine is (l*R*,2*R*,3*S*,5*R*)-3-(benzoyloxy)-8-methyl-8-azabicyclo[3·2·1]octane-2-carboxylic acid methyl ester. There are three other possible stereoisomers of cocaine, each existing as an enantiomeric pair; all seven compounds have been prepared in the authors' laboratory (figure 1). Cocaine and two of the isomers (table 1) have been evaluated in the dopamine transport receptor binding assay (Kuhar et al. 1988). Evaluation of the other five isomers will answer the question of stereoselective binding. The cocaine analogs evaluated by Kuhar et al. (1988) have all been 2-carbomethoxytropane derivatives (table 1). In order to gain information concerning the effect of the size, volume, and oxidation state of this group on binding, compounds *1-9* listed in figure 2 have been prepared.

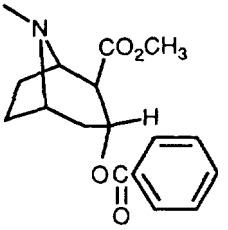
In order to obtain information about the location of the ionic/hydrogen bonding site of the cocaine receptor, it was decided to compare the binding affinities of tropacocaine (figure 3) which showed moderate binding to the dopamine transporter labeled with ^{3}H-cocaine (Reith et al. 1986) and its isomer 6-methyl-6-azabicyclo[3·2·1]octan-3 β·-ol benzoate (*10*). Molecular modeling studies were used to compare the structural features of *10* to tropacocaine. The molecular models of *10* and tropacocaine were constructed by using x-ray fragments and normal computer-supplied connections available from the SYBYL computer graphics system (Tripos Associates, St. Louis, MO 63117). The geometry was optimized with the MAXMIN2 module of SYBYL. The results, shown as an overlay of compound *10* and tropacocaine, both with endomethyl conformations (figure 4) show a high degree of similarity between the two structures, so that the effect of the position of the nitrogen on binding can be assessed.



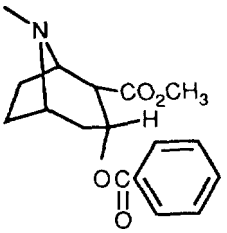
(-)-Cocaine



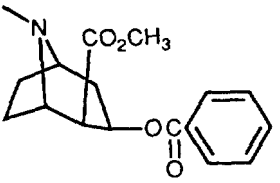
(+)-Pseudococaine



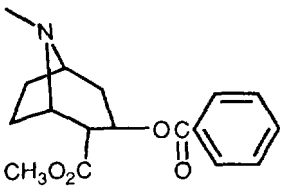
(-)-Allococaine



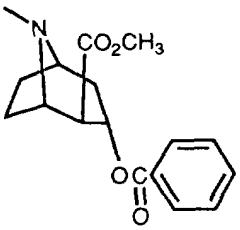
(-)-Allopseudococaine



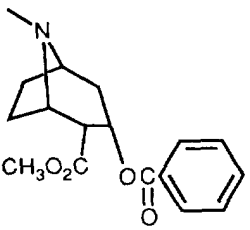
(+)-Cocaine



(-)-Pseudococaine



(+)-Allococaine



(+)-Allopseudococaine

FIGURE 1. *Stereoisomers of cocaine*

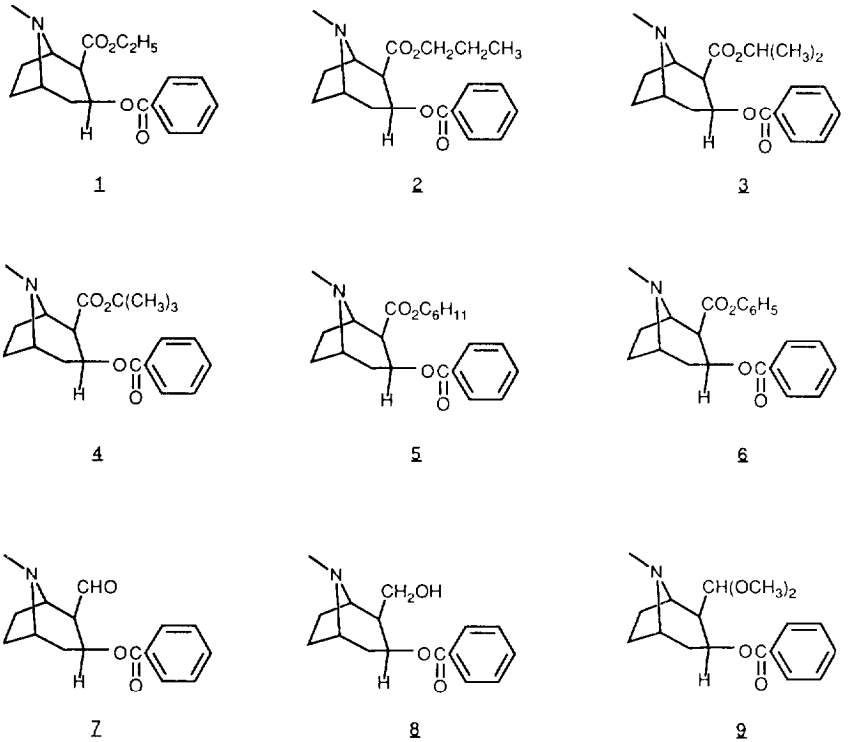
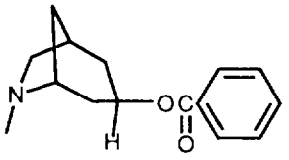


FIGURE 2. *Cocaine analogs modified at the 2 position of the tropane ring*

Cocaine and WIN-35,065-2 have good affinity for the dopamine transporter (table 1). even though the phenyl ring is closer to the tropane ring in WIN-35,065-2. Structures for cocaine and WIN-35,065-2 were constructed using the SYBYL software system. Systematic conformational searches using the SEARCH module of SYBYL were used to locate the global and other low-energy conformations of both compounds. Figure 5 shows an overlay of the global energy minimum conformation of WIN-35,065-2 and a low energy conformation of cocaine. Inspection of figure 5 suggests that the 3-naphthyl group of compound *11* (figure 3) would possess aryl groups in the space occupied by the electron-rich portions in both cocaine and WIN-35,065-2.

The p- and m-isothiocyanate derivatives of cocaine, *12* and *13* respectively, have been prepared as potential irreversible binding ligands for the dopamine transporter. In addition, compound *14,* the tritium-labeled analog of WIN-35,065-2, was prepared as a new radioligand for the cocaine receptor.



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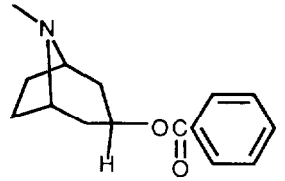
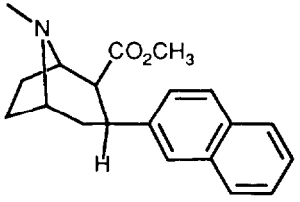


Tropacocaine



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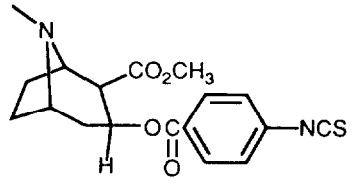

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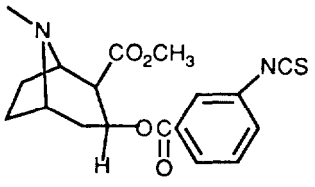

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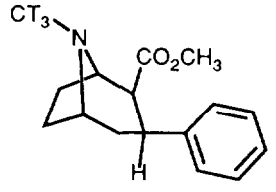

14

FIGURE 3. *Cocaine and WIN-35,065-2 analogs*

CONCLUSION

The potency of cocaine and cocainelike drugs in self-administration studies correlates with their potencies in inhibiting ^{3}H-mazindol binding to the dopamine uptake site in rat striatum. Since there is no correlation for binding to a large number of other pre- and postsynaptic binding sites, the cocaine receptor related to substance abuse appears to be the one associated with dopamine uptake inhibition.

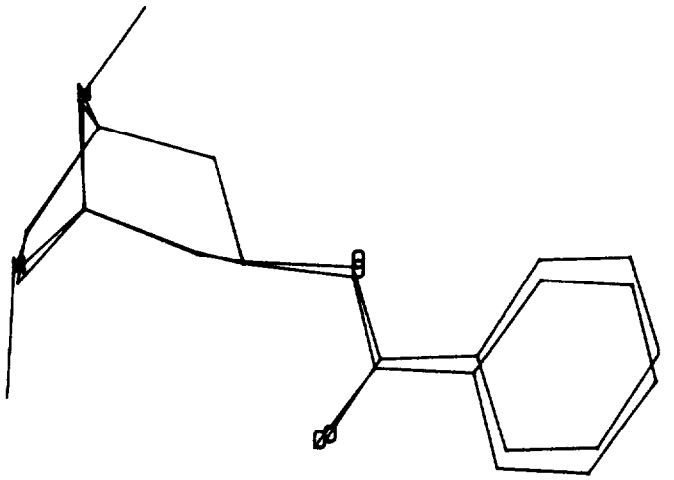

FIGURE 4. *Computer-generated molecular overlay of compound 10 and tropacocaine*

NOTE: Hydrogens have been removed for clarity

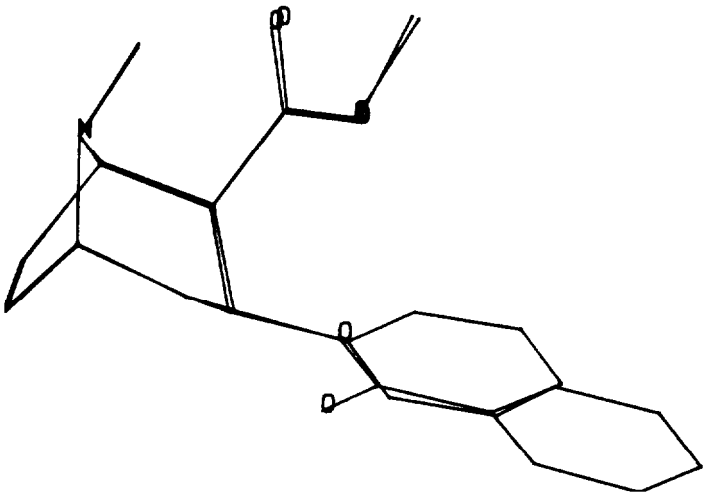

FIGURE 5. *Computer-generated molecular overlay of WIN-35,065-2 (global minimum conformation) and cocaine (one low-energy conformation)*

NOTE: All hydrogen atoms have been removed for clarity.

Several rigid azabicyclo analogs of cocaine. in which the size, stereochemistry, and/or the relative location of the amine functionality, carbomethoxy group, and aromatic ring were varied, have been prepared. The examination of these compounds in the dopamine transporter receptor binding assay will provide information about the cocaine pharmacophore. In addition, other cocaine analogs were prepared that may serve as new biochemical tools for the study of the cocaine receptor.

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Javitch, J.A.; Blaustein, R.O.; and Snyder, S.H. [^{3}H] Mazindol binding associated with neuronal dopamine and norepinephrine uptake sites. *Mol Pharmacol* 26:35-44, 1984.

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Structural and Functional Similarities of Opiate Receptors of Neural and Immune Origin

Daniel J. J. Carr and J. Edwin Blalock

INTRODUCTION

Opioid peptides, originally described as interacting with specific receptors found in neural tissue (Lord et al. 1977; Cuello 1983), have been reported to modulate various immune system parameters (Weigent and Blalock 1987). The immune modulatory capacity of opioids has, in many cases, been shown to be reversible or inhibited by opioid receptor antagonists, such as naloxone, leading investigators into hypothesizing the existence of opioid receptors on cells of the immune system. In addition, the recent observations describing the production of opioid peptides by cells of the immune system (Blalock et al. 1985) has led to the hypothesis of bidirectional pathways shared between the immune and neuroendocrine systems, based on the commonality of ligands and receptors (Blalock 1984). In an effort to further characterize the interaction between the immune and neuroendocrine systems, a comparison of the physicochemical properties of opioid receptors on cells of the two systems was initiated.

CHARACTERISTICS OF OPIOID RECEPTORS OF NEUROENDOCRINE ORIGIN

Classically, opioid receptors are defined according to their selectivity for various opioid ligands. Thus far, three predominate species of receptor classes have been defined, including μ, δ,, and κ (Barnard and Demoliou-Mason 1983). Of these, the μ and δ subtypes have been studied structurally as well as functionally at the cellular and molecular levels (Simonds 1988). Structural analysis reveals the S-class opioid receptor, isolated from a neuroblastoma x glioma hybrid cell line, to have a molecular weight of 58,000 daltons (Klee et al. 1982; Simonds et al. 1985; Carr et al. 1987).

The μ-class opioid receptor reportedly possesses a molecular weight of 58 to 65 kilodaltons, depending on the type of neural tissue used (Simon 1987; Cho et al. 1986). These observations, along with previous investigations (Bowen et al. 1981), suggest the neural opioid receptor may be a single glycoprotein species with specific regions associated with ligand binding influenced by ionic strength.

Molecular mechanisms by which neural opioid receptors transduce messages appear to be linked to G protein-adenylate cyclase pathways and ionic conductance channels (Simonds 1988). Opioids that interact with both μ- and b-class receptors have been shown to inhibit adenylate cyclase activity through G_i proteins. In addition, electrophysiologic analysis of opioid receptor-ligand interactions shows a modulatory capacity on both Ca^{2+} and K^+ ion conductance. For example, opioids reportedly activate inwardly rectifying potassium currents in various neuronal systems. They also reduce voltage-dependent calcium channels (Simonds 1988). Thus, much information as to the characteristics of opioid receptors on neural tissue has been obtained.

PIIARMACOLOGIC AND PHYSICOCHEMICAL CHARACTERISTICS OF IMMUNE-CELL OPIOID RECEPTORS

As previously mentioned, early evidence suggesting opioid receptors on cells of the immune system (immunocytes) came predominately from functional assays on immune responsiveness. Human T-cell rosetting was shown to be modulated by [Met]-enkephalin and morphine (Wybran et al. 1979). Moreover, this effect was reversed with the addition of naloxone, an opioid receptor antagonist. Other naloxone-reversible effects of opioids have been well documented for both the humoral and cellular arms of the immune system (Sibinga and Goldstein 1988). Furthermore, biological responses arc by no means the only methods that have been employed to show opioidlike receptors on immunocytes. By pharmacologic techniques, early work demonstrated opioidlike binding sites on immunocytes that were, in some cases, saturable and of relatively high affinity (Lopker et al. 1980; Mehrishi and Mills 1983; Ausiello and Roda 1984). Recently, specific binding sites for the κ ligand bremazocine were demonstrated on EL-4 thymoma cells with a Kd of 60 nM and B_{max} of 2,700 fmols/10^6 cells (Fiorica and Spector 1988). Similarly, using the K-selective ligand U69,593, the authors have determined that the P388d_1-macrophage cell line possesses κ opioid receptors with a Kd of 17 nM and B_{max} of 54 fmol/10_6 cells (Carr et al. 1989). Thus, it seems apparent that cells of the immune system (as determined by pharmacologic techniques) possess opioid receptors that appear to have some selectivity for specific classes of ligands and that possess high-affinity binding sites. In an effort to further define immunocyte-receptor characteristics, immunologic and biochemical techniques were employed. Documentation of the mass of the opioid receptor(s) from neural tissue was originally determined using detergent-solubilization procedures followed by molecular sieve

chromatography and radioreceptor assays of the various fractions (Simon 1987). With the advent of more sophisticated peptide ligands and class-specific alkaloids as well as the introduction of chemical crosslinkers with short linker arms, it became possible to selectively label opioid receptors in situ and determine their molecular weight via polyacrylamide gel electrophoresis. In an effort to determine if an opioid receptor from immunocytes could be specifically labeled in this manner, ^{125}I-β-endorphin was incubated with immunocytes in the presence or absence of unlabeled naloxone. Bound ligand was then covalently attached to the receptor and subsequently analyzed by gel electrophoresis and autoradiography. In the presence of a reducing agent (2-mercaptoethanol), a β-endorphin-labeled protein with an apparent molecular weight of 46 kilodaltons was observed (Carr et al. 1988b). The labeling was specific, since in the presence of unlabeled naloxone, no band was observed. Interestingly, a 46-kilodalton protein has reportedly been observed to be associated with opioid receptors of neuroendocrine origin as well (Yeung 1987: Roy et al. 1988). Since β-endorphin binds with near equal affinity to both μ- and δ-class binding sites (Barnard and Demoliou-Mason 1983), a more selective ligand was sought. To this end, cis-(3)-methylfentanylisothiocyanate (SUPERFIT), a δ-class opioid receptor ligand was employed. *In situ* labeling of T-cell-, B-cell-, and macrophage-enriched populations revealed the specific labeling of a protein that migrated with an apparent molecular weight of 58,000 daltons under nonreducing conditions on polyacrylamide gels (Carr et al. 1988c). Under reducing conditions, the molecular weight shifted to 70,000 daltons, indicative of intradisulfide chains, which have previously been reported in neural opioid receptors (Gioannini et al. 1985; Kamikubo et al. 1988). Interestingly, the same procedures when applied to the neuroblastoma x glioma hybrid cell line NG108-15 yielded identical results (figure 1). Moreover, the labeling of the protein species on immunocytes was nearly identical, as one might predict using neuroendocrine receptors. That is, the δ-selective ligand [D-Ala^2,D-Leu^5]-enkephalin (DADLE) was able to block SUPERFIT labeling, whereas the p-selective ligand [D-Ala^2,N-Me-Phe^4,Gly^5-ol] enkephalin (DAGO) did not block binding of SUPERFIT (Carr et al. 1988c). In addition, naloxone was able to block greater than 70 percent of the binding of SUPERFIT, suggesting a classical opioid receptor-ligand interaction. Taken together, these results indicate an opioid binding site on cells of the immune system that demonstrates selectivity in ligand preference as well as biochemical characteristics similar to neuroendocrine-derived receptors.

FUNCTIONALITY OF IMMUNOCYTE OPIOID RECEPTORS

Mechanistically, the immunocyte opioid receptor appears to be coupled to the same second-messenger pathways. Investigations into the immunocyte cAMP pathway have determined that opioid peptides such as β-endorphin and [Met]-enkephalin are able to suppress adenylate cyclase activity in a naloxone-reversible fashion at 10^{-7} to 10^{-9} M (Fulop et al. 1987; Carr et al. 1988a). In addition, prostaglandins, which are known to elevate cAMP in

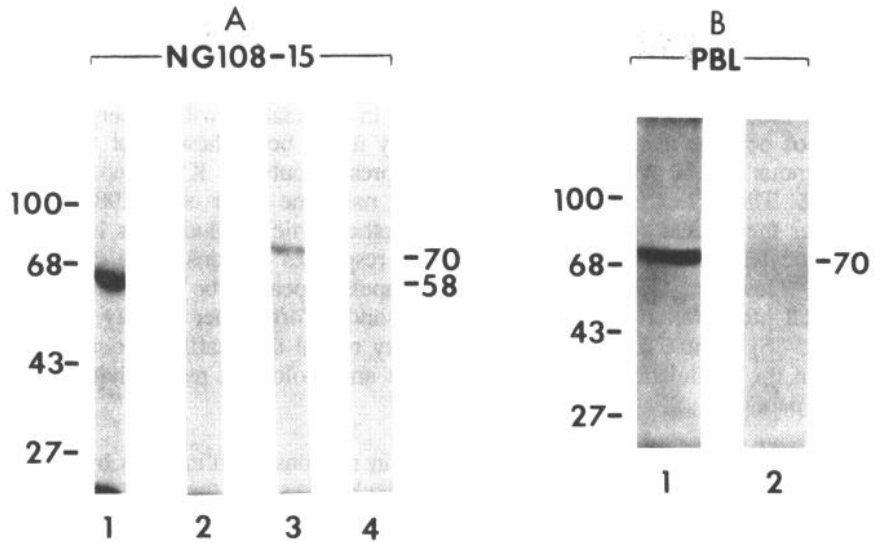

FIGURE 1. *Part A. Autoradiogram of electrophoresed [^{3}H]-SUPERFIT-labeled opioid receptors from NG108-15 cells under reducing (lanes 3 and 4) and nonreducing (lanes and 2) conditions. Part B. Autoradiogram of murine peripheral blood lymphocytes labeled with [^{3}H]-SUPERFIT.*

NOTE: Part A. NG108-15 cells were incubated (45 minutes, 4 °C) with [^{3}H]-SUPERFIT in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of unlabeled SUPERFIT. Cells were washed once and lysed in denaturing buffer (in the presence or absence of 2-mercaptoethanol and 6 M urea. Cell lysates were electrophoresed on a 10-percent polyacrylamide gel, and the resultant gel was dried and subjected to autoradiography for 5 days at -70 °C. Intensifying screens were employed to amplify the signal. Part B. Approximately 5x10^6 murine peripheral blood lymphocytes were incubated (45 minutes, 4 °C) with [^{3}H]-SUPERFIT in the presence (lane 2) or absence (lane 1) of unlabeled SUPERFIT. Cells were washed and lysed in denaturing buffer (2-percent SDS, 0.062 M Tris-HCI, lo-percent glycerol, 5-percent 2-mercaptoethanol). Cell lysates were electrophoresed on a 10-percent polyacrylamide gel, and the resultant gel was dried and subjected to autoradiography for 5 days. Enhancing screens were used to amplify the image of the bands.

SOURCE: Carr et al. 1988c, copyright 1988, Academic Press, Inc.

neural tissue, are reportedly antagonized by opioids in immunocytes (Farrar 1984). It is interesting to note that an increase in lymphocyte cAMP has a general inhibitory effect on the immune response (Boume et al. 1974). Thus, observations that show opioids to inhibit adenylate cyclase activity in lymphocytes are in agreement with their stimulatory effects on immune functions, such as enhancement of natural killer activity (Mathews et al. 1983) or enhancement in the generation of cytotoxic T cells (Carr and Klimpel 1986).

Another pathway of intracellular signaling is ionic conductance channels. Two predominate ion channels, the K^+ and Ca^{++} channels, have been identified and defined not only in neuronal cells but in cells of the immune system as well (Chandy et al. 1985). In addition, similarities in channel profiles have been found in cells of both systems. It is well known that opioids modulate both K^+ and Ca^{++} currents in association with hyperpolarization of neurons (Simonds 1988). Recently it has been shown that the endogenous opioid peptide β-endorphin suppresses outward K^+ currents in T cells. This effect is partially reversed by naloxone (Carr et al. 1988b). Whether the effects opioids have on K^+ or other ionic conductances in immunocytes are associated with biological. responses remains to be determined. However, leukocyte potassium channels appear to be associated with cell proliferation (Chandy et al. 1984) and natural killer activity (Sidell et al. 1986). Thus, future investigations may reveal or identify associations between the modulation of potassium fluxes and biological responsivity to opioid peptides.

As mentioned earlier, calcium conductance in neurons is influenced by opiates. Investigations into calcium fluxes in leukocytes and the ability of opioids to modulate such function have recently begun using the highly fluorescent (Ca^{2+} indicator FURA-2AM. The introduction of the κ-class opioid receptor ligand U-50,488H to lymphocytes loaded with FURA-2AM causes a rapid influx of Ca^{2+} ions into the cell, as measured by spectrofluorometry (Carr and Blalock 1989). Interestingly, Concanavalin A also induces a rise in intracellular Ca^{2+} over nearly the same time interval. This relationship between a T-cell mitogen and the κ ligand's ability to increase Ca^{2+} suggests Ca^{2+} flux as a common and early event associated with proliferation and/or potentiating immune responses.

These results have led us to define other features of the lymphocyte opioid receptor(s). In an attempt to purify the receptor(s), an anlibody to the opioid receptor(s) was produced by a procedure based on the molecular recognition theory (Bost et al. 1985). Antibody to the opioid receptor was able to block specific binding of β-endorphin to the δ-class opioid receptor as well as to compete with β-endorphin and naloxone for binding to receptors on the neuroblastoma x glioma cell line NG108-15 cells (Carr et al. 1986). Moreover, this antibody was used to purify the b-class opioid receptor from NG108-15 cells, which was then shown to retain its binding specificity and capacity (Carr et al. 1987). Likewise, the antibody was shown to block specific binding of dihydromorphine to leukocytes. In addition the antibody possessed agonist activity, similar to β-endorphin in reducing cAMP content in leukocytes in a naloxone-reversible fashion (Carr et al. 1988a). Recently the antibody was used as an affinity absorbant for the purification of the opioid receptor from leukocytes. The protein species purified by affinity chromatography was analyzed by polyacrylamide gel electrophoresis and was determined (similar to the *in situ* data) to have a molecular weight of 58,000 daltons under ronreducing conditions and

70,000 daltons under reducing conditions (Carr et al., in press). In addition, purified receptor was shown to possess the capacity to specifically bind the δ-class ligand SUPERFIT (Carr et al., in press). Future endeavors are directed to the purification of the opioid receptor from both neuronal and immunocyte sources for the eventual comparison of amino acid sequence. It is anticipated that these sequences will be very similar if not identical.

CONCLUSION

The authors have investigated immunocyte opioid receptors at both the cellular and molecular levels and found them to possess many traits similar to their neuroendocrine counterparts. For example, saturable, high-affinity receptors appear on leukocytes, which in the case of the δ--class receptor exhibit specificity in their ligand preference like neuronal receptors. In addition, the molecular weight of the δ--class receptor isolated from leukocytes is identical to receptors purified from neuronal and neuronal-derived cell lines. Furthermore, the delta class receptor possesses intrachain disulfide bridges that cause a migratory shift on polyacrylamide gels under reducing versus nonreducing conditions. Moreover, the molecular mechanisms that appear to be affected upon receptor activation are parallel between the two systems, suggesting them to be composites of one another. Future work will undoubtedly revolve around *in vivo* manipulations of these receptors in an attempt to understand their physiologic function (Shavit et al. 1986; Weber et al. 1986). It is anticipated that this will lead ultimately to a better understanding of the dynamic relationship between the immune and neuroendocrine systems and how such a relationship impinges on diseases such as AIDS.

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Immunomodulatory Effects of Chronic Morphine Treatment: Pharmacologic and Mechanistic Studies

Henry U. Bryant, Edward W. Bernton, and John W. Holaday

INTRODUCTION

Morphine and its related alkaloids produce a myriad of pharmacological activities, including the antinociceptive, antidiarrheal, euphoric, and addictive properties that have been well characterized. However, a number of other pharmacologic responses following opiate administration have been described, including effects on endocrine function (Holaday and Loh 1979), thermoregulation (Clark 1979), behavior (Lowy and Yim 1983), metabolism (Lamb and Dewey 1981), respiration (Holaday and Malcom 1987) urine output (Huidobro-Toro and Huidobro 1981), and the cardiovascular system (Holaday 1983). Data from a number of laboratories indicate that immunomodulatory effects should be added to the list of activities attributed to morphine and its related congeners.

The concept that opiates might affect immune function is not entirely a recent development. In reports as early as 1967, an increased incidence of bacterial and viral infection was demonstrated in heroin abusers (Louria et al. 1967). While this phenomenon was at first attributed to the sharing of infected drug paraphernalia and contaminated needles, there is now a considerable volume of evidence suggesting that the opiate itself is involved, via suppression of a variety of immunologic endpoints. Lymphocyte proliferative responses to mitogens such as concanavalin A (Con A), phytohemagglutinin, and pokeweed mitogen are suppressed in heroin addicts (Brown et al. 1974). T-cell rosette formation (Wybran et al. 1979) and the total number of circulating lymphocytes are also reduced in this population (McDonough et al. 1980). In animal models, opiates can be shown to affect a number of important immunologic correlates. Lymphocyte

proliferative responses (Bryant et al. 1987a), natural killer-cell cytotoxic activity (Shavit et al. 1986), and the phagocytic and killing properties of polymorphonuclear leukocytes (Tubaro et al. 1985) are all attenuated following morphine exposure. Morphine also inhibits antibody (Lefkowitz and Chiang 1975) and serum hemolysin formation (Gungor et al. 1980) in mice inoculated with sheep etythrocytes. Poly I:C-induced interferon production is suppressed in morphine-treated animals (Hung et al. 1973), as is the development of delayed hypersensitivity in response to mycobacterium bovis (BCG).

Immunomodulatory effects of morphine have been described following injection of a single dose (Weber and Pert 1989) and after daily injections with escalating doses (Gungor et al. 1980). The issue of chronic exposure to morphine with regard to its effect on immune function was of particular interest. One of the most common methods of continuous exposure to morphine in rodents is the subcutaneous (SC) implantation of a pellet containing morphine base. This technique allows for the induction of tolerance and dependence within 12 to 24 hours. This chapter focuses on this work and the findings of other laboratories that have utilized this model to better understand the effects of chronic exposure to morphine on immune function. The generation of an "immunologic profile." in morphine-pelleted mice, pharmacologic studies, and possible mechanisms for the phenomenon are presented in this chapter.

EFFECTS OF MORPHINE-PELLET IMPLANTS ON VARIOUS IMMUNE ENDPOINTS

The initial, and perhaps most striking, observation with regard to the effects of morphine-pellet (NIDA) implantation on the immune system was the marked atrophy of the spleen and thymus that occurs within 72 hours of SC implantation of a 75-mg morphine pellet (figure 1). This atrophy of the spleen and thymus was observed following correction for the loss in overall body weight associated with chronic morphine treatment and was accompanied by reduced cellularity of these organs (Bryant et al. 1987a).

