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Assessment of Environmental Contaminant-Induced Lymphocyte Dysfunction

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Although it has been established that environmental