In addition to the reduction of immune organ weight and cellularity, a suppression of mitogen-induced lymphocyte proliferation was observed in morphine-pelleted mice. Blastogenic responses to Con A, a T-cell mitogen, or to lipopolysaccharide (LPS), a B-cell mitogen, were measured by assessment of tritiated thymidine incorporation into the DNA of the proliferating lymphocytes. As with spleen and thymus size, 72 hours following implantation of the morphine pellet, blastogenic responses to both Con A and to LPS were significantly suppressed (Bryant et al. 1987a). Lymphocytes stimulated to proliferate by cells carrying foreign histocompatibility antigens in a mixed lymphocyte response were less responsive when taken from morphine-pelleted mice (figure 2). It was also found that, in mice immunized with sheep erythrocytes, morphine-pellet implants suppressed the

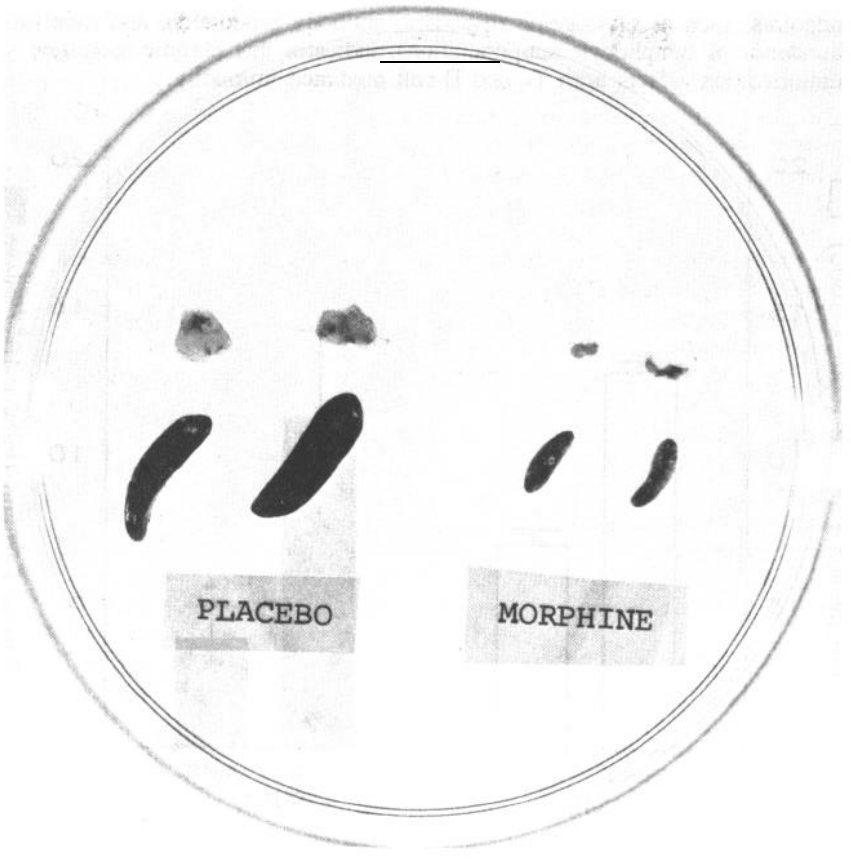

FIGURE 1. *Spleens and thymi removed from C3H/HeN mice 72 hours following implantation of an inert placebo pellet or a pellet containing 75-mg morphine*

NOTE: Pellets supplied by the National Institute on Drug Abuse.

ability of B-lymphocytes to produce antibody in response to sheep erythrocytes in a plaque-forming assay (figure 2). This latter finding is consistent with the results of Weber et al. (1987), who utilized a similar morphine-pellet model and assessed antibody formation *in vivo*. Interestingly, TNP-ovalbumin-induced antibody production was preferentially suppressed in morphine-pelleted mice, suggesting a T-cell dependent effect (Weber et al. 1987). Flow cytometric analysis has shown that this morphine-pellet regimen leads to a significant reduction in the relative abundance of B cells without significantly affecting the total T-cell population, although there is a

slight elevation in the relative abundance of the T-suppressor subpopulation (Peritt et al. 1988). Therefore, evidence from a variety of immunologic endpoints, such as blastogenic responses, antibody production, and relative abundance of lymphocyte subpopulations, idicates that chronic morphine administration affects both T- and B-cell mediated immunity.

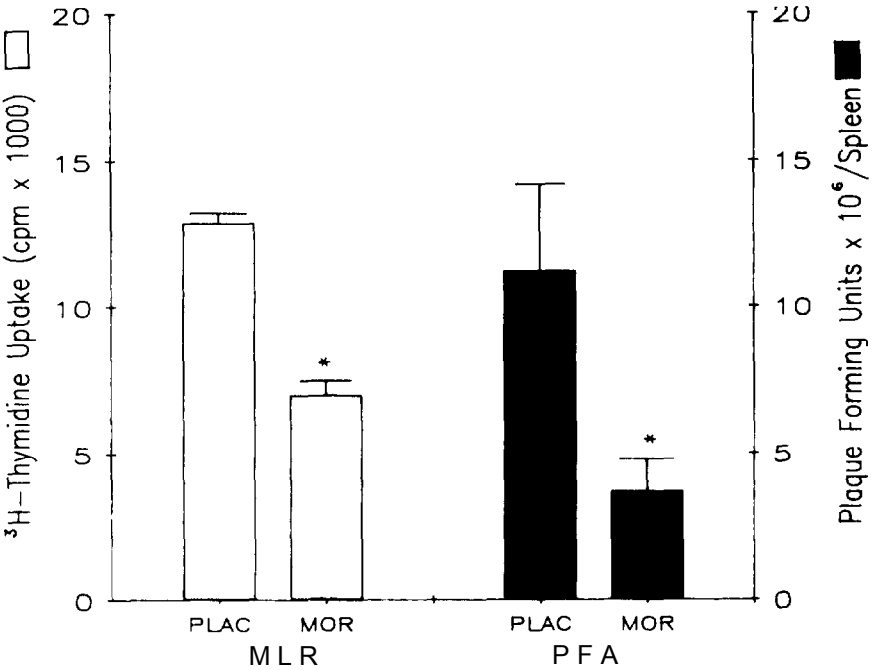

FIGURE 2. *Effect of morphine-pellet implants (72 hours) on a mixed lymphocyte response (MLR) and on antibody production as assessed by a plaque-forming assay (PFA)*

*p<.05 vs. appropriate placebo control

The effects of this morphine regimen on another major group of cells in the immune system, the macrophage, have also been examined. Agents such as BCG, when injected into the peritoneum, elicit macrophage migration into the peritoneal cavity. These macrophages are activated as evidenced by their tumoricidal activity vs. tumor cell targets *in vitro.* Morphine-pellet implantation attenuates the tumoricidal activity of BCG-elicited peritoneal macrophages (Bryant et al. 1988d). Interestingly, exogenous administration of gamma-interferon *in vitro* to peritoneal macrophages obtained from morphine-pelleted mice restores tumoricidal activity. This suggests that

suppressed gamma-interferon production, as has been reported by others (Hung et al. 1973), is a likely contributor to the suppression of macrophage activation. These studies also showed that Ia expression on peritoneal macrophages induced by BCG is reduced in morphine-pelleted mice.

These effects of morphine-pellet exposure on in vitro indices have some relevance *in vivo* as evidenced by the finding that morphine-pelleted animals also show increased risk to bacterial infection. A sublethal dose of *Listeria monocytogenes* in nonpelleted mice produced a 75-percent mortality rate in morphine-pelleted mice vs. 12-percent mortality in noninfected, morphine-pelleted mice (Bryant et al. 1988d). The timecourse of the toxicity suggested that the increased lethality was related to the bacterial infection, as opposed to a morphine-related toxicity. This finding is in agreement with the work of Tubaro et al. (1983), who found that continuous morphine administration via an implanted osmotic minipump increased lethality due to infection with *Candida albicans.*

PHARMACOLOGIC ANALYSIS OF MORPHINE-PELLET-INDUCEID IMMUNOSUPPRESSION

An essential pharmacological issue to establish with any opiate-induced effect is reversibility or blockade by an opiate-receptor antagonist. This is a particularly critical issue when discussing effects of opiates on the immune system, since, in certain instances, opiate modulation of immune function is not naloxone reversible (Schweigerer et al. 1982). In order to determine sensitivity of the immunologic deficits induced by morphine-pellet implantation to reversal by an opiate antagonist, the morphine-pellet was coimplanted with a 6-mg naloxone pellet in one series of experiments. As would be expected in a typical opiate-receptor-mediated phenomenon, the splenic and thymic atrophy and suppression of Con A- and LPS-induced blastogenesis caused by morphine were prevented by chronic cotreatment with naloxone (Bryant et al. 1987b) (table 1). A 48-hour exposure to the naloxone pellet alone was largely ineffective, although a slight reduction in spleen size was noted in these animals. Weber et al. (1987) demonstrated that morphine-pellet-induced suppression of antibody production *in vivo* also is prevented by naloxone and that the effect is stereospecific, offering further evidence that this effect is mediated by an interaction at an opiate receptor.

Another important concern, particularly when dealing with chronic treatment regimens, is the timecourse of the effect. Again, this issue is of particular relevance when examining immune endpoints, as changes in immune function are often dependent upon the time at which the response is assessed following challenge. Macrophage activation by endotoxin is a good example of this point. While endotoxin is an excellent stimulus for activation of macrophages following acute exposure, continuous stimulation results in a suppression of the response (Friedman 1972). Furthermore, physiologic responses following acute exposure to opiates arc often quite different from

TABLE 1. *Effect of naloxone and tolerance on morphine-pellet-induced immunomodulation*

Parameter	Morphine[a]	Morphine + Naloxone[b]	Naloxone	Morphine + Morphine PRE-TX[c]
Spleen Weight	55±3*	94±1	85±4*	72±5*
Thymus Weight	36±2*	82±3	90±7	32±4*
Con A-Induced Proliferation				
0.25 µg/ml	32±21*	121±23	101±13	72±12
0.5 µg/ml	47±7*	102±9	100±8	100±14
1.0 µg/ml	52±10*	102±9	96±9	97±19
2.0 µg/ml	60±16	94±10	90±2	85±24
LPS-Induced Proliferation				
0.1 µg/ml	51±27*	103±17	99±8	117±11
0.5 µg/ml	44±20*	89±10	82±6	108±26
5.0 µg/ml	74±17	102±7	94±4	96±23

*p<.05 vs. placebo group; values represent percent of mean placebo response ± SEM.
[a]48-hour morphine-pellet (75 mg) exposure period.
[b]6-mg naloxone pellet coimplanted with morphine or placebo pellet.
[c]72-hour pre-exposure period to 75mg morphine-pellet, followed by a second pellet for 48 hours.

those observed in tolerant animals (Morley et al. 1982). Therefore, extensive temporal analyses were conducted of the effects of morphine-pellet implantation on immune function. T-lymphocyte proliferative responses were largely unaffected at 6 or 24 hours following morphine-pellet implantation, but were reduced at 48 and 72 hours. Interestingly, the response was essentially normal by 96 hours and a rebound stimulation was observed at 120 hours (Bryant et al. 1988a), a time when antinoiceptive tolerance to a morphine challenge is still increasing (figure 3). B-lymphocyte proliferative responses to LPS and the thymic and splenic atrophy followed a similar pattern of suppression, followed by a return toward normal at the later time points. The suppression of B-cell blastogenesis and thymic atrophy appeared to be the most sensitive endpoints.

The loss of immunosuppression at the later time points is most easily explained by either (1) exhaustion or encapsulation of the morphine pellet leading to a decreased bioavailability or (2) to the development of tolerance to the effect. Blood morphine levels were assessed in morphine-pelleted mice in a collaborative effort with Dr. Byron Yobum of St. John's University. Following an early surge at 6 and 24 hours, serum morphine levels

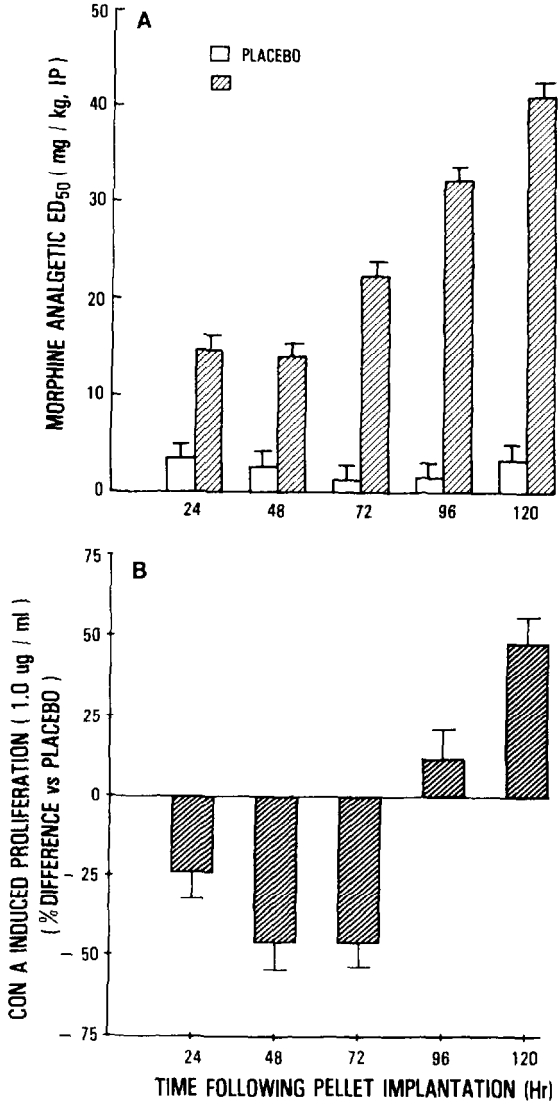

FIGURE 3. *Timecourse for the development of tolerance to antinociception following morphine challenge in morphine-pelleted mice (panel A) compared to the timecourse for the effect on concanavalin A (Con A)-induced lymphocyte proliferation (panel B)*

SOURCE: Bryant et al. 1988a, copyright 1988, American Society for Pharmacology and Experimental Therapeutics

plateaued and remained fairly constant (approximately 600 ng/ml) over the following 4 days (Bryant et al. 1988b). Therefore, the difference in immunomodulatory effects at 72 and 120 hours postpelleting are not due to an altered bioavailability of morphine from the morphine pellet. This is consistent with the early findings of Patrick et al. (1975) who demonstrated that the analgetic ED-50 and morphine levels in the brain are elevated for as long as 144 hours following morphine-pellet implantation.

Since altered release of morphine was not a satisfactory explanation for the altered immunomodulatory effects, additional studies were conducted to evaluate the development of tolerance to these effects. In these experiments, animals were pre-exposed to a 75-mg morphine-pellet for 72 hours, then given an additional pellet for a 48-hours period and the immune variables evaluated. In the morphine-pretreated animals, the degree of thymic atrophy was comparable to nonpretreated mice, but the severity of splenic atrophy was not as great as in 48-hour morphine-implanted animals not pre-exposed to morphine (table 1) (Bryant et al. 1987b). The suppression of Con A- and LPS-induced blastogenesis was completely lost in the morphine-pretreated group. Therefore, it is apparent that tolerance does develop to the immunomodulatory effects of chronic morphine treatment and that the loss of splenic and thymic atrophy and mitogen-induced lymphocyte proliferation at later time points in the model reflects the development of tolerance to the morphine pellet.

The investigators have only scratched the surface of important pharmacological questions regarding morphine-induced immunomodulation. Receptor-subtype involvement, a central vs. peripheral site of action, and effects of other opiate agonists in a similar model are all important questions yet to be addressed. The role of withdrawal in the immunomodulatory effects has been addressed in some preliminary investigations. ‘Temporal factors again appear involved, as at early time points (e.g., 48 hours), precipitated withdrawal has no effect on the suppressive effects of morphine-pellet implantation (Bryant et al. 1987b). However, at later time points (e.g., 144 hours), increases in Con A-induced blastogenesis may be observed in withdrawing animals (Kenner et al. 1988a).

MECHANISTIC STUDIES

Studies into possible mechanisms for the immunosuppressive effects observed 48 hours following morphine-pellet implantation have focused on three areas: (1) direct effects of morphine on lymphocyte proliferative responses; (2) the level of the morphine-induced deficit in the blastogenic cascade; and (3) possible mediation via alterations in neuroendocrine systems, particularly of prolactin and corticosterone.

Direct Effects

Several reports have suggested the presence of opiate receptors on lymphocytes (Carr 1988). Therefore, morphine interaction with these receptors is one possible hypothesis that might explain the immunomodulatory effects in the model. However, exposure of lymphocytes obtained from normal mice to a wide range of morphine concentrations *in vitro* did not suppress Con A-induced blastogenesis. In fact, concentrations in the micromolar range (comparable concentration to serum levels recorded in the morphine-pelleted mice at the 48-hour time point (Bryant et al. 1988b) produced an elevation in lymphocyte proliferation in response to Con A (figure 4). Furthermore, the opiate antagonist naloxone produced a similar profile *in vitro* (i.e., stimulation at micromolar concentrations). These observations argue against a direct mode of action of morphine at the lymphocyte level as an explanation for the immunosuppressive effects.

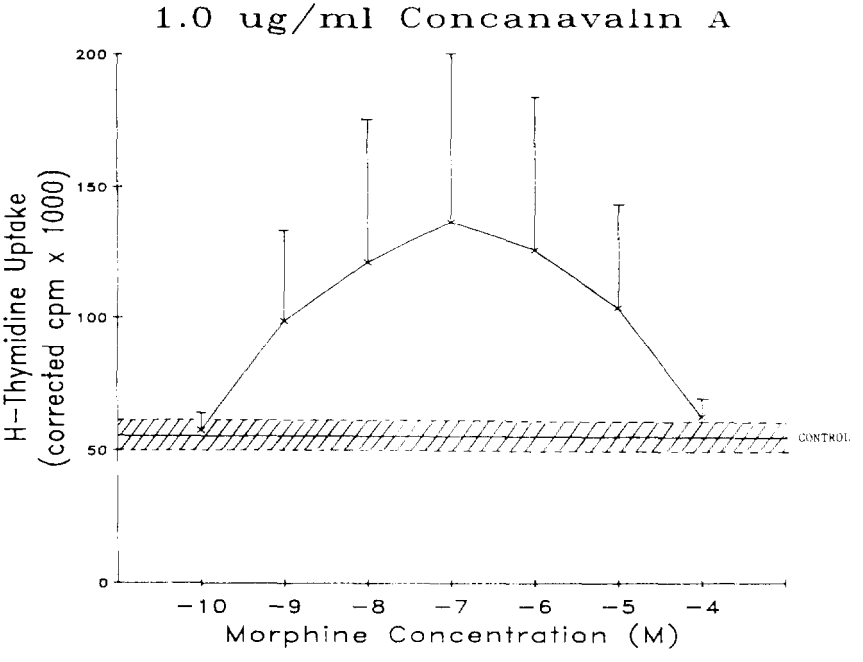

FIGURE 4. *Effect of morphine addition* in vitro *to normal mouse (C3H/HeN) lymphocyte cultures stimulated by 1.0 mcg/ml Con A*

Level of Morphine-Induced Immunosuppression

The blastogenic response of lymphocytes depends upon the interplay of events, which include the recognition and processing of foreign antigen by antigen-presenting cells, production of monokine and lymphokine mediators for amplification of the response, and. ultimately, the proliferation and differentiation of antigen-responsive lymphocytes capable of cell-mediated cytotoxicity, antibody production, and macrophage activation. The lymphocyte product interleukin-2 (IL-2) plays a central role in the proliferation of activated T cells.

In work done with Dr. Thomas Jerrells (University of Texas Medical Center, Galveston, TX), it has been found that the attenuated lymphocyte proliferative responses to Con A in the morphine-pelleted mice are directly linked to a deficiency in production of IL-2 by the lymphocytes. A cell line (CTLL) that is highly sensitive to IL-2 was used as a bioassay for IL-2 levels in the supernatant of proliferating cells from either placebo or morphine-pelleted mice. A marked reduction in the IL-2 level in these supernatants was observed in cell cultures taken from morphine-pelleted mice as compared to those taken from placebo-pelleted mice (Peritt et al. 1988). As observed with the proliferation data, IL-2 levels were not reduced in cell suspensions taken from morphine-pelleted mice that were also given a naloxone pellet.

Further evidence was provided by experiments that demonstrated that addition of exogenous IL-2 to the media elevated the proliferative responses of lymphocytes obtained from morphine-pelleted mice to mitogen, although not to the same magnitude as was observed with lymphocytes from placebo-pelleted mice. Addition of supernatants from normal Con A-stimulated lymphocytes (which contain IL-2 in addition to other important growth factors) also reversed the attenuation of the response in lymphocytes obtained from morphine-pelleted mice. Possible changes in lymphocyte responsiveness to IL-2 were ruled out with an assay developed by Dr. Jerrells and David Peritt. In this procedure, lymphocytes from morphine- and placebo-pelleted mice were bulk stimulated with Con A. Following a sufficient period of time to allow for blastogenesis, those lymphocytes that had responded to the Con A stimuli to form blastocytes were separated from the remaining cells on a Ficoll gradient. As might be expected based on the proliferative data, the number of blastocytes was significantly reduced in cultures obtained from morphine-pelleted mice. Equal numbers of blast cells from morphine and placebo mice were then exposed to IL-2 in culture. Following incubation, proliferation of the blast cells was then determined. The blast-cell procedure is a very useful tool to allow comparison of cells with fully expressed IL-2 receptor. Interestingly, blast cells from morphine-pelleted mice showed no reduction in IL-2-elicited proliferation when compared to blast cells originating from placebo-pelleted mice (Peritt et al. 1988). These studies indicate that the morphine-induced “defect” most

likely occurs prior to receptor recognition of IL-2 and that it can be accounted for at least in part by attenuated production of lymphokine mediators of the mitogen-induced growth response.

These studies suggested that an earlier event in the proliferation cascade might be responsible for the reduced IL-2 production and ultimate inhibition of proliferative responses. Therefore, a separate series of experiments was aimed at determining possible effects of chronic morphine treatment on the macrophage. Macrophages play an important role in the processing of antigen and the production of monokines that eventually lead to the production of IL-2 by lymphocytes. A possible effect of morphine-pellet implantation on the macrophage was suggested by two preliminary observations. The first was that, as previously mentioned, morphine-pellet implantation greatly increased the lethality of a sublethal injection of *L. monocytogenes* in mice. The second observation was that, in addition to inhibiting Con A-induced lymphocyte proliferation, chronic morphine treatment also inhibited the mixed lymphocyte response in mice. The macrophage seemed to be a possible common denominator in this case based on the knowledge that *L. monocytogenes* is primarily a macrophage pathogen and that the mixed lymphocyte response requires antigen processing (i.e., by macrophages) since lymphocytes from an allogeneic animal serve as the proliferative stimuli in this assay.

To assess possible effects of morphine treatment on macrophage involvement in these proliferative defects, macrophages and T-lymphocytes were isolated from spleens obtained from placebo- or morphine-pelleted mice and then combined in a crossover design (figure 5). With this approach, it was demonstrated that when lymphocytes from chronically morphine-treated mice are incubated with macrophages from placebo-pelleted mice, only a mild reduction in Con A-induced proliferation is observed. However, when lymphocytes from placebo-pelleted mice are incubated with macrophages from morphine-pelleted mice, the more familiar marked reduction of Con A-induced proliferation is observed (Bryant et al. 1988d). These findings suggest a critical role for the macrophage in morphine-induced immunosuppression. The possibility has been ruled out that prostaglandins originating from the macrophage are involved in the immunosuppressive effect, since the suppression cannot be blocked by indomethacin added *in vitro*. Therefore, future investigations will examine the effects of chronic morphine treatment on the production of macrophage activation factors (e.g., gamma-interferon) and on monokine production (e.g., interleukin-1) by the macrophage itself.

Since a direct effect of morphine appeared unlikely, possible indirect effects were assessed. Cold exposure is known to suppress the immune system (Hardy et al. 1986), and morphine produces hypothermia. However, this possible indirect mechanism was ruled out by the timecourse studies since,

at the later time points, morphine-pelleted mice were hypothermic but were not immunosuppressed (Bryant et al. 1988a).

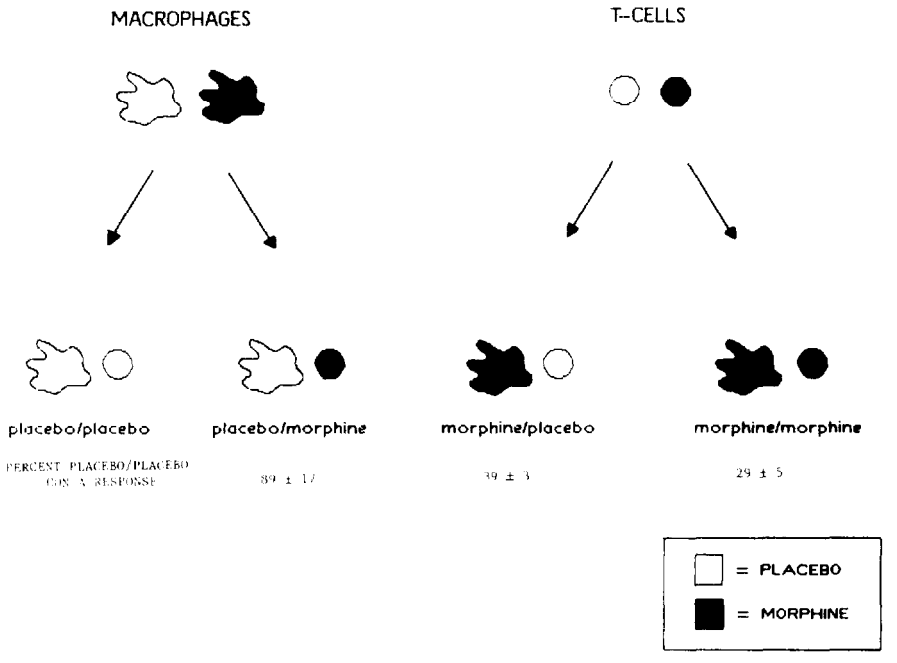

FIGURE 5. *Design for macrophage/T-cell crossover study*

NOTE: Numeric values represent tritiated thymidine uptake induced by 0.5 mcg/ml Con A as a percentage of the placebo macrophage/T-cell control combination.

Neuroendocrine-Based Mechanisms

Possible effects of morphine on neuroendocrine correlates have also been examined as a possible mechanism for the immunosuppressive effect. Based on the germinal research efforts by Dr. Bernton, investigation of the immunopermissive role of prolactin has taken place (Bemton et al. 1988). Since morphine is known to affect prolactin release (Holaday and Loh 1979), this link was first examined as a possible mechanism for the immunosuppressive effect of chronic morphine treatment. Implantation of pellets containing ovine prolactin or injection of prolactin-releasing drugs such as the dopamine antagonist metoclopramide prevented the suppression of mitogen-induced proliferative responses induced by morphine. Interestingly, the splenic and thymic atrophy subsequent to morphine-pellet

implantation were not reversed by these prolactin-modifying regimens. Prolactin levels were assessed in the morphine-pelleted mice, and, surprisingly, no significant differences were noted when compared to placebo-pelleted mice at 6, 24, 48, 72, 96, or 120 hours postimplantation, indicating an apparent difference in the effects of morphine on prolactin release between mice and rats (Bryant et al. 1988b). Recent findings by Julie Kenner (Kenner et al. 1988) and by others (Montgomery et al. 1987) have demonstrated the presence of a lymphocyte prolactin or prolactinlike protein that is elevated following mitogenic stimulation. Levels of lymphocytic prolactin are also unaltered by morphine-pellet implantation. When considered with the rest of the data, these findings suggest that, while prolactin-modifying regimens can reverse certain aspects of morphine-pellet-induced immunosuppression, a role for alterations in prolactin as a possible mechanism for the effect of morphine is unlikely. These findings also indicate that this morphine-pellet model may be useful in identifying immunopotentiating substances.

Glucocorticoid mobilization subsequent to stress is known to be one mediator of stress-induced immunosuppression (Solomon and Amkraut 1981), since glucocorticoids are well-recognized immunosuppressants (Parrillo and Fauci 1979). Morphine is also known to activate the hypothalamo-pituitary-adrenal (HPA) axis (George and Way 1955; Holaday and Loh 1979). Indeed, several lines of evidence suggested involvement of glucocorticoids in the immunosuppressive-type effects following chronic morphine treatment: (1) timecourse studies indicated a significant degree of adrenal hypertrophy in the morphine-pelleted mice (Bryant et al. 1988a); (2) the thymic atrophy produced by morphine-pellet implantation is primarily of the thymic cortex, a particularly sensitive area to the lytic effects of glucocorticoids (Bryant et al. 1988b); (3) the profile of events blocked by morphine in the lymphocyte proliferative cascade closely parallels steps blocked by glucocorticoids (figure 6); and (4) corticosterone levels are elevated in morphine-pelleted mice, with a timecourse that roughly resembles the suppression of mitogen-driven proliferative responses in these animals (Bryant et al. 1988b). Therefore, the immunosuppressive effects of 48-hour morphine-pellet implants were evaluated in adrenalectomized mice and in mice treated with the glucocorticoid receptor antagonist RU-486 in order to determine the contribution of adrenal corticosteroids to chronic morphine-induced immunosuppression. As reviewed in table 2, the magnitude of splenic and thymic atrophy was less pronounced in the adrenalectomized, morphine-pelleted mice. The relative degree of the attenuation of LPS-induced lymphocyte proliferation was also smaller in the adrenalectomized morphine-pelleted mice and morphine-induced reduction of Con A-stimulated proliferation was absent in the adrenalectomized animals (Bryant et al. 1988b).

Adrenalectomy represents a rather severe manipulation to the organism. Complete adrenalectomy also removes adrenergic outputs from the adrenal medulla, which may have some impact on immune function (Johnson et al

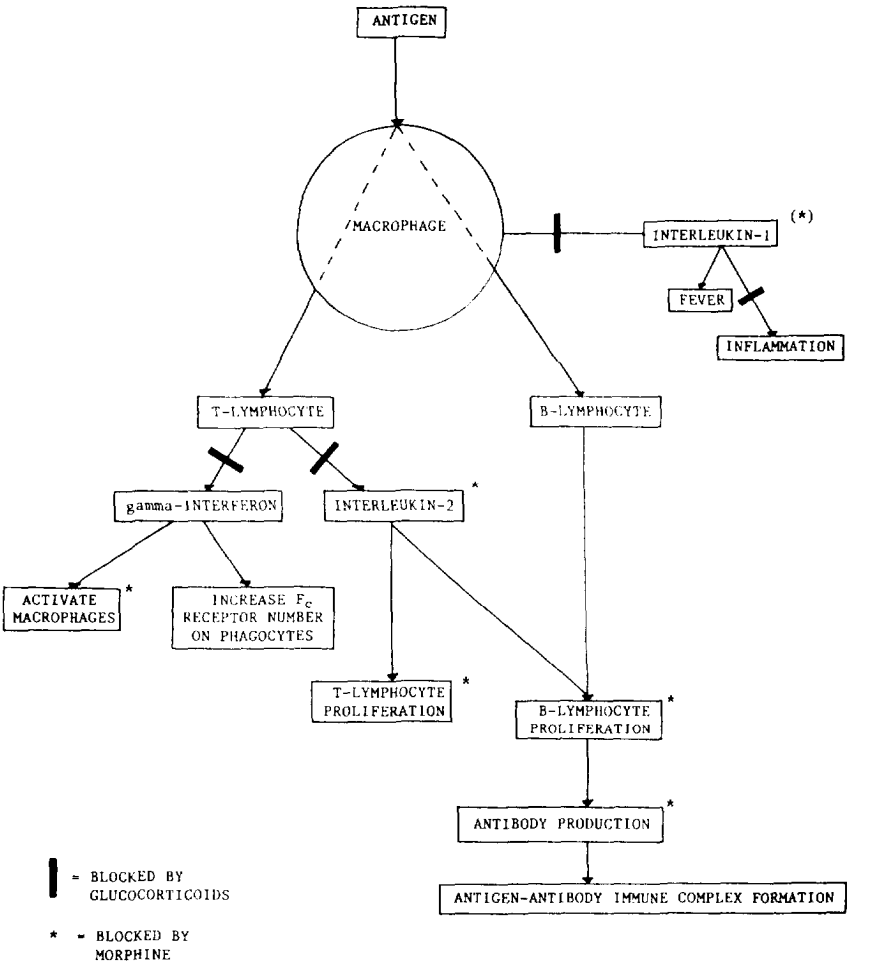

FIGURE 6. *Comparison of steps in the lymphocyte proliferative cascade that are sensitive to glucocorticoid administration (bars) and to chronic morphine treatment (asterisks)*

1981). Therefore, as a corollary to the adrenalectomy studies, the effects of RU-486, a steroid congener known to act as an antagonist at the glucocorticoid receptor were also examined (Moguilewsky and Philibert et al. 1984). Overall, effects similar to adrenalectomy were observed in morphine-pelleted mice given 20 mg/kg RU-486 twice per day of morphine exposure (table 2) (Bryant et al. 1988b). As with adrenalectomy, splenic atrophy was less pronounced with RU-486 administration. RU-486 had only marginal effects

on thymic atrophy and the suppressed proliferative responses of splenocytes from morphine-pelleted mice to Con A or LPS. The suppressed mixed-lymphocyte response in morphine-pelleted mice was also less severe when RU-486 was administered. These studies indicate that morphine-induced immunosuppression is at least in part due to morphine-induced activation of the HPA axis. In addition to direct activation of the HPA axis, chronic morphine treatment may lead to inflated circulating corticosterone levels indirectly, since morphine and corticosterone compete for the same metabolic enzymes for degradation and ultimate clearance (Holaday and Loh 1979).

TABLE 2. *Comparison of immunosuppressive effects in adrenalectomized (ADX) and RU-486* treated *mice*

Parameter	Morphine-pellet*		Morphine-pellet	
	Sham	ADX	Saline	RU-486
Spleen Weight	6.5±2	83±5	60±1	82±5
Thymus Weight	44±2	78±5	45±3	52±5
Con A-Induced Proliferation (0.25 µg/ml)	44±4	123±12	52±8	70±7
LPS-Induced Proliferation (0.5 µg/ml)	62±6	74±10	68±14	92±8
Mixed Lymphocyte Response	—	—	56±1	84±7

*48-hour morphine-pellet (75 mg) exposure period

NOTE: Values represent percent of mean placebo response ± SEM.

SOURCE: Bryant et al. 1988b.

Several lines of evidence suggest that the immunosuppressive effect of chronic morphine administration is related to its stimulatory effect on the HPA axis and subsequent mobilization of glucocorticoids. Traditional stress models are known to result in analgesia, adrenal hypertrophy, thymic atrophy, increased levels of circulating corticosterone and cholesterol, and hyperphagia (Neill et al. 1970; Stewart and Eikelboom 1981; Hulse et al. 1982; Morley et al. 1983; Rothfeld et al. 1983; Terman et al. 1984). Stress also results in attenuated blastogenic responses to mitogenic stimulation (Solomon and Amkraut 1981). Chronic morphine treatment produces a remarkably parallel pattern of responses (Pang et al. 1977; Holaday and Loh 1979; Lowy and Yim 1983; Bryant et al. 1987c). Morphine-induced

immunosuppression represents another effect of the opiate that is shared with chronic stress. This similarity has prompted the suggestion that chronic morphine treatment may be considered as a pharmacologic mimic of more traditional stress models. Finally, the immunosuppressive effects of morphine may have potentially important clinical relevance, particularly in individuals at a hign risk for infection or in immunocompromised patients. It has been speculated that the increased incidence of acquired immunodeficiency syndrome in narcotic drug abusers may have some basis in the immunomodulatory effects of the opiate. Indeed, research efforts reported in this monograph support this conjecture (Watson et al., this volume). Effects on immunocompetence should represent an additional area of concern regarding untoward effects in the development of novel opiate-related pharmaceuticals.

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Morphine-Induced Immune Modulation: Does It Predispose to HIV Infection?

Prince Kumar Arora

INTRODUCTION

While the prevalence of HIV seropositivity in the United States is highest for homosexual males (ranging from 20 percent to 70 percent), the incidence of seropositivity among intravenous drug abusers can be even greater, ranging from about 5 percent in certain areas to 75 percent in others (Centers for Disease Control 1984). Of all seropositives, intravenous drug abusers are generally regarded as posing the most substantial risk of spreading the disease to heterosexuals, with the risk being greater in urban areas (Centers for Disease Control 1984). Also at risk are infants born to parents who are intravenous drug users. Current statistics suggest that one out of two children born to seropositive women develop and almost certainly die of AIDS before the age of 5. There is growing evidence that drug abuse leads to an increased susceptibility to infections (Chetubin and Millian 1968; Sapira et al. 1970) and widely abused drugs such as the opiates have been shown to produce profound effects on the immune system (Cherubin and Millian 1968; Sapira et al. 1970; McDonough et al. 1980; Donahoe et al. 1985; Donahoe et al. 1986; Shavit et al. 1986; Yahya and Watson 1987; Bryant et al. 1988; Arora et al., in press). These findings prompted the study of the relationship(s) between drug abuse and increased susceptibility to infection.

This chapter will report the finding that opiate addiction causes marked atrophy of immune organs and a robust and sustained elevation of $CD4^+$:$CD8^+$ (T helpet/inducer:T suppressor/cytotoxic) and $CD5^+$:$CD8^+$ (T helper:T suppressor/cytotoxic) cell ratios in rodents implanted subcutaneously (SC) with a single morphine pellet. These observations demonstrate that morphine-induced changes in immune competence may predispose for the higher incidence of HIV infection in opiate abusers.

MATERIALS AND METHODS

Morphine Pellet Implantation

Male B10.BR mice (Jackson Laboratory, Bar Harbor, ME) were implanted SC with pellets containing either morphine (75 mg, free base) (Gibson and Tingstad 1970) or microcrystalline cellulose (placebo). Morphine and placebo pellets were prepared by K. Sones and G. Grimes of the National Institutes of Health (NIH) pharmacy. This regimen of morphine produced a mortality rate of 10 to 30 percent. These deaths occurred usually within the first 24 hours of pellet implantation and occasionally as late as 48 hours. The appearance of an abstinence syndrome following naloxone challenge (2 mg/kg, intraperitoneally (IP)) was used to assess the efficacy of pellet implantation. Both morphine- and placebo-treated mice were challenged with naloxone HCl 24 to 96 hours after implantation, and their behavior was assessed for an additional 15 minutes. An abstinence syndrome, including hyperactivity, vocalization, jumping, and wet-dog shakes, was apparent less than 3 minutes after naloxone treatment in morphine-treated mice. In contrast, naloxone produced only a transient hyperactivity in placebo-treated mice. Morphine treatment also resulted in a slight weight loss (less than 10 percent of the total body weight) at 72 hours compared to placebo-treated animals.

Organ Weight and Viable Cell Count

Mice were killed by cervical dislocation at 24-hour intervals following pellet implantation. Immune organs were removed, weighed, and placed in sterile Hanks balanced salt solution (HBSS) (GIBCO, Grand Island, NY) containing 10 percent heat-inactivated fetal bovine serum (FBS) (Hazleton Laboratories Inc.) (HBSS+FBS). Single-cell suspensions from individual organs were prepared as previously described (Arora and Shearer 1981). Cells were treated with ACK-lysing buffer (NIH media unit) to remove erythrocytes and washed twice in HBSS+FBS. Viable cell counts of the spleen and thymus were determined by trypan blue exclusion. Each data point is the mean plus or minus the standard error of the mean of five animals. These results represent three to four separate experiments.

Histopathology of Immune Organs

Histopathology of immune organs was done by Dr. Kim Waggie (Comparative Pathology Section, Division of Research Services, NIH). At 72 hours, both the spleen and thymus were removed from mice implanted with either morphine or placebo pellet. The immune organs were fixed in 10 percent formalin, embedded in wax, and slices cut by microtome. Single slices of the immune organs were stained with hematoxylin and eosin and examined under microscope.

Flow Cytofluorometric Analysis

At various time intervals following morphine-pellet implantation, single-cell suspensions from individual organs were prepared and stained with monoclonal antibodies (mAb) specific for cell-surface markers (sIg, Thy 1, CD4, CDS, and CD8) as described previously (Arora et al. 1987b) (table 1). In brief, $2x10^6$ cells were incubated on ice (in the dark) with 5 µl of appropriate fluorescent isothiocyanate (FITC)-conjugated mAb for 45 minutes. After two washes with HBSS+FBS (2 percent), the cell pellet ($2x10^6$ cells) was resuspended and analyzed by flow cytofluorometry. In some cases, cells were incubated with mAb, washed, and then incubated with a 5 µl of FITC-conjugated goat F(ab')-anti-rat IgG antibody specific for k-chain on ice for 30 minutes, washed, and analyzed. FlTC-conjugated monoclonal antibodies specific for sIg^+ cells were obtained from Organon-Teknika Corporation, West Chester, PA. FITC-conjugated antibodies specific for Thy 1^+lyt 1^+ ($CD5^+$). and lyt 2^+ ($CD8^+$) cells were obtained from Becton Dickinson, Inc., Mountain View, CA. $L3T4^+$ ($CD4^+$)-specific antibody was obtained from Becton Dickinson, Mountain View, CA). Goat anti-rat k-chain mAb was obtained from Organon-Teknika Corporation, West Chester, PA.

TABLE 1. *Surface markers used to define cell types*

Cell Surface Marker	Cell Type
sIg^+	B lymphocytes
$Thy1^+$	T lymphocytes
$CD4^+$	Helper/Inducer T lymphocytes (H/I TL)
$CD5^+$	All T lymphocytes
$CD8^+$	Suppressor T lymphocytes (STL)
	Cytotoxic T lymphocytes (CTL)

RESULTS

Time Kinetics of Morphine Effect on Immune Organ Weights

As shown in figure 1, implantation of a single morphine pellet had a marked effect on the weight of immune organs. A 40-percent reduction in splenic weight was observed within 24 hours of implantation of morphine pellet (figure 1, top panel). The magnitude of spleen atrophy became greater, with maximum suppression at 72 hours (65 percent reduction relative to placebo). This effect waned and then was undetectable after 6 to 8 days. Atrophy of the thymus in morphine-treated mice (figure 1, bottom panel) displayed a similar pattern. Thymic atrophy was most pronounced during 48 to 72 hours postimplantation (70 to 80 percent reduction relative

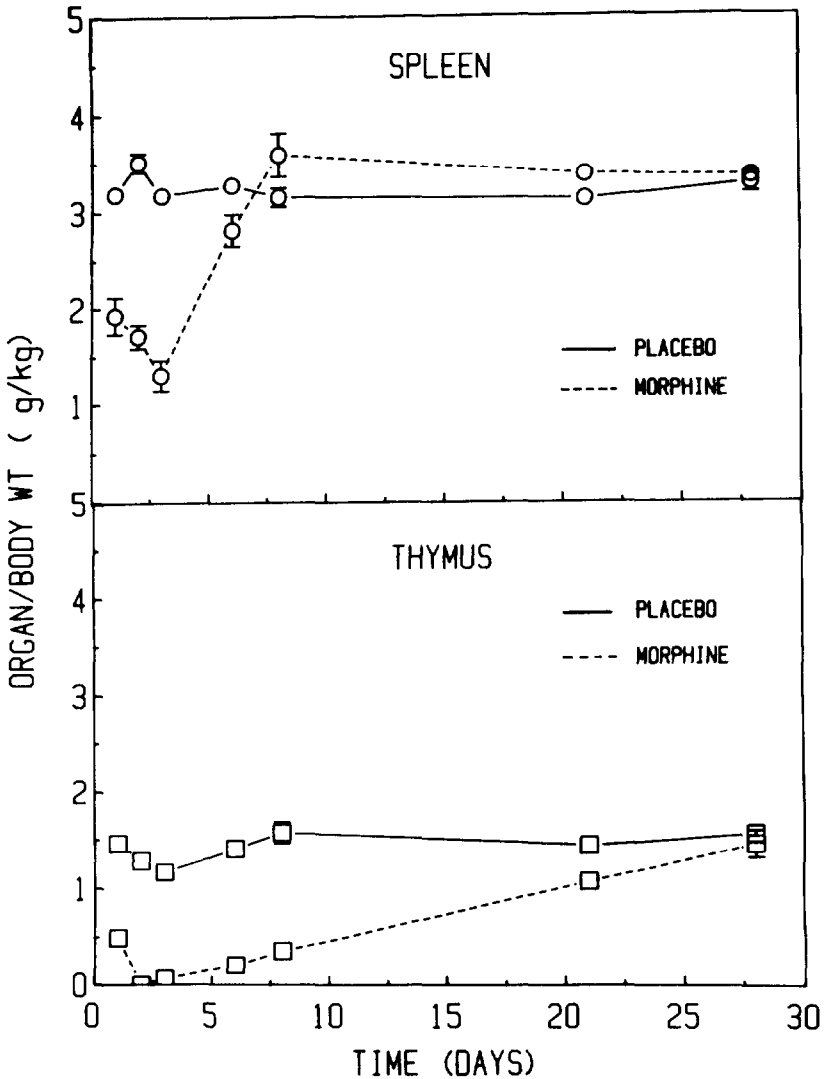

FIGURE 1. *Time course of morphine-induced effects on spleen and thymus wet weights in mice*

NOTE: Mice were sacrificed at various time intervals after implantation of a 75-mg morphine pellet (----) or a placebo pellet (—). The spleen and thymus were removed and weighed, and the weights corrected for the individual animal's body weight. Each data point represents the mean organ weight in g/kg of body weight ±SEM for five animals.

SOURCE: Arora et al., in press.

to placebo), but, unlike spleen, thymus size remained significantly smaller for a longer period (17 to 21 days postimplantation of the morphine pellet). A 10- to 30-percent rate of lethality was observed in mice given morphine-pellet implants. These deaths occurred usually within the first 24 hours of pellet implantation and occasionally as late as 48 hours.

Time Kinetics of Morphine Effect on Viable Cell Count in Spleen and Thymus

The reduction in spleen weight was associated with a decrease in spleen-cell number as shown in figure 2, top panel. As with splenic weight, the greatest reduction of spleen-cell number was observed at 48 to 72 hours postimplantation intervals (90 percent at 48 hours to 62.5 percent at 72 hours relative to placebo). At subsequent time points (17 to 21 days later), the spleen-cell number of morphine-pelleted mice returned toward that of the placebo group. Thymic atrophy was also associated with a decrease in cell number (90 percent at 72 hours to more than 9.5 percent at 96 hours relative to placebo group) in the thymus (figure 2, bottom panel), the magnitude of which paralleled the reduction in thymus weight. The recovery of cell numbers in immune organs occurred at different rates, but the reduced cell number persisted after normalization of organ weights had occurred (compare figure 1 with figure 2). Cell viability as assessed by trypan blue exclusion was unaffected by morphine treatment. Histopathological analyses of immune organs (spleen and thymus) demonstrated a significant depletion of the number of periarteriolar lymphoid sheaths (PALS) in the spleen of morphine-implanted (figure 3B) compared with placebo-implanted mice (figure 3A). In addition, a significant depletion of lymphocytes in the white pulp area was observed in spleens of morphine-implanted mice (figure 3D) compared to controls (figure 3C). Morphine treatment also caused a significant depletion of cells in the thymus (figure 4B compared to figure 4A). Thc localization pattern of cells in the thymus revealed depletion of thymocytes in the cortex region (figure 4).

Flow Cytofluorometric Analysis

Spleen. Figure 5 summarizes data on the cytofluorometric analysis of spleen cells. At various time periods following implantation of morphine pellet, cells were stained with FITC-conjugated monoclonal antibodies specific for cell surface markers. Morphine did not cause any significant change in the number of B (sIg^+) cells (figure 5B to figure 5E) when compared with the placebo group (figure SA). Within 24 hours following morphine-pellet implantation, there was a sharp increase in the number of T (Thy 1^+) cells in the spleen (figure 5F compared with figure 5G). The number of Thy 1^+ cells remained high for an additional 3 days (figure 5H to figure 5J). Figure 6 shows data on the number of $CD4^+$, $CD5^+$, and $CD8^+$ cells in the spleen of both placebo-pellet implanted animals (figure 6A, 6F, and 6K) and morphine-pellet implanted animals (figure 6B to 6E, 6G to 6J, and 6L to 60) at different time periods. The occurrence and

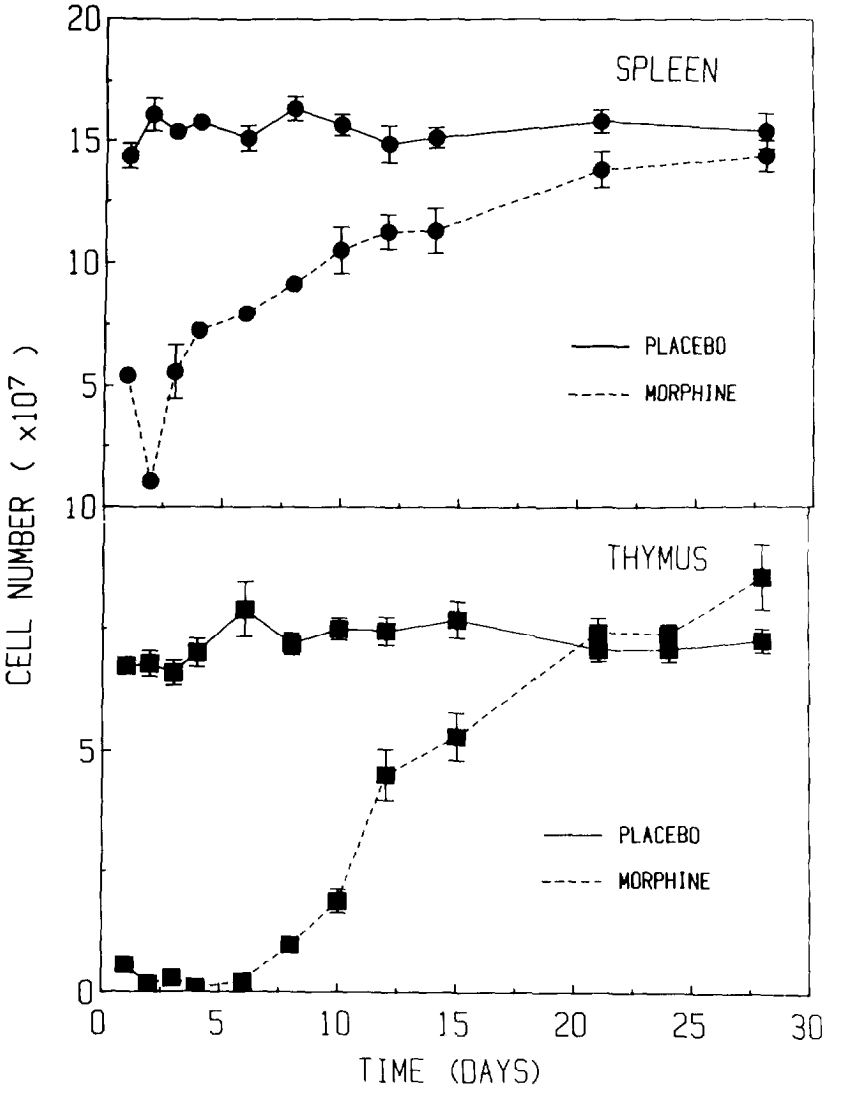

FIGURE 2. *Time coarse of morphine-induced changes in viable cell number in spleen and thymus at various time intervals after implantation of a 7.5-mg morphine pellet (----) or a placebo pellet (—)*

NOTE: Each data point represents the mean variable cell count ± SEM of five animals. these results are representative of 3 to 4 separate experiments.

SOURCE: Arora et al., in press.

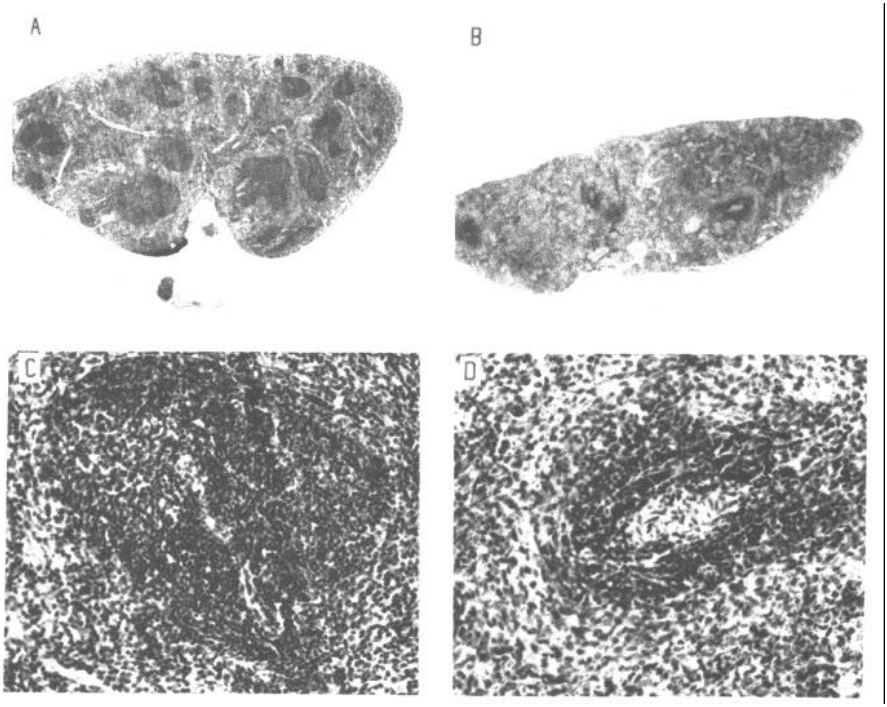

FIGURE 3. *Hermatoxylin and eosin staining of spleen 72 hours after implantation of mice with placebo pellet (A and C) or morphine pellet (B and D)*

NOTE: Note depletion of the number of periarteriolar lymphoid sheaths (white pulp areas) and loss of lymphocytes in the white pulp area. Magnification X 50 (A and B); X 200 (C and D).

SOURCE: Arora et al., in press.

significant increase in the number of $CD4^+$ and $CD5^+$ cells are readily evident in figures 6B to 6E and 6G to 6J, respectively. There was little or no change in the number of $CD8^+$ cells in the morphine-pelleted group (figure 6L to 60) when compared to the placebo group (figure 6K). The observed increases in the number of $CD4^+$ and $CD5^+$ cells occurred in 100 percent of the animals in the morphine-pelleted group.

Thymus. The cytofluorometric data on thymocytes shown in figure 7 indicatc that morphine caused a significant drop in the number of Thy 1^+ cells (figure 7B to 7E) when compared to the placebo group (figure 7A). Morphine also had an effect on the number of $CD4^+$ (figure 7G to 7J) and $CD8^+$ (figure 7L, to 7O). but the drop in the number of $CD8^+$ cells was

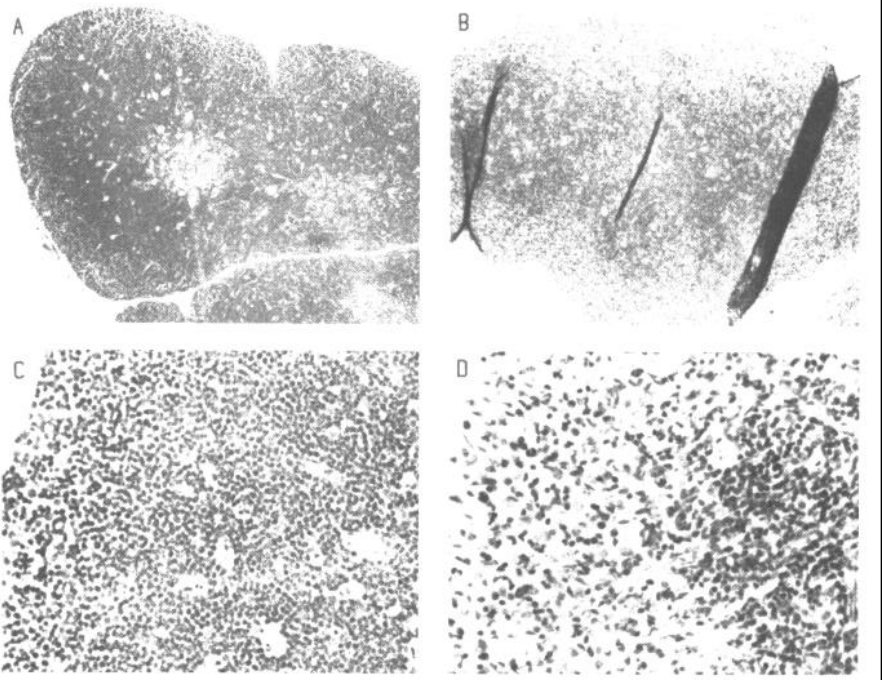

FIGURE 4. *Hematoxylin and eosin staining of thymus 72 hours following implantation of mice with placebo pellet (A and C) or morphine pellet (B and D)*

NOTE: Note the depletion of thymocytes in both the cortical and medullary regions. Magnification X 80 (A and B); X 310 (C and D).

SOURCE: Arora et al., in press.

more marked when compared to the drop in the number of $CD4^+$ cells at all time points examined.

Morphine Effect on Cell to Cell Ratios

As shown in table 2, morphine markedly increased the ratios of T:B, $CD4^+$:$CD8^+$, and $CD5^+$:$CD8^+$ cells in the spleen and $CD4^+$:$CD8^+$ cells in the thymus. The increased $CD4^+$:CDS^+ and $CD5^+$:$CD8^+$ ratios were due to increases in the number of $CD4^+$ and CDS' cells respectively, compared with placebo-implanted animals.

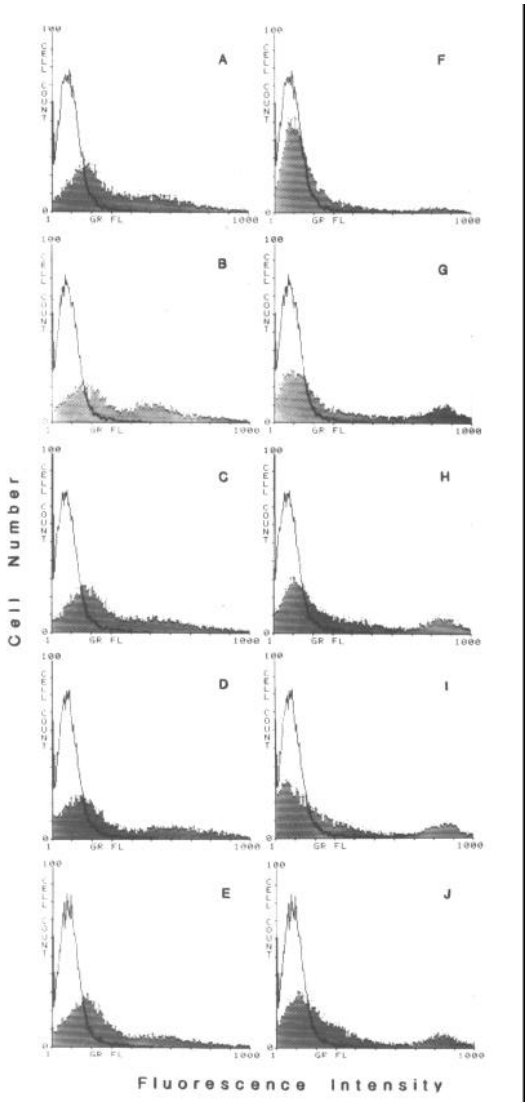

FIGURE 5. *Flow cytofluorometric analysis of spleen cells obtained from mice implanted with placebo or morphine, stained with FITC-conjugated goat anti-mouse or anti- Thy 1.2 antibodies*

NOTE: Cells obtained on day 1 (1B and 1G). day 2 (1C and 1H). day 3 (1D and 1I), and day 4 (1E and 1J) from mice implanted with placebo (1A and 1F) or morphine (1B to 1E and 1G to 1J) pellets were stained with FITC-conjugated goat anti-mouse IgG (1A to 1E) or anti-Thy 1.2 (1F to 1J) antibodies. Each cytofluorogram shows the fluorescence of stained (shaded area) vs. unstained (solid line) cells.

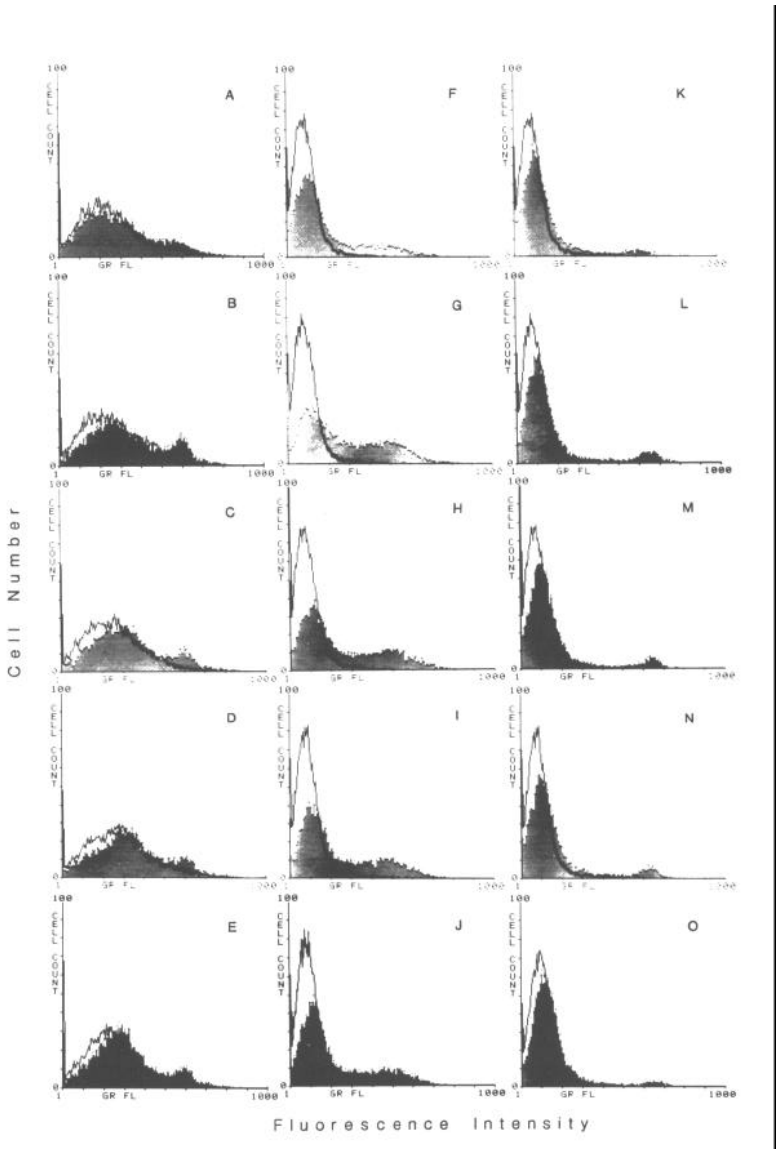

FIGURE 6. *Flow cytofluorometric analysis of spleen cells obtained from mice implanted with placebo or morphine, stained with CD4, CD5, or CD8 monoclonal antibodies*

NOTE: Cells obtained on day 1 (2B, 2G. and 2L), day 2 (2C, 2H. and 2M), day 3 (2D. 2I, and 2N), and day 4 (2E, 2J, and 2O) from mice implanted with placebo (2A, 2F, and 2K) or morphine (2B to 2E, 2G to 2J, and 2L to 2O) pellets were stained with $CD4^+$ (2A to 2E), $CD5^+$ (2F to 2J), or $CD8^+$ (2K to 2O) monoclonal antibodies. Each cytofluorogram shows the fluorescence of stained (shaded area) vs. unstained (solid line) cells.

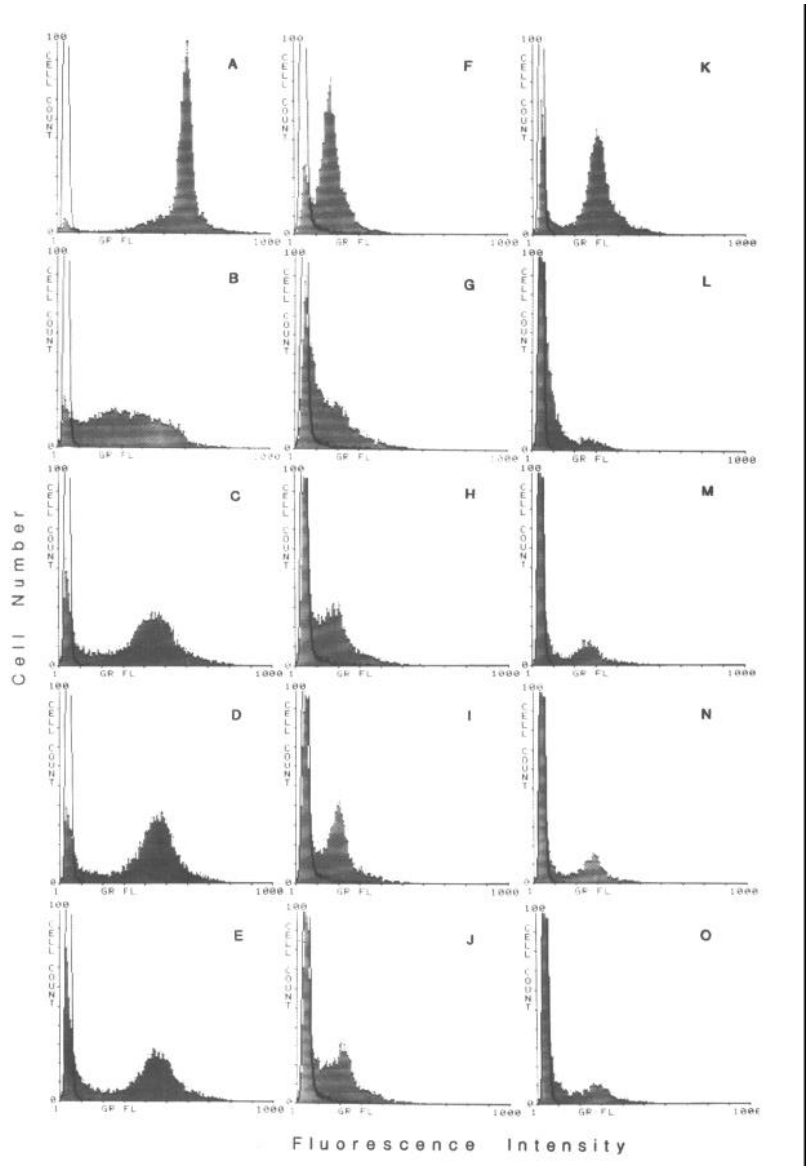

FIGURE 7. *Flow cytofluorometric analysis of thymocytes obtained from mice implanted with placebo or morphine*

NOTE: Ihymocytes obtained on day 1 (3B, 3G, and 3L). day 2 (3C. 3H, and 3M). day 3 (3D, 3I, and 3N), and day 4 (3E, 3J, and 3O) from mice implanted with placebo (3A, 3F, and 3K) or morphine (3B to 3E, 3G to 3J, and 3L to 3O) pellets were stained with Thy 1^+ (3A to 3E), $CD4^+$ (3F to 3J), or $CD8^+$ (3K to 3O) monoclonal antibodies. Each cytofluorogram shows the fluorescence of stained (shaded area) vs. unstained (solid line) cells.

TABLE 2. *Immunofluorescent analysis of spleen cells and thymocytes*

	Cell:Cell Ratio			
	Spleen			Thymus
Groups	Thy 1^+:sIg$^+$	$CD4^+$:$CD8^+$	$CD5^+$:$CD8^+$	$CD4^+$:$CD8^+$
Placebo	0.44	1.52	3.28	1.01
Morphine				
24 hr	0.82	2.56	5.21	2.54
48 hr	0.86	2.56	4.37	2.22
72 hr	0.84	2.16	4.24	2.11
96 hr	0.89	2.70	4.65	2.03
120 hr	0.34	2.66	3.39	N.D.

SOURCE: Arora et al., in press

DISCUSSION

While the mechanisms responsible for these morphine-induced changes in immune function are unknown, they may be mediated directly through opiate receptors present on lymphocytes (Sibinga and Goldstein 1988), indirectly via opiate receptors in the central nervous system, or by activating the hypothalamic-pituitary-adrenal (HPA) axis (George and Way 1955) to release immunosuppressive glucocorticoids (Parrillo and Fauci 1979). Morphine-induced changes in immune function have also been postulated to mimic the effects of stress (Bryant et al. 1987). However, while chemically induced stress (Arora et al. 1987a) and behaviorally induced stress (Laudenslager et al. 1983; Stein et al. 1985) can affect T-cell function, these procedures do not produce the rapid and profound changes in cell number or organ weight reported here.

The reduction in spleen and thymus size may suggest possible morphine-induced changes in lymphocyte trafficking because a decrease in organ weight was associated with a decrease in cell number. However, the observation that cell numbers in immune organs recovered at rates different from the normalization of organ weights would argue against the above possibility. Since opiate receptors are found on the surface of lymphocytes (Sibinga and Goldstein 1988), a direct action is also possible. *In vitro* studies, however, have generally found opioids to be stimulatory in nature (Wybran et al. 1979; Johnson et al. 1982; Gilman et al. 1982; Miller et al. 1984).

Cold stress has long been known to suppress immune function (Hardy et al. 1986). The hypothermia often observed in opiate-treated animals therefore

might act as a "cold stress" to suppress immunity. In the present study, morphine-treated mice did demonstrate a significant reduction of the body temperature at each time interval. However, at later time points, when body temperature of morphine-treated mice was back to normal, the cell numbers in the spleen and thymus were still reduced. These results would suggest that morphine-induced hypothermia may not be responsible for the immunomodulatory effects.

Chronic morphine treatment in itself may represent a "stressful" stimulus to the organism. Stressful stimuli have been known to have marked effects on immune function (Kelley et al. 1985; Arora et al. 1987a), and many of the effects observed in stressed animals, such as analgesia, hypcrcholesterolemia, hyperphagia, changes in thermoregulation and a variety of endocrine parameters, and prevention of the preovulatory leutinizing hormone surge are also observed in morphine-treated animals (Pang et al. 1977; Holaday and Loh 1979; Lowy and Yim 1983; Bryant et al. 1987). Morphine-induced immune modulation may thus represent another effect of this drug shared with chronic stress.

Tne morphine-induced changes in B cells; T cells; and $CD4^+$, $CD5^+$, and $CD8^+$ cells are temporally dissociated from the alterations in both cell number (figure 2) and organ weight (figure l), which suggests that these phenomena are regulated by separate mechanisms. Determination of B-cell; T-cell; and $CD4^+$-, $CD5^+$-, and $CD8^+$-cell numbers involves several steps, e.g., white-blood-cell count, differential counts to determine the percentage of lymphocytes, monoclonal antibody staining and fluorescent analysis to measure the percentage of B- and T-cell subpopulations. and then appropriate calculations. As a consequence, there is more variability in measured T-subset numbers than occurs with a single measurement. The difference between control populations and those just described in morphine-pelleted mice was nevertheless significant and was confirmed in several sequential periods of observations. Moreover, these changes in T-cell populations did not correspond to the development of either tolerance or dependence to morphine (Bryant et al. 1987). That these changes may have clinical relevance is supported by recent findings of opiate-induced increases in the $OKT4^+$ population that were positively correlated with the period of heroin use (Donahoe and Falek 1988).

The infection of $CD4^+$ (T helper/inducer) cells by HIV has been well described (Popovic et al. 1983; Chen et al. 1983; Klatzman et al. 1984; Kalish and Schlossman 1985; Fauci 1988), resulting in modulation of CD4 antigen, virus production, cytopathologic changes, and cell death (Klatzman et al. 1984; Kalish and Schlossman 1985). The CD4 antigen has been reported to be the HIV viral receptor (Dalgleish et al. 1984) and viral infection can be blocked by antibody to the CD4 antigen (Klatzman et al. 1984; Dalgleish et al. 1984). This unique viral tropism may explain many of the immunologic manifestations of AIDS. Moreover, HIV infectivity is

probably enhanced in proliferating $CD4^+$ cells (McDougal et al. 1985), suggesting that susceptibility to HIV may be increased under circumstances in which $CD4^+$ cells are elevated relative to other cellular elements. The presence of elevated levels of $CD4^+$ cells in the immune organs of animals chronically exposed to morphine suggests that this environment may facilitate access for HIV infection of these cells in opiate abusers.

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A Murine Retrovirus Model for Studies of the Role of Cofactors and Ethanol in AIDS Development

Ronald R. Watson

INTRODUCTION

Promising opportunities for the study of immunological changes, cofactor involvement in disease, and immunostimulatory drug testing involve a murine retrovirus model that causes many immunopathological functional changes similar to those seen in acquired immunodeficiency syndrome (AIDS). The effects of the LP-BMS murine leukemia virus infection on immune systems will be reviewed in this chapter, with special emphasis on T and B cells, and macrophages.

LP-BM5 infection suppresses cell functions while causing polyclonal lymphocyte activation. Murine immunological characterization, availability of inbred mouse strains, economy of using mouse vs. primate or human models, and similarity of the immune changes caused by murine retroviruses compared to those seen in AIDS caused by human immunodeficiency virus (HIV) encourage rapid development of the LP-BMS murine leukemia model. Ethanol consumption suppresses, while dietary vitamin A stimulates, immune functions during murine retroviral infection. High dietary vitamin A (retinyl palmitate) caused increased numbers of activated macrophages. It also increased the percentage of cells with markers for Ia^+ cells and macrophages in the retrovirally infected mice compared to infected controls. In uninfected mice, retinyl palmitate stimulated the percentage of cells with activated lymphocytes bearing IL-2R, and T cytotoxic cells. These were associated with a retarded death rate during infection with LP-BM5 murine leukemia in C57/BL6 mice. Thus, vitamin A stimulation may play a role in the slower death rate for retrovirally infected mice. Morphine treatment also increased the percentage of cells with markers for macrophages and activated macrophages in virally infected mice, while suppressing them in uninfected mice. Thus, these drugs of abuse can modulate the peripheral blood lymphoid subsets, which are suppressed by retroviral infection.

MURINE RETROVIRAL MODEL FOR STUDIES OF IMMUNO-MODULATORY COFACTORS IN AIDS

To understand AIDS and develop treatments, a range of animal models have been proposed (Mosier 1986). Models that simulate many aspects of AIDS, yet have the benefits of large numbers of subjects, low cost, and a well-defined immunological system, are critically needed for studies of the effects of cofactors such as alcohol, nutrition, and drugs of abuse. An animal system that meets the criteria of well-developed immunological reagents, well-characterized immune systems, and a variety of genetically inbred strains that can be infected with retroviruses, is the mouse. Just as individual retroviruses may transform different cell types and infect different organisms, it is likely that they impair the immune systems to some extent by differing routes. Understanding the precise cellular and subcellular mechanisms that lead to immunodeficiency in murine AIDS would represent a considerable step forward in the understanding of retrovirus-induced immunodeficiency in man and the role of cofactors, and a step forward in the development of appropriate therapeutic approaches. At least 60 percent of the research literature on retroviruses has dealt with a murine model, improving its value as a model system.

The family Retroviridae classifies all RNA viruses known to replicate by way of a DNA intermediate integrated in the host cell genome, with oncogenic ones grouped in the subfamily Oncovirinae. Many animal retroviruses are not associated with cancer, while some are agents of chronic degenerative diseases of animals, and others are entirely apathogenic. Immunodeficiency is among the major, nonneoplastic disorders associated with murinc retroviral infection. The ability to immunosuppress the host is shared by so many exogenous retroviruses infecting such widely divergent species that the property might be regarded as having significant survival value for such viruses. Viruses, including murine and human retrovituses, can potentially cause disease by at least four general methods involving immunomodulation: (1) as a result of viral replication, perhaps lysing or functionally impairing the lymphoid cells they infect; (2) immunosuppression from the activity of soluble factors of viral or host origin released from infected cells; (3) damage due to infection of cells involved in phagocytosis, antigen presentation, and nonspecific effector aspects of cell-mediated immunity; and (4) suppression of immunity from viral triggering of an imbalance in immune regulation with overactivity of T-suppressor or helper cells.

There are clearly some characteristics shared by murine and human retroviruses. For example, both virions are enveloped by a lipid bilayer derived from the host-cell membrane and contain a surface glycoprotein spike as well as an internal spherical core. They contain the genome and several enzymes, including the reverse transcriptase. The genomes are RNA resembling eukaryotic mRNA and present in two copies. Finally, all major events in replication are unique to retrovituses: synthesis of double-stranded

linear viral DNA in the cytoplasm of infected cells by a process that leads to the formation of long terminal repeats; integration of viral DNA into cellular DNA to form a provirus; and transcription of the provirus using cellular machinery to form new RNA genomes and mRNAs. The genome contains genes for the group-specific antigen *gag,* for the reverse transcriptase *pol*, and for envelope proteins *env.*

LP-BM5 MURINE RETROVIRUS INFECTION OF MICE

Mosier, Yetter, and Morse have characterized a murine retroviral disease model, LP-BM5 murine retrovirus, that approaches the immunological damage of AIDS in man (Mosier 1986; Salzman 1986; Buller et al. 1987; Mosier et al. 1987; More et al. 1988; Klinken et al. 1988; Yetter et al. 1985). Its characteristics form a syndrome similar to AIDS and will be described in some detail as a superb murine model. Quantitative loss of T-cell function occurs far in advance of reduction in the numbers of $L3T4^+$ ($CD4^+$) cells. Natural killer (NK) cell function is abolished early, but lymphocyte-activated killer function remains until 8 to 10 weeks after initiation of the infection (table 1). H-2-restricted cytotoxic T-lymphocyte (CTL) killing of allogeneic cells is depressed starting 3 to 4 weeks after infection. In the early stages of disease (through about 6 to 8 weeks after infection), addition of interleukin-2 (IL-2) restores CTL activity. After that time, addition of exogenous IL-2 has only a very small restorative effect, if it has any effect at all (Mosier et al. 1985; R. Yetter, personal communication). Therc are few early changes in other hematopoietic elements in the mouse model. LP-BM5 MuLV infection does not result in a general depression of bone marrow function, as seems to occur in other animal models for AIDS.

The virus mixture LP-BM5 was originally isolated by Latarjet and Duplan (1962) and produces nonneoplastic lymphoproliferative disease in adult mice. The ecotropic component of the LP-BM5 mixture seems to function to promote replication of the poorly replicating, pathogenic mink cell focus-forming component. LP-BM5 was obtained from a nonthymic lymphoma that had been induced by irradiation of mice. Then bone marrow stromal cells were isolated from the infected mice and, grown as cell lines, yielded the agent. It is a cluster of viruses that includes a B-tropic ecotropic virus and a B-tropic mink cell focus-inducing virus (Watson 1989). The latter virus alone does not cause immunological disease, but mice infected with a nonpathogenic ecotropic virus and then infected with the cell focus-inducing virus do develop disease. Thus, this virus or one that passages with it is the etiologic agent in the disease, with the ecotropic virus functioning to spread it in mouse tissues. The characteristics of the infection are that the animals develop hypergammaglobulinemia (IgM, IgE, and IgG), lymphadenopathy, T-cell functional immunodeficiency, and later neurological signs, including paralysis as well as opportunistic infections (Buller et al. 1987; Mosier et al. 1987; Salzman 1986). The virus infects macrophages, B cells, and, to a lesser extent, T cells. Lymphomas of monoclonal B cell origin

TABLE 1. *Comparison of changes in immune cells and functions induced by HIV-1 infection in man and LP-BM5 murine leukemia infection in mice*

Immunological Parameter	Presence During Retroviral Infection With HIV	LP-BMS MuLV
Abnormalities of B Cells		
Increased lmmunoglobulin Production	+	+
Increased Spontaneous Proliferation	+	+
Reduced Responsiveness to Antigens From Immunization and Infection	+	+
Abnormalities of T Lymphocytes		
Reduced Resistance to Opportunistic Infections and Neoplasms	+	+
Reduced Numbers of $CD4^+$ Cells (T Helper)	+	
Decreased Cytotoxic Lymphocyte Functions for Nonrettovitus, Vitally Infected Cells	+	+
Increased Spontaneous Proliferation	+	+
Diminished T-Helper Functions	+	+
Abnormalities of Mactophages		
Decreased Numbers of Activated Mactophages	variable	+
Reduced Numbers of Mactophages	+	+
Diminished *In Vitro* Pathogen Killing	+	
Spontaneously Increased PGE and IL-1 Secretion	+	+
Abnormalities of NK Cells		
Reduced Tumor Cell Cytotoxicity	+	+
Increased Activity With IL-2 Incubation *In Vitro*	+	+

SOURCE: Modified from Watson 1989d.

have occurred in a small percentage of infected mice, and a few mice develop neurologic symptoms near the end of the clinical course of disease (Mosier 1986). Molecular and genetic analysis of spleen cells showed that early in LP-BM5 polyclonal, there was proliferation of both B and T lymphocytes (Klinken et al. 1988). These cells infiltrate various organs including the brain, and they may be associated with the retrovirus induced, late-developing, neurological changes (Klinken et al. 1988). Derived cell lines continued to produce LP-BM5 and were B cell in origin. This is evidence of transformed B cells. The demonstration of terminal B-cell lymphomas caused by LP-BM5 MuLV infection extends the similarities of that infection to human AIDS (Klinken et al. 1988). Although lentiviruses were associated with human AIDS, and C-type retroviruses appear causative in murine AIDS, both are very similar in causing reduced resistance to pathogens, more neoplasias, and profound immunosuppression.

EFFECTS OF MURINE LP-BM5 RETROVIRUS INFECTION ON LONGEVITY AND DISEASE RESISTANCE

Infected C57/BL6 mice live 5 to 6 months before succumbing to disease or constriction of airways by expanding lymphoid tissues. They frequently die from respiratory failure due to mediastinal lymph node enlargement (Buller et al. 1987), while AIDS patients die from a variety of opportunistic infections and tumor-associated conditions. Exposure to an environment with many potential pathogens and normal flora in the animal rooms results in death much earlier from LP-BM5-induced immunosuppression by infectious disease (R. Yetter, personal communication). On the other hand, mice without functional T cells, infected with the retrovirus, survived past 11 months when the experiment was terminated. Infection of adult C56/BL6 mice with LP-BM5 MuLV leads to the rapid induction of clinical symptomatology with virtually no latent phase. Elevations in serum IgM levels can be detected 1 week postinfection, and lymphadenopathy and splenomegaly are apparent within 3 weeks. Although AIDS patients are susceptible to a variety of pathogens of the immunosuppressed, including *Cryptosporidium* and *Cryptococcus,* laboratory mice live in significant isolation from exposure to many pathogens, so the information on disease resistance is limited. Nonetheless, there is evidence of increased colonization by *Candida albicans* (R. Yetter, personal communication), with spontaneous secondary infections mainly at 3 to 4 months postinfection. These have involved primarily mouse hepatitis virus, and, more rarely, *Pneumocystis carinii* (Mosier 1986). Natural horizontal transmission of LP-BM5 MuLV infection has not been observed (Mosier 1986). The infection is experimentally transmitted by intraperitoneal (IP) injection of virus into either neonatal or adult animals. Initially uninfected mice cohoused with LP-BM5 MuLV-infected mice for up to six months show no signs of disease or viral infection (Mosier 1986).

Ectromelia virus (mousepox) is a naturally occurring agent that is transmitted via skin abrasions. While some mouse strains succumb to mousepox

readily, B6 replicates it at low levels and survives. Adult B6 mice have resistance to mousepox infection, which was significantly suppressed by the retrovirus LP-BM5. LP-BM5 MuLV *in vitro* and *in vivo* suppressed responses to antigens, so that no antibodies developed to subsequent infection with Ectromelia (Buller et al. 1988). Combined Ectromelia and MuLV infection resulted in increased numbers of deaths when mousepox infection occurred for a prolonged time after exposure to the retrovirus. Cytotoxic T-lymphocyte defenses did not develop to mousepox when its infection followed LP-BM5 MuLV infection by several months. This suggests that the retrovirus significantly suppresses directly or indirectly both B- and T-cell specific responses to this pathogen. Lethality due to mousepox was likely induced by immunosuppression due to the mink focus-forming cell component of the LP-BM5 virus mixture (Buller et al. 1988). Some mice resisted Ectromelia challenge when infected with the retrovirus, which may have been due to the continuing functioning of macrophages in some animals. Necrosis of the spleen similar to that in susceptible mice suggested that death was due to Ectromelia.

T CELLS DURING LP-BM5 MULV RETROVIRAL INFECTION

AIDS in man progresses to significant immunosuppression, as does the LP-BM5 MuLV infection in mice (table 1). The role of T cells is critical to AIDS development in man. HIV causes abnormalities in the immune system by depleting the $CD4^+$ T-cells and helper cells and by changing their functions. In addition, HIV activates B cells and infects macrophages, which can be critical to antigen presentation (Fauci 1988). LP-BM5 MuLV does not increase number or activity of T cells, nor are viral components or the various mixtures directly immunosuppressive (Mosier 1986).

While T cells are not the main source of viral replication, they are required for early pathogenesis and disease. LP-BM5 MuLV decreases T-helper cell functions. Although the number of T-helper cells stays relatively constant during early stages of infection, there is some suppression of T-suppressor cells, yielding an increased T-helper:T-suppressor ratio in mice (table 1). However, there is rapid T-cell activation (1 month postinfection) with reduction in response to viral antigens, *in vitro* mitogens, and MHC self-restricted antigens (Salzman 1986; Mosier et al. 1986). This is similar to humans infected with HIV who gradually lose T-cell functions (Fauci 1988). Loss of some of these functions may be due to reduced ability of cells from MuLV-infected mice to produce IL-2. However, mitogenesis is not restored by exogenous addition of IL-2, while responsiveness to MHC self-restricted antigens occurs early in the retrovirus infection (Morse et al. 1988). The mouse model diverges also from AIDS in humans in the extent of B-cell lymphoproliferation and in the absence of a long latent phase of infection (Fauci 1988).

The model has recently been further developed by studies with T-cell deficient C57B1/10 (mu/mu) nude mice (Mosier et al. 1987). In the absence of helper T cells, the mice were not killed by the effects of the retroviral infection during the 5 to 6 months as is usual and survived for 11 months, the length of the experiment. They did not develop many of the characteristics of the disease. The murine system makes possible studies requiring special strains and inbred mice for such immune reconstitution studies. Since viral replication of components of the LP-BM5 MuLV occurred to a similar extent (Mosier et al. 1987), the reduced development of the disease was not due to reduction in virus production. Rather, the development of murine AIDS in the mice required the virus mixture and functional T cells. The requirement for other cells such as B cells or macrophages was not determined. Since the virus was produced in similar levels, however, by the nude mice without mature T cells, it appears that lymphoid cells such as B cells, null cells, and macrophages/monocytes are the major sites of infection by the retrovirus (Mosier 1986). This is logical, as the polyclonal B-cell activation seen in human and murine AIDS requires several lymphokines, at least in normal B-cell activation in uninfected animals. The model was further extended by depleting the $L3T4^+$ helper T cells with the appropriate antisera, which also prevented development of murine AIDS (Mosier et al. 1988). Immunodeficiency in this murine model seems to significantly involve modification of B-cell activation via participation of T cells.

B-CELL FUNCTIONS DURING MURINE LP-BM5 MULV RETROVIRAL INFECTION

B cells and their production of immunoglobulins show a significant number of alterations due to retroviral infection in man (Fauci 1988). As mentioned above, there are substantial changes in B-cell functions due to the murine retroviral infection, in part due to changes in T- and accessory-cell functions (table 1). Activation of both T and B lymphocytes can be detected within 1 week of infection, as judged by flow cytometric analysis of the size of T and B cells and measurements of the percentage of B cells secreting IgM. There is an absolute increase in the number of B cells in the spleen and lymph nodes, and a threefold increase in the fraction of B cells in cell cycle (Mosier 1986). The proliferative response to both T- and B-cell mitogens is depressed by more than 80 percent; $L3T4^+$ helper function for T-dependent antibody responses *in vitro* and *in vivo* are virtually absent; and responses to T-independent antigens are vastly diminished. While T cells from LP-BM5 MuLV-infected mice will not provide helper activity to normal B cells for a specific antibody response *in* vitro, they do stimulate elevated polyclonal IgM secretion by normal B cells (Mosier 1986). During the early part of the MuLV infection, the B cells produce large amounts of immunoglobulin, with an increased number of plasma- or immunoglobulin-producing cells. There is widespread polyclonal activation of B cells (Salzman 1986; Mosier et al. 1985). The cells can become unresponsive to new antigens, either due to B- or T-cell defects. As the disease progresses,

there is a shift to production of only a few antibodies, even monoclona1 proliferation of B cells with development of B-cell lymphomas. Interestingly, responsiveness to mitogens (Mosier et al. 1985) *in vitro* and *in vivo* is suppressed and cannot be restored by T cells from uninfected mice. The later stages of the murine AIDS-like disease involve continued expansion of members of the B lymphocyte lineage, including all stages from pre-B cells to plasma cells, increased numbers of the monocyte- macrophage lineage, and appearance of immature T cells in the periphery (Mosier 1986). Although there is functional evidence of an early depletion of $L3T4^+$ cells, T cells bearing the L3T4 marker continue to be present throughout the course of the disease. The similarities between this murine model for AlDS and early stages of AIDS are striking in many respects. There is an early phase of B-cell hyperactivity and polyclonal activation in both, and the order of deletion of T-cell function is similar, with helper T-cell function lost well before cytotoxic/suppressor T-cell function (Watson 1989d).

The LP-BM5 mouse model diverges from AIDS in humans in the extent of B-cell lymphoproliferation and in the absence of a long latent phase of infection (Mosier 1986). This model also diverges in genomic composition, which is still incompletely known, LP-BM5 does produce an envelope protein *env*, *gag* antigen, and RNA-dependent DNA polymerase *pol*, with some similarities in function to similar proteins produced by HIV-l. It apparently lacks, however, the other genes of HIV-l. Information on whether the infection increases thymosin alpha-l, produces circulating immune complexes, alters delayed hypersensitivity responses, or reduces serum thymosin levels is lacking for LP-BM5. In addition, LP-BM5 does not stimulate production of antilymphocyte antibodies or suppressor substances. As knowledge of the cellular and subcellular mechanisms of immunosuppression retroviruses increases through use of murine models, even though they diverge from HIV-l genetically and functionally to some extent, general understanding will be expanded, with opportunities for more rapid application to human retroviral infections and treatment. Mice infected with LP-BMS do not pose a known risk of transmission of the agent to man (Mosier 1985; Mosier et al. 1985; Mosier et al. 1988).

Although murine retroviral infections are not complete models for human retroviral disease, the LP-BM5 MuLV model has many benefits for immunological studies (table 1). The viral infection simulates well key aspects of AIDS-related complex (ARC) and AIDS. LP-BM5 virus infects inbred mice, yet does so differently with the few strains tested to date, allowing mechanistic studies with the many immunological parameters in mice that are well defined. Although the virus has a rapid clinical course with no latent phase, it produces lymphadenopathy, hypergammaglobulinemia, and late neurological changes. Even though the murine virus is not very closely related to HIV, it does cause T-cell defects and polyclonal B-cell activation and infects and acts on macrophages, whereas in mice without T cells, there is no clinical disease. In both human and murine retroviral infection, there

are multiple alterations of lymphoid cells, and impairment of macrophages seems to be critical. Alteration of these cells, especially of their accessory functions, represents a unifying determinant in the genesis of many manifestations of retroviral-induced immunodeficiency. Mosier et al. (1985; Mosier et al. 1987) concluded that the LP-BM5 MuLV-induced disease is a good mouse model for profound immunosuppression by a retrovirus and that the model has considerable relevance for similar retrovirus-induced syndromes in humans.

CHANGES IN LYMPHOCYTE AND MACROPHAGE SUBSETS DUE TO MORPHINE AND ETHANOL TREATMENT DURING A RETROVIRUS INFECTION CAUSING MURINE AIDS

Dietary materials that suppress immune systems may also be critical in the rate of progression to AIDS after retroviral infection (Watson 1989a; Watson 1989b; Watson 1989c; Watson and Wallace 1989). Possible cofactors that are immunosuppressive include alcohol (Watson 1988b; Mufti et al. 1988; Watson et al. 1988b; Watson and Darban 1988; Watson 1988b) and morphine (Yahya and Watson 1987). Ethanol use has major effects on disease resistance depending upon the amount consumed and the duration. Alcohol-induced cirrhosis suppresses cellular immune functions as well as resistance to viral pathogens. There is evidence that ethanol acts as an immunosuppressive agent *in vitro* and in long-term exposure without liver disease (Watson 1988b). Morphine (Yahya and Watson 1987) also routinely suppresses cellular immune functions and reduces resistance to various pathogens. Retroviral infection in mice is an exciting system to study the effects of such cofactors on immune functions. It has many characteristics of AIDS, such that it has been suggested as an excellent murine model for infection with HIV in man. Therefore, the present investigation examines the effects of ethanol and morphine treatments on changes in numbers of regulatory lymphocytes and monocytes/macrophages during murine retroviral infection.

C57/BL6 female mice, 3 to 4 weeks old, weighing approximately 15 g, were obtained from Simonsen Laboratories, Inc., Gilroy, CA. They were routinely provided with Purina laboratory chow and water *ad libitum.* Thirty mice were injected with morphine sulfate IP every day for 7 days during the same weeks others were exposed to ethanol. The morphine was given at a daily dose of 75 mg/kg body weight IP. For the initial ethanol exposure, 30 mice were subjected to a Carnation Slender liquid diet containing 7 percent (v/v) ethanol (36 percent of calories) for the initial 7 days of the study as described previously (Watson et al. 1988b). Then all mice fed ethanol were again provided the chow diet for 3 weeks, before being fed a 5-percent ethanol diet for 1 week, which was 3 weeks postviral infection. Some mice were sacrificed; the remainder received the chow diet for 3 additional weeks, followed by 1 week of 5 percent ethanol diet. At this time,

8 weeks postinfection, more were sacrificed. Uninfected mice in groups of 20 animals were treated identically with each drug of abuse.

C57/BL6 mice were chosen because they had previously been observed to consume sufficient amounts of ethanol in a liquid diet to develop functional tolerance and physical dependence. Physical dependence on ethanol was achieved as evidenced by the high incidence of convulsions and tremors 4 to 8 hours after the removal of dietary ethanol. Mice used developed signs of ataxia several days before sacrifice. After the first week of exposure to drugs of abuse, each animal (30 mice per group) was injected IP with 0.1 ml of LP-BMS murine leukemia inoculum, which was donated by Dr. R.A. Yetter.

Identification of T cells isolated from the peripheral blood was carried out as described elsewhere (Watson et al. 1988b, Watson et al. 1988a). Ethanol increased the percentage of T-helper cells in virally infected mice compared to infected controls (table 2). However, ethanol consumption did not affect the percentage of any lymphocyte subsets except those with the activation markers, IL-2R, in uninfected mice, while morphine suppressed them.

TABLE 2. *Effects of morphine or dietary ethanol on frequency of lymphocytes identified by surface markers 8 weeks post-LP-BM5 murine leukemia virus infection*[1]

	Control		Ethanol		Morphine	
	Uninfected	Virus Infected	Uninfected	Virus Infected	Uninfected	Virus Infected
T Helper	33.5±4.9	24.3±1.1*	38.0±2.5#	41.0±2.6*#	25.6±1.5*	20.0±1.0*
T Suppressor/ Cytotoxic	20.5±0.7	11.3±0.5*	20.6±1.1#	19.0±2.6*#	9.3±0.6*	8.3±0.5*
Total T Cell	68.0±2.8	73.0±2.6	67.3±0.5	64.3±9.6#	73.6±1.5	59.0± 1.0#
B Cell (M u $^{\pm}$)	17.5±10.6	8.3±0.5*	26.0±1.0#	2.3±1.1#	11.0±2.0#	12.0±1.0#

[1]Mean ± SD (n=4).
*Significantly different from uninfected control.
#Significantly different from virus-infected control.

Similar effects were seen with the T-suppressor cells with both compounds. Neither drug of abuse significantly affected total T cells in uninfected mice, while both tended to suppress total T cells in infected mice. Both morphine and ethanol suppressed the percentage of activated lymphocytes identified by IL-2R on their surfaces in uninfected mice. Morphine had a

tendency to increase IL-2R bearing cells during infection as compared to infected controls (table 2).

As shown in table 3, LP-BM5 MuLV infection reduced significantly the percentage of peripheral blood cells with markers for macrophages and activated macrophages. Both ethanol in the diet and morphine injection reduced the percentage of these cells. Ethanol consumption by retrovirally infected mice had a trend toward a lower percentage of each of these cell types compared to ethanol-fed or virus-infected controls (table 3). However, morphine injection during infection increased the percentage compared to morphine-treated or virally infected controls (table 3). Extremely similar results were seen at 4 weeks postinfection with both compounds.

TABLE 3. *Effects of morphine and dietary ethanol on frequency of macrophages identified by surface markers 8 weeks post-LP-BM5 murine leukemia virus infection*[1]

	Control		Ethanol		Morphine	
	Uninfected	Virus Infected	Uninfected	Virus Infected	Uninfected	Virus Infected
Macrophage ($MAC1^+$)	21.5±14.8	8.0±1.0*	10.6±1.1*	3.0±2.0*#	5.3±1.5*	17.3±2.0#
Activated or Stimulated Macrophages ($MAC2^+$)	23.0±18.3	5.6±2.0*	17.0±2.6#	3.3±1.1*	7.3±2.0*	11.3±3.0#
Ia^+	15.0±7.0	5.6±0.5*	9.0±1.0*#	2.3±1.1*#	15.6±2.0	12.0±1.7#

[1]Mean ± SD (n=4).
*Significantly different from uninfected control ($p<0.05$).
#Significantly different from virus-infected control.

After 25 weeks of infection, there had been mortality in the infected mice, with 45.0 percent surviving. Among the controls not treated with a drug of abuse or infected, none died by 25 weeks. In the ethanol-fed, infected mice, however, there was greater mortality, with 8.6 percent surviving. There were deaths among ethanol-fed uninfected mice, with 41.7 percent surviving. In these mice, deaths occurred at the first two ethanol exposures when mice were small; in the ethanol-fed infected mice, deaths continued throughout the experiment, particularly from 20 to 25 weeks after infection. Among the morphine-injected, infected animals at 25 weeks postinfection, 44.8 percent survived. There were deaths after morphine exposure, and 38.4 percent of morphine-injected uninfected mice survived at 25 weeks postinfection, which was not significantly different. Survival was less in thc virus-infected, ethanol-fed mice than was expected, if deaths due to

exposure to ethanol and virus were combined without interaction (18 percent). On the other hand, survival in mice injected with morphine and retrovirus infected was higher than would have been expected by survival rates from morphine injection alone and virus infection alone (17.3 percent). The altered survival may be in response to several immunological changes. Even though the LP-BM5 MuLV-infected mice are more susceptible to infectious diseases, in clean animal facilities they are presumed to be relatively unexposed to pathogens. In addition, virus-stimulated lymphoid cells yield increased lymphomas and expanded lymphoid tissues, bringing premature death (Salzman 1986). Thus, suppressed or enhanced stimulation of lymphoid cells, particularly B and T cells, would be expected to change survival rates (Mosier et al. 1987). Finally, recent *in vitro* work shows that immunological stimulation of cells increases the likelihood that the retrovirus will be transmitted to other cells. Lymphoid cell activation measured by the percentage with activation markers were changed by these treatments. Thus, any of the above mechanisms could have been significantly involved in death rate changes.

Others have shown that T cells are critical to development of the murine disease with death, even in the presence of continued viral infection (Klinken et al. 1988). Nude mice without mature T cells did not die as early as the immunologically competent mice did. Thus, ethanol, which increased T-helper and T-suppressor cells and reduced the number of T cells in virally infected mice compared to infected controls, may exacerbate immunological changes caused by retroviral infection. Morphine may not produce these effects, as it tended to reduce the number of T cells, while relatively increasing macrophages in infected mice. The increase in activated macrophages due to morphine occurred concomitantly with an increase in macrophages, so that the ratio was similar to that for infected mice and lower than that for controls, particularly ethanol-fed mice. The effect of morphine to increase the percentage of activated lymphocytes and macrophages could increase the cells that can be infected by retrovirus. LP-BMS MuLV infects T cells, and such changes suggest that drugs of abuse modulate aspects of the cellular immune systems, particularly in retrovirally infected mice (Watson et al. 1988b). They could be cofactors in modifying the progression to AIDS by retroviruses.

CONCLUSION

The chronic, occasional exposure to dietary ethanol during retroviral infection significantly affected the numbers of various T subsets in the peripheral blood, while not affecting uninfected mice. This may be the first demonstration of a dietary component acting to uniquely alter T cells during a retroviral infection. Changes in numbers and activation of T cells and macrophages indicate potentially decreased host defenses. The fact that ethanol exposure similar to binges in man caused significant functional changes during retroviral infection needs more detailed studies. The levels

of ethanol in the blood were similar to levels attainable in man and were associated with withdrawal signs in the mice. Therefore, the existing evidence indicates that high levels of ethanol and/or its metabolites have the potential, during retrovirus infection, to alter the numbers of cells that can participate in cellular immune responses.

LP-BM5 MuLV infection resulted in suppression in the numbers of T-helper cells and macrophages, confirming previous results (Mosier et al. 1985; Mosier et al. 1987). It was found, in addition, that activated macrophages were reduced by the infection. The loss of both macrophages, as well as activated macrophages (MAC-2) and lymphocytes (IL-2R positive cells), may be critical in the eventual death and altered immune functions associated with the virus. It can explain changes in immunoregulation and reduction in disease resistance to pathogens caused by this retrovirus.

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Drug Abuse and AIDS: Causes for the Connection

Robert M. Donahoe

INTRODUCTION

The principal demographic factor connecting intravenous (IV) drug abuse with blood-borne pathogens like HIV1 is the needle sharing habits of addicts. Addicts who contract AIDS, in turn, serve as the major reservoir for its heterosexual and pediatric transmission. Because of these circumstances, over 25 percent of current AIDS cases in the United States are either directly or indirectly connected to needle sharing by IV drug abusers. Since public health measures have been largely unsuccessful in reducing the spread of AIDS in addicts, as compared with other demographic groups at high risk for AIDS, this percentage is on the rise.

Factors other than needle sharing are also relevant to the spread of AIDS by addicts. Prominent among these is the influence of the drugs themselves. By altering host immunocompetence, drugs of abuse represent a source of acquired immunodeficiency independent of HIV1, with the potential to alter susceptibility of addicts to HIV1, and the development of AIDS. This chapter explores potential causes for immunomodulation by drugs of abuse and their influence on the course of AIDS in addicts.

Opiates are the primary drug abused intravenously. However, because of increased availability and reduced cost, IV administration of cocaine has recently increased dramatically, particularly among heroin users. Therefore, most of the ensuing discussion focuses on the role of opiates and heroin addiction in modulating host immunocompetence, although much of this discussion is relevant to the immunological effects of behaviorally active drugs of abuse in general.

HISTORICAL PERSPECTIVE ON IMMUNOMODULATION BY OPIATES

A chronological documentation of observations that support the linkage between opiates and altered immune function appears in table 1. These and

related findings have recently been reviewed by several authors (Yahya and Watson 1987; Sabinga and Goldstein 1983). The evidence indicates that the functions of most, if not all, circulating cells of the immunological system are influenced by opiates, which clearly supports the notion that IV drug abusers can suffer from generalized drug-induced immunodeficiency (DII), which may influence the course of HIV1 infection. Importantly, the observations in table 1 also suggest that a specific target for opiate effects may be the HIV1 viroreceptor itself, the CD4 molecule, and that opiates affect immunological cells both directly and indirectly through effects on the neuroendocrine system.

TABLE 1. *Chronological history of key reports linking opiates with immunomodulation and AIDS*

Year	Observation	Authors
1909	Morphine depresses phagocytosis *in vivo* and *in vitro.*	Archard et al.
1928	Morphine addiction alters leukocyte differentials in dogs.	Pierce and Plant
1970	Infection and cancer are elevated in heroin addicts.	Sapira et al.
1972	Cytogenetic damage is elevated in lymphocytes of heroin addicts.	Falek et al.
1974	Heroin addicts exhibit depressed mitogenesis and elevated immunoglobulin levels.	Brown et al.
1979	Morphine depresses T cell E-rosette formation *in vitro* in naloxone-reversible fashion.	Wybran et al.
1980	Heroin addicts exhibit naloxone-reversible depression of T cell E-rosette formation.	McDonough et al.
1980	Phagocytes have opiate receptors.	Lopker et al.
1980	Morphine injected into mice alters immune function.	Gungor et al.
1983	Heroin addicts are a high-risk group for AIDS.	Wormser et al.
1983	Rodents injected with morphine are more susceptible to infection and cancer.	Tubaro et al.
1984	Opioid-mediated stress depresses NK function.	Shavit et al.
1985	Inhibition of receptor microdisplacement defined as cause for opiate effects on E-receptors.	Donahoe et al.
1985	Th/Ts ratios depressed in heroin addicts in the absence of HIV1.	Des Jarlais et al.
1986	Morphine modulates CD2, CD4, and CD8 *in vitro.*	Donahoe et al.
1987	Purified human T cells have opiate-binding sites	Madden et al.

GENERALIZED IMMUNOLOGICAL EFFECTS OF OPIATES: THEIR ROLE IN AIDS

There is no clear understanding of the role of immunity in protection of a host against infection with HIV1 or containment of the infection thereafter. The membrane-budding nature of the virus suggests that protection or containment will require both cell-mediated and humoral branches of the immune response. However, the types of immune response that are most pertinent are unknown. Therefore, despite evidence that opiates modulate the function of T and B cells, macrophages, polymorphonucleocytes, and natural killer (NK) cells, it is largely uncertain how such effects might influence susceptibility of addicts to HIV infection and development of AIDS. A possible exception to this generalized uncertainty relates to the finding that opiates modulate expression of the HIV1 viroreceptor, the CD4 molecule. This exception is detailed later in this discussion.

One approach to defining the relationship between generalized immunological effects of opiate addiction and AIDS is to use experimental animal models. Investigations with rodent models (Tubaro et al. 1983) have shown that morphine alters animal susceptibility to bacterial infection and tumor transplantation. However, these findings are difficult to relate directly to the AIDS situation, since the pathogens and hosts involved in the rodent model systems are far removed from the circumstances inherent to AIDS. Rodent models directed at opiate effects on AIDS-like viruses would of course be more appropriate. However, the relevance of such models to human AIDS is open to debate from at least three different aspects of experimental design: neuroendocrine/pharmacological relevance; immunological relevance; and virological relevance.

To maximize the relevance of an animal model to the circumstances of human AIDS, the author has established a rhesus monkey model. Unquestionably, a monkey model is phylogenetically close to the human situation. Especially relevant is the shared susceptibility of monkeys and man to very closely related immunodeficiency viruses. Use of a monkey model is aimed at establishing the effects of morphine on the immunological status of monkeys subjected to a standard addiction paradigm and at determining their subsequent susceptibility to infection with the M-9 strain of simian immunodeficiency virus isolated from sooty mangabey monkeys (SIV/SMM). This virus crossreacts serologically with HIVl, infects human lymphocytes, and causes an AIDS-like syndrome in rhesus monkeys (Fultz et al. 1986). Two separate experimental approaches are being taken: (1) direct examination of the pathogenicity of SIV/SMM in animals simultaneously being addicted to morphine; and (2) examination of the long-range immunological effects of morphine addiction before testing animals for susceptibility to SIV/SMM. Preliminary data from the first approach suggest that intercurrent morphine addiction does not alter the ability to isolate SIV/SMM from the blood of infected monkeys, unless the animal is stressed by withdrawal from opiates.

Data from the second approach indicate that cell-mediated immunity is altered in addicted monkeys in comparison with controls, within weeks of injection of opiates. The main parameters affected are T-cell numbers and percentages of various T-cell subtypes, blastogenic responsiveness, mixed-lymphocyte responsiveness, and NK activity. Interestingly, the effect of morphine, to some extent, appears to be one of aggravating stress-mediated immunodeficiency inherent to the manipulation of the monkeys and their subsequent adaptive response. Further study with this model will reveal whether pathogenicity associated with SIV/SMM infection will be altered by morphine addiction.

THE AIDS VIRORECEPTOR CD4 IS MODULATED *IN VIVO* AND *IN VITRO* BY OPIATES

In 1980, McDonough et al. reported that heroin addicts typically have depressed levels of T cell E-rosettes, which are reversible *in vitro* by naloxone. This observation led to the demonstration that heroin addicts also experience depressed ratios of their T-helper and T-suppressor lymphocytes (Th/Ts) (Donahoe et al. 1987) in the absence of infection with HIV1, a finding corroborating that of Des Jarlais et al. (1985). Notably, addicts who do not exhibit depression in their Th/Ts, as a group, exhibit increasing percentages of circulating Th cells corresponding to the duration of their addiction. While substantiating further the immunomodulatory potential of addiction, such observations also suggest that addict susceptibility to HIV1 may be affected by alteration of the expression of Th cells, the principal target cells for HIVl.

To better establish the relevance of these *in vivo* findings suggestive of opiate effects on T-cell functions and to identify possible mechanisms involved, the *in vitro* effects of morphine on E-rosette formation and expression of the antigenic markers of Th (CD4 molecules) and Ts (CD8 molecules) lymphocytes as well as their CD2 markers were investigated. Since the nature of the E-rosetting phenomenon was incompletely understood at the time these investigations started, experiments were devised to assess kinetics of E-rosette formation to define the processes involved and the effects of opiates thereon. Multiphasic kinetics of E-rosette formation were observed (Donahoe et al. 1985) under certain thermal conditions (reciprocally increasing and then decreasing). These kinetics were indicative of cyclical processes of E-rosette formation and loss. They suggested that the effect of opiates on these processes was to inhibit conformational changes in the E-receptor molecule, known as microdisplacement, that are responsible for allowing dormant or cryptic E-receptor molecules within the plasma membrane to be up-regulated.

Adaptation of the kinetic procedures for assessment of E-rosette formation to cytofluorometric types of analyses resulted in procedures capable of measuring the effects of morphine on CD2 expression at the molecular level.

Initially, a single-color, antigenic-marker-staining paradigm was used to detect effects of morphine on CD2. Later, assessment of CD2 was done simultaneously with either CD4 or CD8 by using standard double-staining paradigms. These studies (Donahoe et al. 1987) showed that morphine modulated all three T cell surface markers (CD2, CD4, and CD8). Their expression was affected by cyclical processes of receptor modulation as had been seen with the E-rosetting procedures. Transmodulation between receptor molecules and endocytic processing appears to be the primary process involved in this modulation. Similar, longer range *in vitro* effects of endorphin on CD4 expression have also been reported by Puppo et al. 1985. These observations indicate that the modulation of surface markers observed with T cells from heroin addicts to some extent must be mediated through their direct interaction with opiates. This conclusion is supported by numerous reports documenting the presence of opiate-binding sites on lymphocytes and other cells of the immune system (Sabinga and Goldstein 1988), including a study done with the author's colleague (Madden et al. 1987) incorporating rigorous pharmacological criteria and preparations of highly enriched T cells.

In a separate study (Bueso-Ramos et al. 1988), it was also found that selected anti-CD2 and anti-CD4 monoclonal antibodies reciprocally down-regulate the mutual expression of CD2 and CD4 molecules in both the presence and absence of morphine. This mutual down-regulation was evident for the Leu3a but not the OKT4 epitope of CD4. This is notable because the Leu3a epitope is positioned near the NH, terminus of the CD4 molecule on the "outermost" part as it protrudes from the cell membrane and is responsible for HIV1 binding (figure 1). Thus, such Leu3a modulation could be projected to interfere with HIV1 infectivity by directly altering viroreceptor expression. Since *in vitro* data suggest that a long-range (hours-days) effect of opioids is depression of CD4 expression (Puppo et al. 1985), it seems logical to project that such effect would reduce chances of HIV1 infection of Th cells *in vivo*. However, studies using monoclonal antibodies to block HIV1 infectivity for Th have shown that lack of a full complement of CD4 receptors is insufficient cause to reduce HIV1 infectivity (Weber and Weiss 1988). In lieu of stoichiomctry of CD4 as the deciding factor in determining susceptibility of Th for HIV1, it may be that generalized immunomodulation by opiates, including its effects on CD4 expression, is a more pertinent factor. Another factor may be that modulation of CD4 expression by morphine affects feedback mechanisms controlling circulating levels of Th. Such effect would be harmonious with the previously discussed findings (Des Jarlais et al. 1985; Donahoe, et al. 1987) that heroin addicts frequently experience elevated circulating levels of Th cells. In this way, chronic addiction leading to increases in Th could be expected to increase the likelihood of HIV1 infection. Obviously, further study is needed to separate strictly immunological effects from viroreceptor effects of opiates as contributing factors in the pathogenesis of HIV1 infection in heroin addicts.

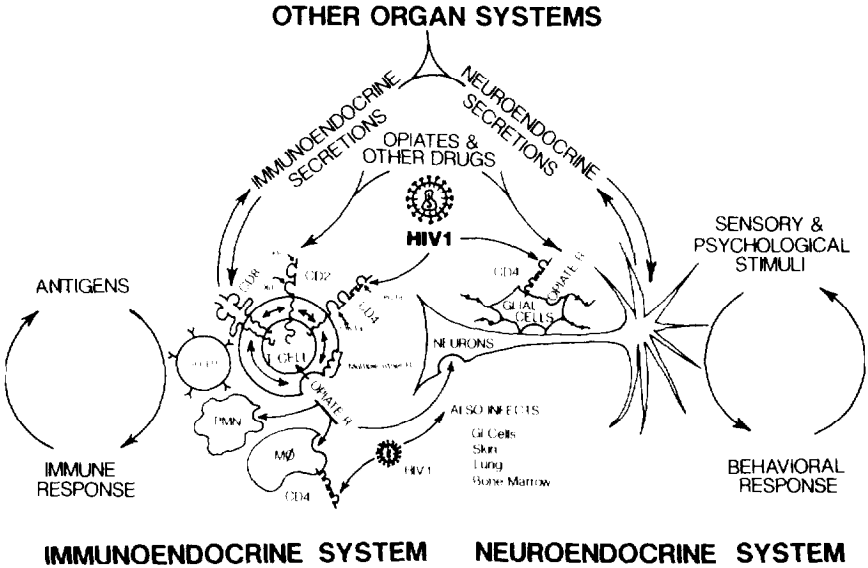

FIGURE 1. *Effects* of *opiates and HIV1 on the intersystemic network connecting the neural and immune systems*

NOTE: Opiates and other drugs of abuse are exogenous modulators of this network. These drugs, their endogenous congeners, and the hormonal products influenced by them work together to set the homeostatic tone of the various physiological response systems in the network. A common focus of these effects is the plasma membrane of affected cells. Here, they modulate expression of surface receptor molecules relevant to both behavioral and immunological functions. As illustrated in the T cell, this receptor modulation occurs within the membrane itself via transmodulation as well as via second-messenger effects (note that the T cell is represented as a thymocytic precursor of CD4+ and CD8+ circulating T cells). Since the CD4 molecule is both an HIV1 viroreceptor and a key regulatory component of immunoresponsivity. its modulation in an addictive setting is bound to be critical to the immunocompetence of addicts and their susceptibility to AIDS. According to this network theme, any perturbation in the network including psychological stressors, may affect CD4 expression, immune function, and susceptibility to HIV1 and its complications.

THE EFFECTS OF OPIATES ON IMMUNOCOMPETENCE MAY BE BOTH DIRECT AND INDIRECT

Opiates are also likely to affect susceptibility to HIV1 infection and AIDS in more indirect ways than those outlined above. One way is to moderate immunoresponsiveness indirectly through alterations in the neurocndocrine system. Another way is to affect the production of, and response to, peptides and hormones secreted by leukocytes that are connected to endogenous opioid circuitry, such as immunosecreted enkephalins and endorphins and other analogs of classical neuroendocrine hormones produced by T cells and other leukocytes (Weber, this volume).

In regard to the latter consideration, there is presently no specific information to determine how heroin addiction might be influenced by such factors. Nonetheless, it is inconceivable that secretion of, or response to, enkephalins and endorphins secreted by T cells and other leukocytes (and, consequently, immunoresponsiveness affected by these factors) would not be influenced by heroin addiction. Regarding the possibility of indirect neuroendocrine influence, the study of Shavit et al. (1984) probably best illustrates that the immunological effects of heroin addiction are also mediated to some extent by opiate effects on the central neuroendocrine system. The study showed that splenic NK activity in rats is depressed by a naloxone-reversible type of footshock stress but not by a nonreversible type. Shavit et al. (1986) also showed that NK activity is depressed after both systemic and intracerebral administration of morphine to rats but not with a systemically administered morphine analog that does not traverse the blood-brain barrier. Both studies led to the conclusion that NK activity of rat spleen cells was centrally mediated by opiates, although a precise neuroanatomical site regulating immune function has proved elusive. Weber and Pert (in press) and Weber (this volume) have extended these findings. Using the technique of focal microinjection of morphine through guide cannulae aimed at various brain structures, they show that opiates act in the periaqueductal gray matter (PAG) of the mesencephalon, but not in other opiate-receptor-containing neuroanatomical sites, to produce suppression of NK cell activity. The observed suppression is mediated through opiate receptors, since it can be blocked by naltrexone, an opiate antagonist. These findings suggest that opiate release and subsequent action in the PAG may be components of an important neural pathway involved in stress-induced immunosuppression and illness and neoplasia associated with it. A complexity is introduced into these circumstances, however, when the data of Kay et al. (1984) that show that morphine and endorphin enhance NK activity directly *in vitro* are considered. This finding suggests that systemic regulation of NK activity differs from the *in vitro* circumstance.

Interestingly, studies with rhesus monkeys indicate that NK activity is enhanced 2 weeks after initiation of four-times-daily injections of morphine. The enhancement observed is counter to depressed effects in a number of T cell functional parameters. Since the data from these experiments indicate that control levels of T cell responsivity are rebounding to historical norms, while levels for morphine-exposed animals are not, it appears that the control animals are rebounding from the effects of an undetermined stressor common to the experimental conditions, but that the animals exposed to morphine are not. This suggests that the NK enhancement observed is related to effects of opiates on some stress-mediated phenomenon and may, therefore, be centrally mediated. It is, however, unclear how these data relate to those of Shavit et al. (1986), since their findings indicated depression of NK activity by morphine, not enhancement. Kraut and Greenberg (1986) have also shown enhanced NK activity in response of mice to morphine injection, so the reasons for such differences are unclear.

Clearly, differences in species and experimental protocols used must be considered when assessing the immunological effects of opiates.

AIDS AND OPIATES: CAUSES FOR THE CONNECTION

The foregoing discussion suggests that opiates govern immunocompetence in human addiction in a variety of ways. The possibilities in this regard are represented schematically in figure 1, which illustrates the interdependencies of the immune system with other organ systems as they are influenced by opiates and HIV1. Since both opiates and HIV1 directly influence both the immunoendocrine and neuroendocrine systems, and these systems are networked between themselves and other organ systems, there is an understandable uncertainty about the exact role of opiates in AIDS.

The complexities of this issue are extended by the uncertain relevance of *in vitro* and animal-model studies to the situation of human addiction and by the uniqueness of the HIV1 pathogen, which is the final determinant in host susceptibility to AIDS. Unquestionably, innate interindividual variation is also relevant to determination of host response to pathogens and drugs, as are other environmental factors that interdict in the intersystemic network impacted by addiction, as in figure 1. Yet, despite the complexities of the intersystemic interdependencies illustrated in figure 1, there are some comforting commonalities that allow better understanding of the AIDS-drug connection and provide direction for future research. It is the cells of the immune system themselves that are most pertinent in this regard. In the final analysis, it is the response of these cells to the addictive milieu that decides immunocompetence. Thus, we need to know how drugs affect these cells directly and indirectly through the pathways delineated in figure 1 so that this knowledge can be integrated with that concerning infectivity and pathogenicity of human immunodeficiency viruses.

We have noted that opiates and other drugs of abuse (cocaine and alcohol) have a common ability to modulate surface molecules on T cells (Donahoe et al. 1986). Though this effect is common, the causes arc diverse. With opiates, the effects are presumably mediated through receptor-ligand interaction, whether it is the T cell acting directly with the opiate or some hormonal product resulting from other systemic and centrally mediated points of opiate-receptor stimulation. With alcohol, as another example. the cause for surface molecular modulation appears to principally involve alterations in membrane fluidity, which influence receptor activity within the membrane of both the T cell and the cells with which it interacts within the intersystemic network, as outlined in figure 1 (Madden et al. 1984; Donahoe et al. 1986). With cocaine, a key effect may relate to its anesthetic properties of Na-channel blockade, which could alter surface-receptor expression within the network in various ways (Donahoe et al. 1986). Importantly, it is equally likely that environmental stressors can influence this situation in the same way as drugs, because the intermediaries of the stressors are the

endogenous congeners of the drugs or products of endogenous responses to them. The main point is that by altering receptor expression, which all drugs of abuse do as mimics of endogenous peptides and hormones, behavior of the cells affected will be changed. This is the principal commonality of importance that is to be derived from figure 1, receptor change will lead to behavioral change. Since AIDS viruses and opiates mediate these changes to some extent at the same immunoregulatory molecule, CD4, these factors are inextricably linked in the puzzle that is AIDS.

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Immune Function in Heroin Addicts and Former Heroin Addicts in Treatment: Pre- and Post-AIDS Epidemic

Mary Jeanne Kreek

INTRODUCTION

According to the various surveys of the National Institute on Drug Abuse particularly the DAWN survey, and also the New York State Division of Substance Abuse Services, there are now over 2 million people in the United States who abuse heroin, of whom 500,000 are classified as "hard-core" heroin addicts. "Hardcore" is defined as repeated use of heroin three to six times a day for over 1 year, with development of tolerance, dependence, and drug-seeking behavior (Kreek 1987b; National Institute on Drug Abuse 1983; National Institute on Drug Abuse 1987). There are probably another 500,000 who use heroin regularly, but not necessarily for the length of time or to the extent defined above. Most of this heroin use is by needle. Also, the DAWN survey suggests 22 million persons in the United States have used cocaine at some time, and approximately 6 million have used it recently, suggesting that use may be regular (Kreek 1987b; National Institute on Drug Abuse 1983; National Institute on Drug Abuse 1987). Alcohol is still the most commonly misused agent, with problem drinking occurring in approximately 11 million and alcoholism in approximately 6 million persons in the United States. In New York City, there are approximately 250,000 hardcore heroin addicts at this time. In the DAWN surveys, which are carried out at regular intervals, a change was found between 1985 and 1987 with respect to the top five drugs (excluding alcohol use alone) mentioned in emergency room visits: cocaine now heads the list, alcohol in combination with other drugs is now second. and heroin has dropped to third position. However, in the medical examiners' surveys, alcohol in combination with other drugs still leads the list of deaths due to drug abuse. Heroin is second; cocaine is third; and two other opiates, propoxyphene and codeine, are in fourth place.

In this chapter, a historical survey of what my research group and others found to be immunological abnormalities in street heroin addicts prior to the advent of human immunodeficiency virus (HIV) infection, the acquired immunodeficiency syndrome (AIDS) epidemic, and the rapid increase in the use of cocaine is presented. Although recent retrospective studies of the HIV epidemic suggest that the cocaine epidemic may have occurred even earlier, cocaine abuse escalation was recognized in 1981 when the price dropped, availability increased, and the numbers of regular users rose dramatically over the next 3 years (Novick 1989b). An even greater escalation in the magnitude of cocaine abuse occurred around 1985 when the very potent, free-base form of cocaine, "crack," which is usually self-administered by smoking with inhalation of the drug, became available. AIDS was first diagnosed in 1981 and was recognized regularly in intravenous (IV) drug abusers in New York City by 1982. Retrospective studies have documented that HIV infection reached the parenteral drug-abusing population of New York City around 1977 to 1978 (Des Jarlais et al. 1989; Des Jarlais et al. 1984; Novick et al. 1986b; and Novick et al. 1986c).

PRE-AIDS IMMUNE FUNCTION IN HEROIN ADDICTS

The first historical era for this review of immunological information is 1966 to 1978, prior to the HIV epidemic, prior to common cocaine use, and in fact prior to any large-scale pharmacological treatment of narcotic addiction, although methadone-maintenance treatment had already started as an experimental treatment. From 1966 to 1968, there were a few reports appearing from New York City and also from Lexington, KY, of immunological abnormalities in heroin addicts not in methadone-maintenance treatment either entering detoxification treatment or being seen at post mortem (Cherubin and Milliam 1968; Helpern and Rho 1966; Louria et al. 1967; Sapira 1968). In the late 1960s the medical examiner of the city of New York reported diffuse lymphadenopathy at time of post mortem in essentially all deaths of what was called "narcotism," which was heroin overdose (Helpern and Rho 1966). This is a very important early finding to be reconsidered, because this common physical finding in IV drug abusers prior to the advent of HIV infection means that clinicians and scientists now cannot identify HIV infection in street drug abusers by using lymphadenopathy as one of the physical findings. Next, diffuse abnormalities in levels of serum immunoglobulins were found in heroin addicts, along with a very high prevalence of biologically false-positive tests for syphilis, a test that depends on abnormal amounts of IgM antibodies, in these prepharmacological-treatment study groups; these findings were reported by several workers (Cherubin and Milliam 1968; Louria et al. 1967; Sapira 1968).

METHADONE PHARMACOKINETICS AS USED IN MAINTENANCE TREATMENT: IMPLICATIONS FOR IMMUNE FUNCTION

In 1964, Professor Vincent Dole, the late Marie Nyswander, and I initiated studies of possible long-term pharmacological treatment of narcotic addiction. At the time, it was decided that a drug selected for treatment should have characteristics very different from heroin or morphine. The theoretically desirable attributes for such a drug were that it would be orally effective, have a very long duration of action with little peak effect, and yet would sustain a person in an essentially normal state physiologically (Dole et al. 1966; Kreek 1972). In 1964, analytical technology was not yet available to measure the very low plasma levels of many basic amine drugs, such as heroin, morphine, and methadone; thus, we had to rely primarily on clinical observations to estimate the pharmacokinetic profiles of each agent. Methadone had already been used in the management of addiction, primarily on the street by drug addicts who would use it to self-detoxify or self-maintain in settings of heroin shortages or desire for treatment. It was also used effectively in a few established inpatient treatment resources for short-term (10 to 14 days) detoxification of heroin addicts. Methadone was known to be an orally effective drug. In early clinical studies of methadone used for treatment of pain, apparent cumulative effects, including undesired respiratory depression after repeated doses, were observed despite its relatively short duration of action in pain relief. These findings suggested that methadone might have a much longer duration of action than initially had been reported. At that time (1964) methadone was reported to have the same duration of action as morphine with respect to pain relief.

We now know that heroin (and its major metabolite, morphine) and methadone in tolerant subjects have very different characteristics. Methadone has approximately a 24- to 36-hour duration of action, with withdrawal symptoms initially appearing around 24 hours following dosing (Dole and Kreek 1973; Kreek 1973c; Kreek et al. 1976). This is sharply contrasted with heroin used on a chronic basis; withdrawal symptoms will begin within 3 to 4 hours after the last dose of heroin. The pharmacokinetic profiles are now known to be strikingly different for these two agents. By giving a drug orally, as opposed to parenterally (using hypodermic needles and syringes), one can get away from both the lure and the psychological ramifications of needle use and also from the potential hazards that accompany "dirty" needle usage, such as infection with a variety of viral and bacterial agents that may contaminate shared or unsterilized needles and other injection paraphernalia.

When gas-liquid-chromatographic techniques were developed for measuring plasma levels of methadone, there was found analytically what had been seen clinically much earlier, that is, following oral dosing, there is a very low peak plasma level of methadone, barely a doubling of the nadir or

steady-state level, during chronic high-dose methadone administration to tolerant and dependent former heroin addicts; this small peak is followed by a slow decline, with sustained plasma levels and thus perfusion at critical opiate receptor sites of action for a 24-hour dosing interval (Kreek 1973c; Kreek et al. 1976; Kreek et al. 1978). If the dose of methadone is selected to be less than the degree of tolerance developed by the individual in treatment, there is no narcotic effect perceived, no euphoria, no sleepiness, no "high," nor any other narcotic effect. However, the dose must be selected with the strategy of staying below the degree of tolerance developed by that individual, in sharp contrast to the strategy for the treatment of pain, for which, to achieve analgesia, one must always exceed the degree of tolerance developed by the individual requiring treatment. The steady-state, methadone-maintained patient receiving a steady dose of methadone has no analgesic effects from the methadone administration and thus, in the setting of pain stimulus, will experience pain and will respond as any other person to appropriate usual, or slightly higher than usual, doses of a short-acting narcotic coadministered with methadone; this is the pain management of choice in such patients.

Further studies were performed of the pharmacokinetics of methadone in former addicts in steady-state, methadone-maintenance treatment (Kreek et al. 1976; Kreek et al. 1979; Kreek et al. 1978; Nakamura et al. 1982). Because of the extensive distribution and the resultant very low plasma levels of this drug, it was not possible to use classical radioisotope tracer techniques; the amounts of isotope that would be required exceed the ethical limits in human research (Kreek et al. 1979; Nakamura et al. 1982). Therefore, stable isotope-labeled species of methadone were used to do these studies (Kreek et al. 1979; Nakamura et al. 1982). In collaboration with Dr. Peter Klein and his group, formerly at Argonne National Laboratories and now at Baylor Medical School, three different species of stable isotope-labeled methadone, tri-, penta-, and octadeuteromethadone, were synthesized with one specific label on each of the enantiomers and also on the racemic compound used in therapeutics, to determine the metabolic fate of the two enantiomers simultaneously and measure quantitatively the entire methadone mass using gas chromatography, chemical ionization—mass spectrometry with selected ion monitoring (Kreek et al. 1979; Nakamura et al. 1982). Using these techniques, we have been able to determine that in humans the active R(1;-)enantiomer of methadone has a slower elimination than does the inactive S(d;+)enantiomer (Kreek et al. 1979; Nakamura et al. 1982). Based on studies both with and without use of stable isotope techniques, we have found that the half-life of methadone in humans is 24 hours for the racemic compound and approximately 48 hours for the active enantiomer. This sharply contrasts with the 1- to 4-hour half-life of heroin in humans. Also, methadone has greater than 90 percent systemic bioavailability after oral administration, contrasted to less than a 30-percent bioavailability for heroin. These pharmacokinetic differences arc probably the primary explanation of why one cm transform a formerly behaviorally abnormal person into a

normal well-functioning one during chronic methadone-maintenance treatment and also why physiological indices, profoundly perturbed by the chronic use of the short-acting heroin, may become increasingly normal with time in stabilized methadone maintenance treatment (Kreek 1973b; Kreek 1978).

There are currently approximately 100,000 former heroin addicts now in methadone-maintenance treatment in the United States and abroad. In good, well-staffed programs, usually those that provide counseling, rehabilitation efforts, and access to medical care and behavioral care as needed, the voluntary retention rates range from 55 to 80 percent. If adequate doses of methadone are used, illicit heroin use persists in less than 10 percent of patients (Dole 1988; Kreek 1973c; Kreek 1987b). However, in several followup studies it has been shown that more than 80 percent of former methadone-maintained patients, irrespective of the degree of the rehabilitation, relapse to heroin or other narcotic use within 2 years of leaving methadone treatment (Kreek 1987b). These data are superimposable on outcome data of former heroin addicts treated in drug-free programs; less than 30 percent are able to stay narcotic free after leaving residential drug-free treatment. These findings may teach us something about the biology of addictive disease and emphasize the need to look more deeply into what may be some of the mechanisms of addiction. Also. some of those mechanisms may be very important for an understanding of the immune phenomena associated with drug abuse. Methadone-maintenance treatment has a general 55- to 80-percent long-term success rate; treatment with an opioid antagonist, such as naltrexone, has a 15- to 20-percent success rate in unselected heroin addicts; drug-free treatment is successful in 20 to 30 percent of such patients in a variety of different kinds of programs in which long-term followup studies were performed.

PRE-AIDS IMMUNE FUNCTION IN METHADONE-MAINTAINED FORMER ADDICTS

Starting with the first patient, prospective studies were begun to examine the medical status of heroin addicts at time of entry to experimental methadone treatment in 1964 and during 3 or more years of continuous maintenance treatment (Kreek 1973a; Kreek et al. 1972; Kreek et al. 1979). Within 3 or 4 years, other groups also were beginning to study heroin addicts entering or during methadone-maintenance treatment, either prospectively or at “one point in time” (Adham et al. 1978; Brown et al. 1974; Cushman 1973a; Cushman 1973b; Cushman and Grieco 1973; Cushman et al. 1977; Cushman and Sherman 1974; Dunk et al. 1987; Falek et al. 1972; Falek 1980; Geller and Stimmel 1973; Grieco and Chuang 1973; Madden et al. 1979; McDonogh et al. 1980; Sherwood et at. 1972). This availability of a treatment that had a very high rate of retention permitted study of some of the physiological effects of pharmacological treatment, in addition to study of the medical status before and during treatment (Kreek

1973a; Kreek et al. 1972; Kreek et al. 1986). Some of the very earliest observations included absolute lymphocytosis in 20 to 30 percent of subjects; elevated levels of serum IgM and IgG in 50 to 70 percent of subjects; and diverse serum protein abnormalities (Kreek 1973a; Kreek 1973b; Kreek et al. 1972). Abnormalities in T-cell rosette formation and impaired lymphocyte responsiveness to mitogens were also reported (Brown et al. 1974; Cushman et al. 1977). Abnormal natural killer (NK) cell cytotoxicity in patients who recently entered methadone treatment was found in early studies (Lavie et al., unpublished).

Three sets of workers looked at changes in immunological function with time in methadone treatment: our group at The Rockefeller University, the groups of Stimmel at Mount Sinai Medical Center, and Cushman at St. Luke's-Columbia Medical Center (Brown et al. 1974; Cushman 1973a; Cushman 1973b; Cushman and Grieco 1973; Cushman et al. 1977; Cushman and Sherman 1974; Geller and Stimmel 1973; Grieco and Chuang 1973; Kreek 1973a; Kreek et al. 1972). We all found that lymphocytosis was present in over 30 percent of the patients both at the time of entry and during 3 years or more of high-dose methadone-maintenance treatment (Kreek 1973a; Kreek et al. 1972). We found that even after 3 years or more of methadone-maintenance treatment, 76 percent of patients had elevated levels of IgM, 48 percent had elevated levels of IgG, and 7 percent had elevated levels of IgA. Of those patients with elevated levels of IgM, 52 percent had biological false-positive tests for syphilis (Kreek 1973a; Kreek et al. 1972). The persistent antigenic stimulus for the elevations in immunoglobulin levels remains unknown. Dr. Deborah Doniach in England performed extensive studies searching for autoantibodies in sera of these patients, in collaboration with our group. It was found that the prevalence of autoantibodies was actually less than would have been anticipated, given the prevalence of chronic liver disease in this population. Over 60 percent of all these methadone-maintained patients had evidence of chronic liver disease at lime of entry (Kreek et al. 1972).

Stimmel and coworkers, also studying heroin addicts prospectively from time of entry into methadone-maintenance treatment, but following them for a shorter term in treatment than our studies, found elevated levels of IgM in 87 percent and IgG in 63 percent of subjects entering methadone-maintenance treatment (Brown et al. 1974). After 1 to 18 months of treatment, these abnormalities had lessened. Percentages of patients having abnormalities decreased from 87 to 76 percent for IgM and from 63 to 48 percent for IgG. Possibly more important, the levels of elevations of immunoglobulins observed also decreased significantly (Brown et al. 1974). Cushman similarly found that there was a reduction both in numbers of patients with elevations in levels of immunoglobulins and in the actual degree of those elevations for IgM and IgG during long-term methadone-maintenance treatment (Cushman 1973a; Cushman 1973b; Cushman and Grieco 1973; Grieco and Chuang 1973). Cushman also showed that the

numbers of patients with biological false-positive tests for syphilis decreased with time in methadone-maintenance treatment, from 23 percent of the entire study group of heroin addicts at time of entry to 6 percent after a mean of 23 months in treatment (Cushman and Sherman 1974). Cushman, Grieco, and colleagues showed that abnormal T rosetting was found in most heroin addicts but that the numbers of methadone-maintained patients showing T-cell and B-cell rosetting abnormalities seemed to decrease with time in treatment (Cushman et al. 1977). Stimmel and colleagues showed that lymphocytes from heroin addicts had a significantly reduced ability to respond to mitogenic stimulation *in vitro* using phytohemagglutinin and concanavalin A as mitogens (Brown et al. 1974). Followup studies of these subjects during methadone treatment showed significant improvement in some, but not all, subjects (Brown et al. 1974). In all these studies, those of our group, of Stimmel, and of Cushman, appropriate adequate treatment doses of methadone (60 to 100 mg per day) were administered (Dole 1988). Therefore, all study subjects in long-term methadone-maintenance treatment were being exposed to a higher opioid load than they had been when they were self-administering heroin on the street. Also, since methadone has a plasma half-life of around 24 hours, but heroin only 1 to 4 hours, the area under the plasma concentration-time curve for methadone is significantly greater than that for heroin (Kreek 1973c; Kreek 1989; Kreek et al. 1976). Following heroin self-administration, plasma-level peaks occur three to six times a day, depending on the number of self-administrations, followed by rapid decline to a nadir with resultant low areas under the plasma concentration-time curves.

Also, in each of these three sets of studies, it was found that only 20 to 30 percent of heroin addicts entering treatment had a history of clinically apparent hepatitis, but more than 50 to 70 percent had laboratory evidence of chronic liver disease. When markers for hepatitis B were measured, it was found in each of these studies that 5 to 15 percent of heroin addicts were, and still are, chronic carriers of hepatitis B antigen (Kreek 1973a; Kreek 1973b; Kreek 1978; Kreek 1987b; Kreek et al. 1972; Kreek et al. 1986; Novick 1988b; Novick ct al. 1986b). Hepatitis B is still the major infectious disease of easy transmissibility within the population of IV drug abusers in most regions of our Nation (Novick et al. 1986b). Alcohol abuse also was then and is now a major problem (Kreek 1973a; Kreek 1978; Kreek 1981; Kreek et al. 1972). It was found, in the prospective studies beginning in 1964, that approximately 30 percent of street heroin addicts in New York were abusing alcohol to excess and that this problem continued concomitantly with their heroin addiction (Kreek 1973a; Kreek 1978; Kreek 1981; Kreek 1987b; Kreek et al. 1972). Thus, alcohol abuse, along with hepatitis B and hepatitis delta (identified in the late 1970s and shown by retrospective analysis of our banked sera collection appeared in the New York City drug-abuse population in the mid-1970s), is the major cause of liver disease in this population (Kreek et al. 1987; Kreek et al. 1986; Novick et al. 1988b; Novick et al. 1985; Novick et al. 1986b).

Delta hepatitis virus is an incomplete RNA virus that is dependent upon the hepatitis B DNA virus for its replication, expression, and infectivity; it causes a potentially lethal viral liver disease. Serological evidence of infection with delta virus is now present in between 10 and 50 percent of New York City street heroin addicts (Kreek et al. 1987; Kreek et al. 1986; Novick et al. 1986b; Novick et al. 1985; Novick et al. 1986b).

In all immunological studies from 1964 onward, the question was asked of whether or not the presence of liver disease, which is known to cause many kinds of immune abnormalities, and alcohol abuse, which also causes a variety of immune abnormalities, could account for the abnormalities in immunoglobulins and also in T-cell function observed, including the abnormal responses to mitogens and T-cell rosetting. Cushman, Stimmel, and our own group found that there was no predominant correlation between the immunological abnormalities and the presence of liver disease. Indeed, although liver disease and alcohol abuse may contribute to the abnormalities observed, they are not the primary or sole factors causing these abnormalities.

In progress is a prospective study of young adult heroin addicts who became hardcore addicts before the age of 18, some of whom now have been in methadone-maintenance treatment for up to 20 years (Kreek et al. 1986). Whereas more than 15 percent of these youngsters had hepatitis B antigenemia, and most of these were chronic carriers of hepatitis B virus at time of entry to treatment, when stabilized for 10 years or more in methadone-maintenance treatment, none (0 percent) still had hepatitis B antigenemia (Kreek et al. 1986). The number of patients in this followup study is too small to do meaningful statistical determinations for significance, but the rate of conversion from a chronic carrier state to a "protected" immunized state (with hepatitis B surface antibodies) in this group is at least brisk. This again raises the question: Is immune competency improved during chronic methadone-maintenance treatment?

NEUROENDOCRINE EFFECTS OF HEROIN AND OF METHADONE AS USED IN MAINTENANCE TREATMENT: IMPLICATIONS FOR IMMUNE FUNCTION

In 1973 and 1975, some very exciting and relevant scientific findings were made, that is, the discovery of specific opioid receptors and the endogenous ligands of three distinct classes that bind to these receptors. The distinct groups of endogenous opioids remain three, and each has been defined by both biochemical and molecular biological techniques: the enkephalins, the dynorphins, and beta-endorphin from proopiomelanocortin (POMC). Although all of the opiate-receptor-active endogenous opioids contain the four amino acids characteristic of met- or leu-enkephalin, nevertheless only enkephalins and dynorphins are known to be processed to met- or leu-cnkephalin. Of the three endogenous opioid classes, the one that has

attracted the most attention is the POMC precursor. In humans, ACTH and beta-endorphin are released from POMC in equimolar amounts from the anterior pituitary into blood; POMC also is processed in the hypothalamus in humans to yield beta-endorphin and ACTH.

Much research work has been reported recently, documenting both the expression of the message for genes of the endogencus opioids and synthesis of the peptides of three classes in peripheral sites outside the brain, hypothalamus, pituitary, and spinal cord. The mRNA for the enkephaiin series clearly is expressed in many other sites. For instance, my group, interested in the possible role of the brain-gut axis in the addictive diseases, has been studying the endogenous opioid system of the gastrointestinal tract. We have recently shown that the message for the preproenkephalin gene is extensively expressed in the gastrointestinal tract of the guinea pig, especially in the colon (Zhang et al. 1988). Zurawski and colleagues have recently reported that the message for preproenkephalin is expressed by mouse T-helper cells that have been activated by concanavalin A and also that enkephalin peptidc synthesis by these activated T cells occurs (Zurawski et al. 1986). The message for the dynorphin gene also may be expressed in several different peripheral sites, along with synthesis of dynorphin peptides; work continues on this question.

The precursor peptide POMC is particularly of interest, because it yields one of the major stress hormones, ACTH, which in turn drives the adrenal cortex to produce corticosteronc in rodents and cortisol in humans. Two independent groups of investigators, Westly and colleagucs (1986) and Lolait and colleagues, have reported expression of the message for the POMC gene in Newcastle disease virus-infected mouse splenocytes and in unstimulated mouse splenic macrophages, respectively (Lolait et al. 1986; Lolait et al. 1984).

The endocrine effects for drugs of abuse, such as heroin, and also treatment drugs, such as methadone, arc potentially very important for several reasons (Kreek 1978; Kreek 1987a; Kreek 1987b). One is that endocrine and neuroendocrine function modulate many other important physiological functions, including immune function. Cortisol (corticosterone in rodents), ACTH, and also possibly beta-endorphin may affect specific cellular or humoral elements of the immune system. Our research group is interested in the possible relationship between neuroendocrinc function and the behavior of addictive disease per se and is also interested in the relationship between neuroendocrine function and immune function (Kreek 1987a; Kreek 1989). In man, in sharp contrast to most of the recently reported findings in the rodent model, an acute injection of a short-acting or long-acting opiate, such as heroin, morphine, or methadone, will cause inhibition of release of ACTH and beta-endorphin. with resultant blunted release of cortisol from the adrenal cortex and a flattened circadian rhythm of that release (Kreek 1972; Kreek 1973a; Kreek 1978; Kreek 1987a). During subacute

and chronic injections of short-acting narcotics, intermittent multiple doses of drug must be used to achieve desired effects, with opiate peaks followed by rapidly declining levels of opiate; these declining levels are accompanied by mild or severe withdrawal symptoms several times each day in the addict. In heroin addiction, inhibition of the neuroendocrine peptides at time of peak plasma levels of short-acting opiate continues, but if narcotic withdrawal is allowed to proceed, then the opposite response begins, which is the stress response of increased release of beta-endorphin, ACTH, and cortisol (Kreek 1972; Kreek 1973a; Kreek 1978; Kreek 1987a). Other neuroendocrine effects of exogenous short-acting opioids in man that have been well described include inhibition of release of LH and concomitant lowering of levels of testosterone, along with increased release of vasopressin and prolactin (Kreek 1978; Kreek 1987a).

In a very simplified scheme of the hypothalamic-pituitary-adrenal (HPA) axis in humans, normally a negative feedback control mechanism is operative, with suppression of corticotropin-releasing-factor at hypothalamic sites and also suppression of processing and release of POMC peptides, including beta-endorphin and ACTH, at the level of the anterior pituitary and probably also at the level of the hypothalamus by cortisol released from the adrenal cortex in response to ACTH. This negative feedback loop is altered during long-term heroin addiction, with suppression of release of the POMC peptides and resultant abnormal levels of cortisol. In withdrawal from narcotics, an opposite, different alteration in this loop, with the increased peptide hormone and cortisol release. In the first 2 months of methadone-maintenance treatment, the findings are similar to those during heroin addiction, that is, a suppression of release of ACTH and beta-endorphin, but after 3 months or more of steady dose treatment, when the full treatment dose of methadone has been achieved by slowly increasing the daily dose and then stabilizing the daily dose, the circadian rhythm of release and plasma levels of ACTH and beta-endorphin become normalized, and the release of cortisol and the circadian rhythm of its release also become normalized (Kreek 1972; Kreek 1973a; Kreek 1973b; Kreek 1978; Kreek et al. 1981). In long-term methadone-maintained patients, as in normal control subjects, the plasma levels of both beta-endorphin and ACTH are highest in the morning; then the levels begin to drop, with lowest levels in the late afternoon or early evening, according to the known, normal circadian rhythm (Kreek et al. 1981; Kreek et al. 1983).

We have been using a provocative test, the administration of metyrapone, a compound that selectively blocks 11-beta-hydroxylation in the adrenal cortex, thus blocking the last step in the metabolic biotransformation pathway to cortisol production (Kreek 1972; Kreek 1973a; Kreek 1978; Kreek et al. 1984). Since cortisol is the only steroid operative in the negative feedback loop in humans for the control of the HPA axis, the negative feedback loop is therefore disrupted, which in turn causes hypothalamic and pituitary sites to produce more of the POMC peptide hormones ACTH and beta-endorphin.

This provocative test is used in neuroendocrine evaluation and is called a test of hypothalamic-pituitary reserve. In normal subjects, additional amounts of ACTH and beta-endorphin, and thus increased amounts of the precursors of cortisol, should be produced in response to this cutoff of the negative feedback control loop. In certain endocrine deficiency diseases, in cycles of heroin addiction, and during the first 3 months of methadone treatment, there is an apparent inadequate hypothalamic-pituitary reserve; that is, in response to the cutoff of this negative feedback loop, the pituitary does not put out additional amounts of ACTH and beta-endorphin, and the adrenal cortex does not produce increased amounts of the immediate precursors of cortisol (Kreek 1972; Kreek 1973a; Kreek et al. 1984). However, we have shown that during long-term methadone-maintenance treatment, this abnormality in response to metyrapone disappears, and the responses become normal (Kreek 1973a; Kreek 1978; Kreek 1987a; Kreek et al. 1984).

For the past few years, since sensitive and specific laboratory tests for the quantitative measurements of low plasma levels of the peptides ACTH and beta-endorphin have been developed and validated, the metyrapone test is now carried out by a single-dose administration of metyrapone, which abruptly lowers cortisol levels (Kreek et al. 1984). Direct measurements of peptide hormones released into plasma in response to this abrupt lowering are then carried out (Kreek et al. 1984). In the earlier metyrapone test, multiple doses of mctyrapone were administered over a l-day period, and during that day and the next 2 days, the precursors of cortisol were measured as urinary excretory products (Kreek 1972; Kreek 1973a; Kreek 1978). Using the single-dose test in methadone-maintained patients, a two-fold to fourfold increase in plasma levels of both ACTH and beta-endorphin is observed over the 4 hours following metyrapone administration in response to an abrupt decrease in cortisol production; this result is identical to that defined as a normal response to metyrapone testing in otherwise healthy subjects (Kreek 1987a; Kreek et al. 1984). Thus it has been shown that neuroendorine functions, both baseline levels and responses during various kinds of provocative tests, are normal in stable-dose, long-term, methadone-maintained former heroin addicts.

In studies still in progress of drug-free former heroin addicts and former methadone-maintained patients no longer receiving methadone, preliminary observations indicate a hyperresponsivity of the drug-free former-narcotic-dependent person to metyrapone testing. This contrasts sharply with the hyporesponsivity seen during cycles of heroin addiction (Kreek 1987a; Kreek et al. 1984). Whcthcr or not this excess production of stress-related peptides is related to behaviors observed in drug-free heroin addicts or zero-dose methadone-maintained patients, that is, the relapse to use of illicit narcotic drugs, which would convert this hyperresponsive state into a hyporesponsive state, is not yet known. However, this observed hyperresponsivity of the HPA axis during metyrapone testing, a chemically induced stress,

must now be considered in the context of possible relationships to other kinds of stress in former heroin addicts not receiving opioid medication. This excessive release of HPA-axis peptide and steroid hormones is also similar to that seen when narcotic use is abruptly stopped in narcotic-dependent persons and also, to a lesser extent, between doses of self-administered short-acting heroin. The relationship of this hyperresponsivity of neuroendocrine function to immune function in the setting of relative or absolute narcotic withdrawal is not known.

COCAINE, ETHANOL, AND POLYDRUG ABUSE DURING METHADONE TREATMENT: IMPLICATIONS FOR IMMUNE FUNCTION

Methadone-maintenance treatment per se does not specifically or directly treat alcoholism, cocaine dependency, or any other nonopiate drug dependencies. Howevcr, methadone-maintenance treatment may help engage a patient in drug-free treatments for alcoholism and cocaine dependency, which ideally would be available in all treatment programs for these common, concomitant addiction problems. At this time, even during effective methadone-maintenance treatment, with, essentially, cessation of illicit heroin use, there is continued heavy use of alcohol, a use which usually began before or during heroin addiction, by 25 to 50 percent of patients. Since 1978 to 1983, there is also extensive use of cocaine by 25 to 50 percent of methadone-maintained patients, just as there is by street heroin addicts (Kreek 1973a; Kreek 1978; Kreek 1981; Kreek 1987b). From a treatment standpoint, these findings make it imperative to attempt to combine drug-free treatment for these problems with methadone-maintenance treatment for narcotic addiction (Gordis 1988). In addition, there is an urgent need for the development of pharmacological treatments for cocaine dependency and alcoholism, since drug-free and psychotherapeutic treatments for these addictions have had very limited success rates over the long term (Gordis 1988; Kreek 1987b). Chronic abuse of alcohol, cocaine, and other agents also may have profound implications with respect to the potential normalization of neuroendocrine function, which is hypothesized to be closely linked to the normalization of immune function observed in former heroin addicts in methadone-maintenance treatment (Kreek 1989; Van Dyke et al. 1986; Watson et al. 1985). Preliminary studies by our group and others suggest that both cocaine abuse and alcohol abuse may significantly alter neuroendocrine function as well as immune function in these patients (Kreek 1989; Van Dyke et al. 1986; Watson et al. 1985). Many groups are now studying both the acute and chronic and direct and indirect effects of alcohol and cocaine on specific indices of immune function, primarily because of the possible relationship of any such effects to HIV infection and AIDS (Kreek 1989; Van Dyke et al. 1986; Watson et al. 1985).

NEUKOENDOCRINE EFFECTS OF NALTREXONE AND RELATED OPIOID ANTAGONISTS: IMPLICATIONS FOR IMMUNE FUNCTION

To explore further the role of the endogenous opioid in addictive disease and to determine the effects of any drug used to treat drug abuse, specifically in this case heroin addiction, on both neuroendocrine function and immune function. an additional sequence of studies has been carried out on the effects of chronic treatment with the specific opioid antagonist naltrexone on neuroendocrine function. Former heroin addicts in naltrexone treatment for a mean of 5 months, with at least 5 weeks of naltrexone treatment, were studied in collaboration with Drs. Kostcn and Kleber; it was not possible to study patients in treatment for a longer period of time (Kosten et al. 1986a; Kosten et al. 1986b). It was found that the morning basal levels of beta-endorphin were significantly increased in the naltrexone-treated patients as compared with otherwise healthy control subjects (Kosten et al. 1986b). These findings were not surprising, since acutely, naloxone when given in an IV bolus or naltrexone when given either IV or orally in a single dose will cause a brisk rise in ACTH and beta-endorphin and, in response, a rise in plasma levels of cortisol levels, all of which then fall off rapidly following the pharmacokinetic profile of each antagonist. Cortisol levels were also increased during naltrexone treatment in many patients. We had the opportunity to restudy seven patients after they stopped naltrexone treatment and had been naltrcxone free for a period of time; basal morning levels of cortisol returned to normal range after stopping naltrexone treatment (Kosten et al. 1986a). Naltrcxone-treated former heroin addicts received the drug three or even as infrequently as two times a week for feasibility and compliance reasons; therefore the doses administered were very large because of the relatively short half-life of naltrcxone (4 to 6 hours). A high peak plasma level occurred after dosing, followed by a steady decline, rather than a stabilized, steady-state plasma level of the drug. It was not surprising that no full normalization of neuroendoctine function occurred during chronic naltrexone treatment (Kosten et al. 1986a; Kosten et al. 1986b).

Real levels of beta-endorphin, ACTH, and cortisol became normal in drug-free, former-methadone-maintained patients, but subjects have not responded normally to provocative tests of neuroendocrine function. We have not been able to do provocative tests in chronic naltrexone-treat patients yet. However, abnormal results of provocative testing might be anticipated, since during chronic naltrcxonc treatment, there is no normalization in baseline neuroendocrine function. The three hormones of potential interest with respect to immune modulation, ACTH, beta-endorphin, and cortisol, are at least intermittently elevated during naltrexone treatment (Kosten et al. 1986a; Kosten et al. 1986b).

POST-AIDS IMMUNE FUNCTION IN HEROIN ADDICTS AND IN METHADONE-MAINTAINED FORMER ADDICTS

AIDS has changed many things, and for those of us working in research related to addiction, it has become an additional confounder, beyond hepatitis B, hepatitis delta, alcohol abuse, cocaine abuse, and polydrug abuse, all of which commonly occur both in street addicts and in patients in treatment and also confound or complicate research design, patient selection, and data interpretation. AIDS also has become a confounder for treatment of narcotic addiction, with the former hopes of full recovery now often not possible to realize. As of September 5, 1988, there were 72,645 cases of AIDS in the United States; 23 percent of these were in New York City. Since January 1, 1988, IV drug use has been the primary risk factor for new cases of AIDS in New York City, now accounting for 43 percent of cases. In the Nation as a whole, parenteral drug abuse is the primary risk factor in approximately 2.5 percent of AIDS cases; parenteral drug abusers are the group at second highest risk for the development of AIDS. Both male and female IV drug users arc affected. Children with AIDS are primarily the children of IV drug users at this time (Des Jarlais et al. 1985; Des Jarlais et al. 1989; Kreek 1989). IV drug users are probably the largest contributors to the spread of AIDS to the heterosexual community (Des Jarlais et al. 1985; Des Jarlais et al. 1989; Kreek 1989).

Identifying Initial HIV Infection in New York City

In 1984, studies were carried out on unlabeled banked sera, which had been collected from 1969 onward, to determine when HIV seemed to attack the New York City population of heroin users (who were examined on entry to and during methadone-maintenance treatment) (Des Jarlais et al. 1989; Des Jarlais et al. 1984; Novick et al. 1986b; and Novick et al. 1986c). At the same time, Des Jarlais carried out various studies both in street addicts and in persons entering various kinds of treatment programs for parenteral drug abuse (Des Jarlais et al. 1985; Des Jarlais et al. 1984). It was found that 50 to 60 percent of parenteral drug abusers on the streets of New York were positive for the antibody test, indicating infection with HIV in 1984 (Des Jarlais et al. 1989; Des Jarlais et al. 1984; Novick et al. 1986b; and Novick et al. 1986c). Around 50 percent of former drug abusers who had entered methadone-maintenance treatment after 1982 were also positive for the HIV antibody (Novick et al. 1986b; Novick et al. 1986c). We found, howcvcr, that less than 10 percent of former parenteral drug abusers who had entered and remained in effective methadone-maintenance treatment before the epidemic reachcd New York City in 1978 were HIV-antibody positive (Des Jarlais et al. 1984; Novick et al. 1986b; Novick et al. 1986c). This 10 percent of patients with positive anti-HIV tests could be the cocaine-abusing group of patients in methadone-maintenance treatment; at least 10 to 20 percent of cocaine abusers who arc active heroin addicts or former heroin addicts in treatment use cocaine by the IV route, placing

these persons at continuing risk for HIV infection. The finding that less than 10 percent of patients in effective methadone-maintenance treatment were anti-HIV positive at a time when greater than 50 percent of street addicts were anti-HIV positive was highly significant in 1984 and still has important public health implications in 1988. It is imperative to get hard-core heroin addicts into effective treatment at this time. Data presented by Dr. Olof Blix of the University of Uppsala at the Fourth International AIDS meeting held in Sweden in June 1988 showed that those heroin addicts who had entered Swedish methadone programs prior to 1979 and were still in treatment in 1988 had less than a 5-percent anti-HIV positivity prevalence (Blix et al. 1988). New entries into methadone treatment were not allowed in Sweden between 1979 and 1983, but the government did allow those already in treatment to remain. New patients again were allowed to enter methadone-maintenance treatment programs in 1983; this study showed that more than 50 percent of those who entered treatment from 1983 to 1988 were anti-HIV positive at the time of entry (Blix et al. 1988). These data are therefore essentially superimposable on our data from New York City.

Post-AIDS Problems in Studying Immunological Abnormalities in Active and Former Heroin Addicts in Treatment

AIDS has altered our ability to do large-scale immunological studies in populations of street IV drug abusers in many locations. It has also posed some new problems related to immunological abnormalities in drug abusers (Novick et al. 1988a). Our group has recently described false positive tests for anti-HIV using the standard ELISA tests in 7 percent of unselected parenteral drug abusers entering hospital primarily for treatment of liver disease. Although these patients repeatedly had positive ELISA tests for anti-HIV, on further testing they repeatedly had negative Western blot tests. The question was, who were these false-positive testing subjects. In our study, 5 out of 70 in a group of parenteral drug abusers had false-positive test results; analysis of data showed that the patients with false-positive test results were parenteral drug abusers who also had a very long duration of alcohol abuse and, as a result of that alcohol abuse, had profoundly abnormal laboratory tests reflecting liver function and protein synthesis, with elevated levels of serum globulins and diverse immunoglobulin abnormalities. The abnormal levels of immunoglobulins, in part due to chronic alcohol abuse in these narcotic addicts, may have caused a false-positive anti-HIV test (Novick et al. 1988a).

Another recent study related to immune dysfunction in drug-using populations has been performed by our group in collaboration with Des Jarlais and Trepo (Kreek et al. 1987). We studied a group of unselected parenteral drug abusers, without AIDS but with about a 50-percent positive HIV-antibody test result, and a group of parenteral drug abusers with active AIDS requiring treatment (Kreek et al. 1987). In the non-AIDS group, 5 percent had hepatitis B antigenemia, and 21 percent had positive

antihepatitis delta serological tests, indicating prior delta hepatitis infection (Kreek et al. 1987). Of the 374 subjects without AIDS, only three had hepatitis delta antigencmia. In the study group with AIDS, however, 15 percent had hepatitis B antigenemia, and 6 percent also had hepatitis delta antigenemia (Kreek et al. 1987). Hepatitis delta antigen marker is normally cleared within the first 2 to 4 weeks after hepatitis delta infection, and the nonprotective antibody appears. In the group with AIDS, no subject was found with hepatitis delta antibodies, a highly significant difference from the street drug abusers without AIDS, with or without anti-HIV positivity, suggesting that production of the delta antibody cannot occur or is suppressed in the setting of the immunosuppression of AIDS (Kreek et al. 1987). The infectivity of the antigenemic state for delta hepatitis is much greater than when the antigen, reflecting active viral replication, has cleared. However, the infectivity of the delta hepatitis virus is possible only in those persons who have actively replicating hepatitis B virus, as reflected by hepatitis B antigencmia, since hepatitis delta is a defective RNA virus requiring replication of the DNA hepatitis B virus for its own expression and infectivity. Hepatitis delta virus may still be infectious after the antigenemia has cleared and the nonprotective antidelta antibodies have appeared, but only as long as there is replicating hepatitis B virus. These findings suggest that the natural history of both hepatitis B and hepatitis delta may be significantly altered in the setting of AIDS. They also suggest that the infectiousness of patients with both hepatitis B and combined hepatitis B and delta may be increased in the setting of active AIDS. These findings make imperative a serious consideration of vaccination of all persons in the risk groups for HIV infection and also all health care and laboratory workers working with patients with HIV infection and AIDS.

Important issues to be clearly delineated when considering the possible effects of drugs of abuse and drugs used to treat drug abuse with respect to the immune system are: What are the direct drug effects, and what are the indirect drug effects? When one performs studies in which drugs are added to the experimental system *in vitro*, any effects observed are, by definition, direct drug effects on specific components of the immune system. Such studies of potential direct drug effects can be carried out using cells from otherwise healthy normal subjects or cells from abnormal populations. When a drug is administered to an intact animal or to a human, and then the effects of the drug are studied either *in vivo* or in cells from such a subject *in vitro*, potentially both direct effects and indirect effects are observed. It is impossible in such an experimental design to completely separate the indirect from the direct effects. They may be equally contributory, or one may be primarily contributory. It is therefore essential to consider this also before attempting to interpret data obtained from patients either abusing drugs or receiving a drug in treatment.

NEED FOR CLINICAL STUDIES OF IMMUNE FUNCTION RELATIVE TO DRUG ABUSE AND ITS TREATMENT

Clinical studies of immune dysfunction in humans with drug abuse or addictions of a variety of types, with and without AIDS, and also in humans in treatment for drug abuse or addiction and receiving a variety of therapeutic pharmacological agents, need to be carried out. One of the most important things to be remembered in study design and in data interpretation is that one must define precisely who the study subjects are and what drug is being abused or being administered in treatment. The length of time of abuse or treatment and the doses of drug received as well as the schedule upon which drugs are received are also important factors to be evaluated. We need to conduct studies in humans, because humans arc significantly different from even the closest primates and certainly profoundly different from rodents with respect to neuroendocrinc function, certain specific immune indices, and the pharmacokinetics of many of the drugs which should be studied. *In vitro* studies using both human and animal material and also studies using animal models, preferably in many species, must be carried out. Clinical studies, however, must ultimately be performed. These studies must be as well controlled as possible. When possible, it is very important to have healthy control subjects or contrast groups to answer specific questions. If active street addicts or abusers of specific drugs are to be studied, certain questions should be asked repeatedly, so that they may be answered very carefully and as honestly and correctly as possible, including the length of time of drug abuse or addiction prior to treatment; the types of drugs abused; the route of administration and the, approximate doses of the drug used; the length of time in treatment; the type of treatment being receivcd, including the drug or drugs being used in treatment; the doses of drugs being administered; and the dosing schedule by which these treatment agents arc administered. Also this verbal data. especially with regard to any possible continuing drug abuse, should be confirmed by urine monitoring. Thc medical status and the laboratory status of the potential study subject must be well defined. with a careful definition of liver disease, including the type and relative severity thereof, and the status with respect to HIV infection as well as other indices. The available clinical literature of immune function in drug abusers since the onset of the AIDS epidemic is extraordinarily deficient in this regard. Therefore. rather than presenting the diffuse, often uninterpretable literature, studies will be discussed in which a few of these variables have been controlled.

POST-AIDS IMMUNE FUNCTION IN HEROIN ADDICTS: RELATIONSHIP TO CHRONIC LIVER DISEASE

In a study reported in 1985, Des Jarlais examined both street drug abusers who were HIV negative and those who were HIV positive by ELISA testing and Western blot confirmation (Des Jarlais et al. 1985). He found that there were highly significant differences between the anti-HIV-negative and

anti-HIV-positive subjects with respect to the absolute numbers of cells of the immune system. Total lymphocytes, total numbers of T cells, and total numbers of B cells were all significantly reduced in the anti-HIV-positive group as compared with the anti-HIV-negative parenteral drug abusers; T-cell subsets were also altered, with a significant reduction in T-helper cells in the anti-HIV-positive group, coupled with an increase in T-suppressor cells and a resultant reduced T4:T8 ratio in the anti-HIV-positive group; this reduction in T4:T8 ratio was greater than the minimal reduction in T4:T8 ratio in the anti-HIV-negative group (lower limit of normal T4:T8 ratio for the laboratory used was 1.5) (Des Jarlais et al. 1985). Similarly, Des Jarlais found that IgG was increased in the anti-HIV-positive group to an extent significantly greater than the already significantly elevated and abnormal levels observed in the parenteral drug abusers who were anti-HIV negative (Des Jarlais et al. 1985).

NK cells are the first line of defense in humans against many viral infections and tumor cells. NK cells can be studied immediately in the laboratory, that is, on the same day they are obtained from study subjects, as opposed to studying them after several days of incubation and manipulations that may alter the activity or responsivity from time of acquisition of the cells. We concentrated on studies of NK-cell cytotoxicity activity after drugs had been administered *in vivo* and *in vitro*. At least two other groups reported that NK-cell cytotoxicity activity is significantly reduced in street heroin addicts; we have recently confirmed this (Nair et al. 1986; Novick et al. 1989a; Poli et al. 1985). There is a question whether significant lowering of NK-cell activity in heroin addicts is a direct drug effect, an indirect effect, or an epiphenomenon related to diverse injected foreign substances or to exposure to a variety of diseases, such as hepatitis B, hepatitis delta, undetected HIV infection, or other infections to which heroin addicts are exposed by use of unsterile needles and self-injection paraphernalia. The sexual preference of the heroin addict also must be taken into account.

We have been performing studies in a variety of different populations to address these questions. The first was carried out by Novick and colleagues in England (Novick et al. 1986a). This was a study of 53 heterosexual and homosexual males, all of whom had hepatitis B infection and all of whom were otherwise healthy except for their chronic liver disease; presumably none had HIV infection, given the time when the study was performed (Novick et al. 1986a). NK-cell activity was significantly lower in the homosexual population, independent of hepatitis B virus infection (Novick et al. 1986a).

In a second study, also carried out in England, NK-cell activity was determined in patients with liver disease of a variety of types and degrees of severity (Dunk et al. 1987). NK-cell activity was found to be reduced in patients with cirrhosis and even more reduced in patients with hepatocellular carcinoma (Dunk et al. 1987). However, NK cytotoxicity activity was not

reduced in patients with mild chronic liver disease (Dunk et al. 1987). This is of considerable importance for immunological studies of NK-cell activity in parenteral drug abusers or former addicts in treatment; more than 60 percent of New York addicts in treatment have liver disease of a postviral or postalcoholic type, yet a very low percentage, less than 10 percent, have actual cirrhosis (Kreek 1972; Kreek 1973a; Kreek 1978; Kreek 1981; Kreek et al. 1972; Kreek et al. 1986; Novick et al. 1985; Novick et al. 1986b). Therefore, NK-cell activity is not apt to be compromised by the mild chronic liver disease that is present in most study patients and indeed most former heroin addicts in treatment or active heroin addicts.

POST-AIDS STATUS OF IMMUNE FUNCTION IN METHADONE-MAINTAINED FORMER ADDICTS: RELATIONSHIP TO TIME AND STABILIZATION IN TREATMENT

We hypothesized that whereas lowered NK-cell cytotoxic activity has been documented in heroin addicts and may be due to one or more of the causes enumerated above, in former heroin addicts receiving steady-state, stable-dose, long-term methadone-maintenance treatment, in the absence of or in the presence of otherwise mild chronic liver disease and even possibly in the presence of sporadic use but not regular abuse of another drug or alcohol, normalization of NK-cell activity may occur (Nair et al. 1986; Novick et al. 1989a; Ochshom et al. 1989; Poli et al. 1985).

Standard techniques are used to measure NK-cell cytotoxic activity (Novick et al. 1986a; Novick et al. 1989a; Ochshorn et al. 1989). Initial titrations were done to determine the optimal effector:target ratio with this group; a ratio of 100:1 was selected for many of these studies (Novick et al. 1989a; Ochshom ct al. 1988; Ochshom et al. 1989). The NK-cell cytotoxicity assay has been modified and standardized over the past few years; there is now an intra-assay coefficient of variation of 6.9 percent and intraindividual interassay coefficient of variation of 14.5 percent (Ochshom et al. 1988; Ochshom et al. 1989). We have controlled many factors, including the time of day at which the bloods are drawn. Although it is true that possible circadian rhythm of NK-cell activity in humans has not been well studied; however, because of the well-established neuroendocrine circadian rhythmicity, especially of the HPA axis, all our bloods for NK-cell activity are drawn in the early morning hours, when levels of beta-endorphin, ACTH, and cortisol are the highest. Patients are placed at rest for at least 30 minutes before blood is drawn, because exercise may also change NK-cell activity or actual killer cell number, by redistributing cells into the central circulating pool.

In an initial clinical study, 34 unselected patients in one methadone-maintenance treatment program, which also has abstinence base treatment for other chemical dependencies, were studied (Ochshom et al. 1989). They were unselected with respect to the presence or absence of cocaine abuse,

alcohol abuse, or any other kind of drug abuse, and they were unselected with respect to the length of time in treatment or dose of methadone being administered. The only patients excluded from the study were patients documented to be hepatitis B antigen carriers, because immune dysfunction may be pronounced in such patients. However, patients with all other types and degrees of severe chronic liver disease were included. We found that in the unselected methadone-maintained clinic population studied, 18 patients (53 percent) had normal NK-cell activity; 7 had modestly lowered NK-cell activity, 2 to 3 standard deviations below normal; and 9 had a more pronounced lowering of NK-cell activity greater than 3 standard deviations below normal (Ochshorn et al. 1989). Thus, more than one-half of the unselected methadone-maintenance patients had normal NK activity, and by extrapolation from these studies in heroin addicts, normalized NK-cell activity occurred. In this particular clinic population, where anti-HIV testing has not and cannot be carried out routinely because of staff and patient fears of the possibility of unintentional breach of confidentiality and also because of a lack of sufficient numbers of well-qualified staff to do the counseling needed to prevent any adverse behavioral consequences of routine or mass testing of anti-HIV testing, it is not known whether or not some of the patients with very low NK-cell activity may be anti-HIV positive. Some patients have been tested for anti-HIV; although this is only a small percentage of the total study group, all of these patients showed abnormal lowered NK-cell-activity levels. Therefore, in this particular study, possible HIV infection, which is known to reduce NK-cell activity, cannot be ruled out as a cause for the observed significant reduction in NK activity. This underscores the problem of carrying out immunological studies in parenteral drug abusers at this time. Certainly for this particular study, a reanalysis of data after anti-HIV testing, if and when that is done, will be of great interest. We are also obtaining absolute cell numbers of total lymphocytes, B cells, monocytes, NK cells, T cells, and T-cell subsets in these patients in collaboration with Dr. Neal Flomenberg of the Memorial Hospital-Sloan Kettering Medical Center, New York. In analysis of these data of cell numbers determined to date, it is intriguing that more than 50 percent of the unselected methadone-maintained subjects have normal T4:T8 ratios. Further studies and analyses of data are currently in progress.

A second clinical study performed by Novick, Ochshom, and me has been completed very recently (Novick et al. 1989a). This study was performed using carefully selected study subjects, as contrasted to the clinical study previously described (Novick et al. 1989a; Ochshom et al. 1989). In this study, there were three groups of subjects: (1) active heroin addicts, without cocaine abuse and without significant alcohol abuse and with negative tests for HIV infection; (2) former heroin addicts who are now very long-term methadone-maintained patients, all in treatment for 11 years or more and receiving a methadone dose of more than 60 mg per day; and (3) otherwise healthy, nondrug-abusing, control subjects. NK-cell cytotoxic activity was significantly reduced in the active heroin addict group.

Howevcr, in the long-term methadone-maintained patients, NK-cell cytotoxicity activity was normal, indistinguishable from the normal control subjects (Novick et al. 1989a). Analyses of additional data collected from the three groups show that several other specific indices of immune function have normalized in the long-term methadone-maintained group, as contrasted with the heroin addict group, in which many abnormalities were observed (Novick et al. 1989a).

IN VITRO STUDIES OF EFFECTS OF OPIOID AGONISTS AND ANTAGONISTS ON IMMUNE FUNCTION

The literature on the direct opiate drug effects *in vitro* on NK activity and on the direct effects of endogenous opioid ligands on NK-cell activity is full of conflicting and sometimes contradictory reports, as is, in fact, all of the immune literature with respect to the endogenous and exogenous opioids on specific indices of immune function (Kreek 1989). There are reports suggesting that peptide ligands from each of the three classes of endogenous opioids may increase, have no effect on, or occasionally decrease NK-cell activity.

In a series of laboratory studies, we investigated whether NK cells are normally controlled or significantly modulated by the cndogenous opioids and if so, whether this modulation would be reversed or altered by adding a specific opioid antagonist *in vitro,* thus removing any endogenous opioids bound to specific or nonspecific classical or nonclassical opiate receptors on NK cells. In the first set of *in vitro* studies, the commercially available and clinically used active (1;-)-enantiomer of naloxone was added in increasing concentrations to the buffers used in assays for NK-cell activity (Ochshorn et al. 1988). Cells from otherwise normal healthy subjects and cells from long-term methadone-maintained patients were used in these studies. There was no significant effect of the active naloxone enantiomer on NK-cell activity until a concentration of greater than 10^{-4} M was reached (Ochshorn et al. 1988). These concentrations were greater than any achieved in a therapeutic setting of use of an opiate antagonist and could be considered to be in "toxicological" ranges. They also were greater than the amounts required to remove cndogenous opioids bound to mu, delta, and kappa opiate receptor subtypes. We then carried out precisely the same *in vitro* studies using the inactive (d;+)enantiomcr of naloxone and made exactly the same findings, that is, there were no effects on NK-cell activity until concentrations of greater than 10^{-4} M were reached, and at that point the inactive enantiomer, like the active enantiomer, significantly reduced NK-cell activity (Ochshorn et al. 1988). Examination of the cells at the end of these studies showed that integrity of the cell membranes had been maintained. These findings suggest that although there may be a physical chemical event related to binding or attachment of either the active or inactive enantiomer of naloxone to NK cells, thus altering their activity when very high concentrations of antagonists are added, this, by definition, cannot be due to

"classical" opiate receptor binding, since the altered activity, that is, reduction of NK-cell activity, occurred only at extremely high concentrations, beyond those required for naloxone to act at kappa and delta receptor subtypes as well as at the receptor subtype of its preference, the mu receptor subtype. The same effects were observed when high concentrations of inactive enantiomer were used as when the opiate receptor active enantiomer of naloxone was used. Thus any residual endogenous opioids, bound to any specific subtype of opiate receptors, would have been removed by much lower concentrations of active antagonist. Using cells obtained from methadone-maintained patients, we made exactly the same findings; any specifically bound exogenous opioid would have been removed by the low concentrations of active naloxone, which had no effect on NK-cell activity *in vitro.*

In a second set of *in vitro* studies, we now are studying the effects of the active (R;l;-) and the inactive (S;d;+)enantiomers of methadone NK-cell activity (Kreek 1989). Methadone as it is delivered clinically is the racemic mixture of equal amounts of these two enantiomers. To date, the data resulting from these studies, in which methadone has been added *in vitro* to cells from otherwise healthy persons as well as from methadone-maintained patients, have been essentially superimposable on the data obtained using the opioid antagonist naloxone in similar *in vitro* studies (Kreek 1989). No changes of NK-cell activity, neithcr enhancement nor reduction of activity, are found until methadone concentrations of 10^{-4} M or greater are reached; at concentrations of 10^{-4} M and greater, a significant reduction of NK-cell activity is seen with both enantiomers of methadone. These studies suggest that neither endogenous opioids nor exogenous opioid antagonists or agonists cause direct effects on NK-cell activity until very high concentrations of drugs are used. These studies further suggest that these effects, observed at high concentrations. of potential opioid receptor ligands are not directly linked with any binding to any opioid receptors that may or may not exist on NK cells. Although these very high concentrations of drugs could be considered to cause a toxic effect, such as altering cell-membrane integrity, trypan-blue exclusion by cells remained normal to the end of the study. Therefore the integrity of membranes was Preserved throughout all of these agonist as well as antagonist studies. More subtle alterations in membranes may be altering NK-cell activity at the high concentrations used *in vitro.*

SUMMARY

These studies suggest that specific opiate receptors are not involved significantly in modulating NK-cell activity by any direct effect. The role normally played by the endogenous opioids in directly modulating NK-cell cytotoxic activity may be minimal and certainly is not a clinically relevant controlling factor. These findings also suggest that the repeatedly observed lowering of NK-cell activity in untreated heroin addicts is not due to a direct drug effect. It may be due, however, to an indirect drug effect,

possibly by way of altering neuroendocrine function, which we and others have shown predictably occurs during cycles of heroin addiction, and which, as discussed above, has been shown to become normalized during steady-dose, long-term, methadone-maintenance treatment. The lowering of NK-cell activity in heroin addicts may also be due primarily to the use of unsterile needles, with exposure to and infection with multiple diseases as well as injection of many foreign substances. Clinical studies of all the important immunological indices will have to be carried out in well-characterized human populations, including normal healthy control subjects, drug abusers, drug addicts, and former drug addicts in defined treatment status, before the actual roles of drugs of abuse or drugs used to treat drug abuse in immune function in humans will be fully understood.

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Opioid Peptides, Receptors, and Immune Function

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INTRODUCTION

Thc abuse of drugs in the United States has reached epidemic proportion. Associated with it are the problems of bacterial, fungal, and viral infections. It is believed that patients consuming drugs of abuse are at a much higher risk of contracting AIDS, because drugs of abuse are immunosuppressants (Coleman and Curtis 1988; Farci et al. 1989; Kaplan 1989; Martin and Schoub 1989; Stimson et a1.1988). In this chapter, the effects of chronic administration of opioid agents, particularly morphine and heroin, on clinical complications and biochemical markers of immune function are described. Evidence is provided for the presence of opioids in peripheral circulation (blood and its components) and the existence of opioid receptors on lymphocytes and phagocytes. The effects of opioid peptides, β-endorphin, and methionine-enkephalin, on several *in vitro* parameters of immune functions are described. Finally, preliminary findings on the effects of methionine-enkephalin on biochemical markers of immune function and on clinical symptoms in the acquired immunodeficiency syndrome (AIDS)-related complex (ARC) and AIDS patients are described.

DRUG ADDICTION AND IMMUNE FUNCTION

The modification of immune function by subjects taking drugs of abuse has received considerable attention in recent years. Although it has been recognized for some time that patients using drugs of abuse like morphine or heroin. alcohol, and marijuana have depressed immune function (Watson ct al. 1985; Lundy et al. 1975; Patel et al. 1985) leading to increased risk of infection, the possible mechanisms by which these effects are produced have been studied only recently. Since opiates like morphine and heroin arc self-administered by addicts using intravenous injections, sharing unsterilized needles might be one of the ways the micro-organisms enter the blood stream and produce infections. The other possibility is that drugs of abuse like heroin may have a direct action on the immune function of the

host. For almost half a century, clinical observations of heroin addicts have shown that these subjects have increased incidence of bacterial, protozoal, and viral infections. They include bacterial endocarditis (Luttgens 1949; Hussey et al. 1944; Olsson and Romansky 1962), septicemia (Briggs et al. 1967; Hussey et al. 1944; Cherubin and Brown 1968), pulmonary infarction, tetanus, hepatitis, malaria, thrombophlebitis, and skin sepsis (Hussey and Katz 1950; Louria et al. 1967).

Both clinical and preclinical studies suggest that chronic morphine treatment depresses the immune function, which leads to a higher incidence of infections. Various types of infections in 82 opiate addicts were summarized in a recent study by Lazzarin et al. (1984). The major infections included viral hepatitis, recurrent dental abscesses, subcutaneous abscesses, phlebitis, lues, and respiratory infections.

The factors that can contribute to the immunological abnormalities of heroin addicts are: (1) drugs and/or drug-contaminant-induced defects in both cellular and/or humoral immunity; (2) needle contamination causing viral infections, e.g., hepatitis disturbs cellular and humoral immune mechanisms; and (3) the abuse of other drugs, such as alcohol and marijuana, which are also known to affect the immune function (Lundy et al. 1975; Patel et al. 1985).

THE NORMAL IMMUNE RESPONSE

A brief description of the role of blood cells in the regulation of immune function will be given here. Cellular immunity, which is controlled or regulated by white blood cells, falls into two classes: T cells, which originate in the bone marrow but complete their maturation process in the thymus gland, and monocytes or macrophages. B cells are the source of antibodies, the proteins that bind to antigens and help in their removal or destruction. T cells are more complex in classification and function. In general, T cells cannot recognize free antigen circulating in blood or lymph, and can respond to antigen on a cell surface only under certain conditions. After recognizing antigen, the T lymphocytes, the cytotoxic T cells, actively defend the body by killing infected, foreign, or malignant cells by destroying their cell membrane. There are other kinds of T cells that modulate the immune response by secreting messenger proteins. They include: inducer T cells, which facilitate the maturation of T lymphocytes from precursor forms and the action of other T cells; helper T cells, which facilitate B cells in secreting antibody; and suppressor T cells, which dampen the immune response of B and T cells. Thus, helper and suppressor cells act in opposite directions. Based on the biochemical markers on their surface, the T cells have been classified primarily into two groups: CD4 cells, which function as helpers and inducers, and CD8 cells, which have suppressor and cytotoxic function. The other contributors in the immune response are the macrophages and natural killer cells. These are scavenger cells that develop from monocytes found in the skin and other tissues and act by

engulfing an organism, breaking down its proteins and displaying the antigenic protein fragment on the cell membrane. Macrophages prepare the antigen for recognition by CD4 cells. Natural killer cells destroy virus-infected cells and tumor cells spontaneously without the involvement of lymphocytes or recognizing antigens.

In response to an infection, such as a viral infection, virus-infected cells secrete proteins known as interferons which stimulate the natural killer-killer cells. Macrophages also secrete soluble proteins called monokines, such as gamma-interferon and interleukin-1. The latter activates T cells that have recognized the viral antigen on a cell surface, preparing them to diffcrentiate and divide. Activated T cells produce soluble factors, which are called lymphokines. CD4 and CD8 cells secrete interleukin-2, and stimulate proliferation into cytotoxic suppressor and helper T cells. Cytotoxic T cells lyse virus-infected cells, which are then removed from the body.

MORPHINE OR HEROIN ADDICTION AND IMMUNE FUNCTION

Returning to the effects of chronic morphine treatment on the immune function in addicts, cellular immunity was found to be compromised by functional deficits of polymorphonuclear leukocytes and T lymphocytes (Lazzarin et al. 1984). Immunological function in heroin addicts was studied in 38 patients. The immunological abnormality was evidenced by hypergammaglobulinemia, with higher than normal levels of IgM and IgG, and false positive test for syphilis. Cellular immunity was also found to be impaired, as evidenced by decreased responsiveness of lymphocytes to milogen-induced blastogenesis (Brown et al. 1974). McDonough et al. (1980) also reported a decrease in absolute number of total T lymphocytes in peripheral blood of opioid addicts as measured by the ability of lymphocyte to rosette sheep red blood cells (and an increase in null lymphocyte), but no significant change in B lymphocytes or total white blood cell count was noted. Naloxone was able to reverse these changes. Naloxone not only increased T cell percentage, it also improved the appearance of the E-rosettes. The lymphocytes of addicted patients also had decreased the ability of phytohemagglutin to induce stimulation of ^{3}H-thymidine incorporation into the DNA. Morphine addicts were also shown to exhibit a severe depression of phagocytosis and killing properties of polymorphonuclear leukocytes and monocytes and their ability to generate superoxide anion (Tubaro et al. 1985).

IN VITRO EFFECTS OF MORPHINE

In Vitro, morphine inhibits T cell E-rosetting (Donahoe et al. 1985). Some controversial results have been obtained on the *in vitro* effects of morphine on T, B, and null cells. Thus, Bocchini et al. (1983) reported that at 10^{7} M concentration of morphine and naloxone, the percentage of T cells was increased, that of null cells decreased, and there was no change in B

cells. This was accompanied by an enhanced effect of phytohemagglutinin-stimulated lymphocyte response. It is possible that these effects are dose related, since at high concentration (10^{-3} M), morphine inhibits the lymphocyte response to phytohemagglutinin.

EFFECT OF MORPHINE ADDICTION ON IMMUNE FUNCTION IN ANIMALS

Several studies have been carried out in animals in an effort to understand more precisely the mechanisms by which opioids, particularly morphine, modulate the immune response. Chronic administration of morphine to mice decreased the responsiveness of lymphocytes to concanavalin A stimulation when compared to lymphocytes of nonaddicted mice. This suppressive effect was reversed partially by concurrent administration of naloxonc (Ho and Leung 1979). In morphine-addicted mice, the primary immune response was inhibited, as evidenced by decreases in the spleen to body weight ratio and serum hemolysin production against sheep red blood cells. These effects were naloxone reversible (Gungor et al. 1980). Chronic treatment of mice for 3 days with morphine (75 mg/kg/day) produced immunosuppression, as shown by using a plaque-forming cell assay to evaluate antibody-forming efficiency (Lefkowitz and Chiang 1975) and a rosette-forming cell test (Lefkowitz and Nemeth 1976). In guinea pigs, chronic treatment with morphine affected the lymphocyte metabolism of cyclic nucleotides and lymphocytic responsiveness to concanavalin A stimulation (Law et al. 1978). Many of the above morphine-induced immunosupprcssive effects were either not reversed or partially reversed by naloxone.

Chronic treatment of mice with morphine also resulted in decreased resistance to bacterial and fungal infections. Infections due to *Klebsiella pneumoniae* and *Candida albicans* were enhanced in chronic morphine-treated mice (Tubaro et al. 1983). Morphine decreased reticuloendothelial system activity, phagocyte count, natural cell activity killer and superoxide anion formation in polymorphonuclear leukocytes and macrophages. Additionally, morphine induced a reduction of lymphoid organ weight (Tubaro et al. 1983).

EVIDENCE FOR THE PRESENCE OF OPIOID BINDING SITES ON CELLS CONTROLLING IMMUNE FUNCTION

As discussed above, since morphine causes immunosuppression in both animals and humans, and these effects are at least partially reversed by the opioid antagonist naloxone, it is suggested that opioid receptors may be involved in the immunomodulation. Since the discovery of opioid receptors in 1975, to date at least five major subtypes of opioid receptors have been postulated. They include μ-(preferring morphine), δ-(preferring enkephalins), κ-(preferring ethylketocyclazocine or dynorphins), σ-(preferring N-allylnormetazocine, SKF 10,047), and ε-(preferring β-endorphin). With

the discovery of opioid receptors in mammalian brain and other tissue, a search was started to isolate the endogenous ligands for the receptors, which resulted in the isolation of a family of peptides having opioidlike activity. Three major classes of compounds were enkephalins (methionine and leucine), dynorphin(1-13), and β-endorphin. Endogenous opioids have been implicated in a number of physiological and pathophysiological states involving neural and hormonal actions (Morley et al. 1987). It is thus possible that the endogenous opioids are also involved in the modulation of the immune function via the autonomic nervous system, the endocrine system, and the central nervous system. As noted earlier, T lymphocytes are the major cells in immune function. The first indirect evidence for the presence of opioid receptors on human T lymphocytes was provided by the studies of Wybran et al. (1979) who showed that morphine inhibited and methionine-enkephalin increased the percentage of active T rosettes. This effect was antagonized by naloxone but not by the inactive levomoramide, suggesting the presence of specific opioid receptors on T lymphocytes. Binding sites for ^{3}H-leucine enkephalin on cultured human T lymphocytes were present, but the binding of the ligand could not be displaced or inhibited by naloxone (Ausiello and Roda 1984). The binding sites for ^{3}H-naloxone were deteted on lymphocytes and platelets from peripheral blood of healthy human volunteers. The binding was displaced by unlabeled naloxone and morphine (Mehrishi and Mills 1983). Cultured human lymphocytes were shown to have specific binding sites for ^{125}I-h-[D-Ala^{2}]-ß-endorphin. The binding was not inhibited by opiate agonists and antagonists or by enkephalin analogs, but was inhibited by ß-endorphin or its analogs (Hazum et al. 1979). These studies demonstrated the presence of binding sites for ß-endorphin on lymphocytes that were specific for ß-endorphin but were nonopioid in nature. Similar binding sites have been found on the surface of mouse thymoma cells (Schweigerer et al. 1985). *In vitro* studies using phytohemagglutinin-stimulated human peripheral blood lymphocytes demonstrated the binding of ^{3}H-methionine-enkephalin. and this could be displaced by unlabeled methionine-enkephalin but not with 200 μM naloxone (Plotnikoff et al., personal communication).

The δ- -opiate receptor ligand, cis(+)-3-methylfentanyl-isothiocyanate (SUPERFIT), was found to label a protein from both B- and T-cell enriched murine splenocytes peripheral blood lymphocytes and human peripheral blood lymphocytes (Carr et al. 1988).

EVIDENCE OF THE PRESENCE OF INTACT OPIOID PEPTIDES IN PERIPHERAL BLOOD

ß-Endorphinlike material is present in the serum of rats and, upon stress, it is apparently released from the pituitary into the blood (Guillemin et al. 1977). Although ß-endorphin is present in low concentration in human serum, appreciable concentrations have been found in plasma of patients with certain endocrine disorders (Suda et al. 1978). Methionine-enkephalin

is also present in the human plasma in the intact form in the amount of 55 pg/ml and is possibly secreted by the adrenal medulla (Clement-Jones et al. 1980). Furthermore, the plasma levels of methionine-enkephalin are unrelated to the levels of endogenous adrenocorticotropic hormone (ACTH), β-lipotropin (ß-LPH), and ß-endorphin, which suggests that methionine-enkephalin is derived from its own separate precursor distinct from ACTH, β-LPH, and ß-endorphin (Clement-Jones et al. 1980). Finally, human leukocyte interferon (hIFN-α), which is a group of proteins with antiviral activity, contains recognizable endorphin and ACTH-like activities (Smith and Blalock 1981).

EFFECT OF SOME OPIOID PEPTIDES ON IMMUNE FUNCTION

Several studies have been carried out with opioid peptides, particularly ß-endorphin and methionine-enkephalin in animals, humans, and *in vitro,* to understand their possible role in immune function. Although the concentration of circulating ß-endorphin is very low in the rat, it is increased severalfold following acute stress, producing plasma ß-endorphin concentration to almost 10 ng/ml (Guillemin et al. 1977). The possible relationship between stress, immune function, and endorphins has been reviewed (Amir et al. 1980). The effect of α-endorphin, ß-endorphin, and D-Ala^2-Met^5-enkephalin to modulate the proliferative responses of splenic lymphocytes to mitogenic stimulation was measured. The results indicated that of the three peptides, only ß-endorphin potentiated concanavalin A, and phytohemagglutinin-induced proliferation of T lymphocytes, but had no effect on the response to the B cell mitogen lipopolysaccharide/dextran sulfate (Gilman et al. 1982). The potentiating effect of ß-endorphin was not reversed by naloxone, which suggests that the effect may be mediated by a nonopioid but ß-endorphin-specific mechanism. On the other hand, McCain et al. (1982) reported that ß-endorphin is a potent and efficacious suppressor of phytohemagglutinin-induced blastogenesis in cultured human T lymphocytes that could not be antagonized by naloxone. The reduction in lymphocyte reactivity induced by ß-endorphin did not appear to be due to cytotoxicity, since the lactic dehydrogenase activity of the supernatant fluid, an indicator of cell lysis, was not affected. The differences in the studies of Gilman et al. (1982) and McCain et al. (1982) were that in the former spleenocytes were used, whereas in the latter cultured human leukocytes were used. The influence of ß-endorphin on lymphocyte function has been demonstrated to be dependent on the donor used. The ß-endorphin-induced inhibition of lymphocyte proliferation appeared to be due to fragment ß-endorphin$_{10\text{-}16}$ amino acid sequence, and the effect is probably mediated by interference in the mobilization of intracellular calcium. ß-Endorphin and methionine-enkephalin stimulate chemotaxis of human blood mononuclear cells, an effect which is antagonized by opiate antagonist naloxone (van Epps and Saland 1984), indicating that such an effect is mediated via endogenous opioids. Similar response has also been shown for neutrophils. ß-Endorphin at very low concentration (10^{-14} M) and methionine-enkephalin (10^{-9} M)

have been shown to enhance the activity of natural killer cells from peripheral human blood, and this effect was blocked by naloxone (Matthews et al. 1983). Thus endogenous opioids modify immune function via both opioid and nonopioid receptors. The opioid receptor-mediated effects include increased natural killer cell activity, increased a-interferon and interleukin-2 production, release of histamine from mast cells. enhancement of chemotaxis, and enhancement of T-cell subsets. The effects, which do not appear to involve opioid receptors, include modification of phytohemagglutinin-stimulated proliferation, superoxide production, and binding to terminal complexes of complement (SCSB-9 and CSB-9).

EVIDENCE FOR THE PRESENCE OF METHIONINE-ENKEPHALIN IN PERIPHERAL BLOOD COMPARTMENTS

Methionine-enkephalin is a peptide (Tyr-Gly-Gly-Phe-Met) that has been found in a variety of tissues, including brain, spinal cord, and gastrointestinal tract of several species (Simantov et al. 1977; Yang et al. 1977; Wesche et al. 1977; Gros et al. 1977; Hughes et al. 1977; Miller et al. 1978). It has also been detected in human gut, brain, and cerebrospinal fluid (Polak et al. 1977; Gramsch et al. 1979; Akil et al. 1978).

It has been indicated earlier that methionine-enkephalin. an endogenous opioid peptide, circulates as intact pentapeptide in human plasma (Clement-Jones et al. 1980). Although methionine-enkephalin is degraded rapidly by tissue enkephalinases and aminopeptideases, making its half-life extremely short (Roda et al. 1986), the presence of intact peptides in the circulation raises the question of whether there are protective mechanisms present in the peripheral blood. If so, what are their distribution patterns and what arc their physiological roles? Picogram quantities of immunoreactive methionine-enkephalin were detected in human, rat, and rabbit platelets. The platelets' methionine-enkephalin concentration in the Sprague-Dawlcy rats was not affected by either adrenalectomy or by hypophysectomy (DiGiulio et al. 1982). However, plasma concentrations of methionine-cnkephalin were increased after adrenalectomy in both normal and hypophysectomized rats (Panerai 1988). Therefore, the origin of methionine-enkephalin in blood is still not certain. The distribution and uptake of methionine-enkephalin into human blood cells have also been studied. Blood obtained from normal volunteers was separated into red blood cells (RBCs), white blood cells (WBCs), and platelets, and methionine-enkephalin content of cells was determined by radioimmunoassay. On a per cell basis, WBCs were found to contain about 100-fold greater amounts of methionine-enkephalin than either RBCs or platelets. Similarly, the uptake of ^{3}H-methionine-enkephalin was far greater in WBCs than in RBCs or platelets. These studies suggest that WBCs may act as a compartment of distribution for methionine-enkephalin in blood (Valentine et al. 1988).

Effects of Methionine-Enkephalin on Immune Function: *In Vitro* and Animal Studies

Wybran et al. (1979) provided suggestive evidence for the presence of methionine-enkephalin receptors on human blood T lymphocytes. Human T lymphocytes are easily recognized by their specific ability to form rosettes with sheep red blood cells (SRBC). *In vitro,* methionine-enkephalin increased the percentage of active T rosettes, an effect that was inhibited by naloxone (Wybran et al. 1979). Subsequently, the presence of opioid receptors on human phagocytic leukocytes was reported (Lopker et al. 1980). The distribution and presence of methionine-enkephalin receptors on cells involved in immune function prompted further studies on the possible role of methionine-enkephalin in the host defense mechanisms. Plotnikoff's group has shown that the enkephalins increase phytohemagglutinin-induced lymphocyte blastogenesis (Plotnikoff and Miller 1983) and prolong survival of BDF, mice inoculated with an attenuated L1210 strain of tumor cells (Plotnikoff and Miller 1983; Plotnikoff 1982). Methionine-enkephalin significantly increases the active T cell rosette-forming cells in peripheral blood lymphocytes from lymphoma patients (Miller et al. 1983). Enkepha-Lins also stimulate at low doses and inhibit at high doses T-dependent antibody responses *in vitro* (Johnson et al. 1982). Both methionine- and leucine-enkephalin significantly increased natural killer (NK) cell activity in isolated human peripheral blood lymphocytes (Faith et al. 1984). NK cells belong to the subpopulation of lymphocytes that play an important role in host defense mechanisms against neoplastic disease.

Further evidence for the possible role of methionine-enkephalin in the immune function was provided by the studies of Zurawski et al. (1986), who demonstrated that mitogenic activation of mouse T-helper cells induces preproenkephalin mRNA synthesis. A complementary DNA library prepared from a cloned concanavalin A-activated mouse T-helper cell line was found to encode preproenkephalin mRNA. The supematants from induced T-helper cell cultures were found to have methionine-enkephalin immunoreactivity. These studies suggest that enkephalins like methionine-enkephalin serve a function as neuroimmunomodulators via T-dependent immune functions. With this background information, the effects of methionine-enkephalin in patients with AIDS, ARC, and cancer were determined (Wybran et al. 1987).

Effects of Methionine-Enkephalin on the Immune Function of ARC and AIDS Patients

AIDS is characterized by opportunistic infections and malignant diseases. AIDS is associated with persistent lymphadenopathy and characteristic immunological abnormalities with selective T-cell deficiency, persistent decreased CD4/CD8 ratio, elevated serum immunoglobulins, decreased in *vitro* lymphocyte proliferative responses, decreased NK cell activity and

cell-mediated cytotoxicity, and elevated serum levels of immune complexes. The earlier and less severe form of AIDS is called ARC. Clinical symptoms include fever, fatigue, malaise, night sweats, weight loss, anorexia, oral candidiasis, lymphadenopathy, depression, and diarrhea (Seligmann et al. 1984). The effect of methionine-enkephalin on the immunologic markers and clinical symptoms of ARC patients was determined. Studies were conducted in seven patients with ARC. The patients received methionine-enkephalin three times a week intravenously for a minimum of 21 days. The doses ranged from 20 to 100 µg/kg at each injection. The immunologic results have been summarized in table 1.

TABLE 1. *Immunological changes after 21 days of treatment in patients with ARC and AIDS*

	Day 0	Day 21	P
Lymphocyte Count	1207±186	1129 ± 223	NS
OKT3 Percentage	68 ± 3	77 ± 2	< 0.02
OKT4 Count	174 ± 4.5	271± 71	< 0.05
NK Activity (%)	19 ± 3	24 ± 2	< 0.10
IL-2 Production (U)	0.11 ± 0.05	0.8 ± 0.3	< 0.05
PHA Response (cpm 10")	162 ± 30	261 ± 60	< 0.01

NOTE: The results are expressed as mean ±SEM. The p value is based on Student's t test comparing paired data on day 0 and day 21.

SOURCE :Wybran et al. 1987. copyright 1987, New York Academy of Sciences.

Following the treatment with methionine-enkephalin, there was a significant increase in the number of blood OKT3 (CD3) T lymphocytes and OKT4 (CD4) (T helper cells) lymphocytes without an increase in the absolute number of lymphocytes, significant increase in NK cell activity and interleukin-2 (IL-2) production, and enhancement of the phytohemagglutinin response. The symptoms like weight loss, night sweats, recurrent scrotal infection, depression, malaise, fatigue, and swelling of the lymph nodes regressed. The data clearly indicated that methionine-enkephalin can enhance some immunologic functions in ARC patients, which was also associated with some therapeutic benefits. Obviously, these studies need to be extended to a larger population of ARC patients.

One AIDS patient, who received a single injection of 20 ug/kg of methionine-enkephalin, had his phytohemagglutinin response increase from 4300 cpm to 14000 cpm. Another AIDS patient with Kaposi's sarcoma, who received chronic treatment with methionine-enkephalin, had his lesions

remain stable for 4 months. Thus, methionine-enkephalin administration was associated with the regression of the Kaposi's sarcoma.

SUMMARY AND CONCLUSIONS

The studies have clearly demonstrated that binding sites for opioid peptides, β-endorphin, and methionine-enkephalin exist on T lymphocytes. β--Endorphin appears to be immunodepressant, whereas methionine-enkephalin is immunostimulant. Both *in vitro* and *in vivo* studies have shown that methionine-enkephalin can influence some immune functions. Since *in vitro* modification of immune function requires very low concentrations, it is reasonable to believe that methionine-enkephalin plays a physiological role in the immune system. Although not well established, methionine-enkephalin appears to activate T lymphocytes via opioid receptors and triggers a series of intracellular signals leading to the activation of receptors for interleukin-2 (IL-2), OKT10, and active sheep T red blood cell receptors. Methionine-cnkephalin enhances the activity of NK cells and induces the production of IL-2, which in turn may recruit and activate other T-cell subsets like CD3 and CD4. Methionine-enkephalin also enhances mitogen-induced proliferation of lymphocytes. Since preliminary studies with methionine-enkephalin in ARC patients have provided beneficial effects by the improvements in their symptoms, it will be worthwhile to extend these observations to a larger number of patients with ARC and AIDS. Finally, it appears that some endogenous opioid peptides and their analogs, in addition to methionine-enkephalin, may provide therapeutic benefits not only in ARC and AIDS but also in other immunodeficient states.

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Summary of Recommendations for Future Research

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The National Institute on Drug Abuse was established by law in 1974 as one of three Institutes in the Alcohol, Drug Abuse, and Mental Health Administration, a Public Health Service Agency within the Department of Health and Human Services. Since its inception, the Institute has been the lead Federal agency responsible for reducing the demand for illicit drugs. Through the congressional reauthorization process, the Institute has been directed to carry out its mission utilizing various mechanisms, such as categorical service grants, block grant services, and a wide range of intramural research projects and extramural research grants and contracts. Currently, the National Institute on Drug Abuse is authorized as a research institute, and I emphasize research, to study the causes and consequences of drug abuse and ways of improving our ability to treat and prevent drug abuse and to collect information on the incidence and prevalence of drug abuse.

In recent years, the emergence of widespread abuse of cocaine and its public health consequences have lent a greater immediacy to the mission of the Institute and resulted in significant budgetary increases. However, as Dr. Schuster pointed out. the realization that intravenous or parenteral abuse of drugs is associated with nearly 30 percent of the reported cases of AIDS has created an overwhelming need to attack the problem of drug abuse with all the tools available to society. The main function of the Institute in this regard is to provide the research base on which public health decisions and policies can be made intelligently.

During the course of this technical review meeting, a number of distinguished scientists discussed the "Current Chemical and Pharmacological Advances on Drugs of Abuse Which Alter Immune Function and Their Impact Upon AIDS." Many elegant synthetic and medicinal chemistry studies of the opiates, cocaine, and the cannabinoids were presented. Those present at the meeting also heard about the beginning of systematic studies to determine whether and how the immune system may be affected by the

opioids and other drugs of abuse. Some common themes have emerged, which have led to recommendations for future research and development.

1. There is a need for continued and expanded support for basic chemical. biochemical, and pharmacological research. General suggestions include the following:

   a. the need for highly selective agonists and antagonists for the variety of opioid and other postulated receptors;

   b. the preparation of labeled ligands and affinity labels for positron emission tomography (PET) scanning and the binding and isolation of receptors;

   C. the determination of the physiological role of the purported receptors;

   d. the isolation and determination of the structure of the opioid receptor or receptors, which should obviously be expanded to include other postulated receptor types; and

   e. expansion of our efforts to determine whether there are specific receptors or binding sites for other drugs of abuse, such as the cannabinoids, cocaine. the sedative hypnotics, and so on. Of particular need is the development of specific antagonists and other molecular probes.

2. The exciting discovery that cells of the immune system have specific opioid binding sites calls for expanded research in this area. A number of general recommendations have emerged:

   a. The use of immunologic tools in the study and isolation of receptors for drugs of abuse should be expanded.

   b. Careful and systematic studies of the effect of the opioids and other classes of drugs of abuse on the immune system should be expanded. This is particularly true for cocaine, the cannabinoids, and the inhalants, such as the nitrites, which are associated with populations particularly at risk for HIV infection.

   c. There is a real need for careful studies to determine whether and how the observed effects on the immune system of drugs of abuse in animals and man are related to the spread of the HIV virus and the occurrence of opportunistic diseases. This means that drugs of abuse and the pharmacotherapeutic agents used for treatment of dependence on these drugs should be tested for their

effects on the immune system in the disease state as welt as in the normal.

3. Finally, there are a number of specific recommendations. These include the following:

   a. There is a great need for the pharmaceutical industry to devote more attention to the development of new drugs for the treatment of the addictive disorders. This should not be confined to tne opioids but should be expanded to include the stimulants such as cocaine, the hallucinogens such as PCP, and the abused central depressants.

   b. As a corollary to this, the current development of known possible pharmacotherapeutic agents should be expedited. This includes making (-) α-acetylmethadol (LAAM) rapidly available; possibly developing *nor*-LAAM as a replacement or backup; developing buprenorphine; and producing new long-lasting antagonists or depot preparations of known antagonists. In addition, pharmacotherapies for cocaine dependence should be rapidly developed. A number of current "leads," such as desipramine, calcium channel blockers, and others, exist, and studies should be expanded to determine their efficacy and safety so that they can be made available to the therapeutic community as rapidly as possible.

   C. There is a need for expanded animal and human testing facilities to provide standardized data for feedback to the chemists to aid in the design of new molecules. This should include immunological studies as well as dependence studies.

   d. Efforts should be continued and expanded to make available to interested investigators supplies of new substances that have emerged from chemical and biochemical research. The availability of these new tools will accelerate research and lead to the more rapid development of new and more effective treatments for substance abuse.

   e. A uniform data base should be developed by the National Institute on Drug Abuse to relate chemical structure to pharmacological and immunological findings. The data base should be interactive and easily accessible.

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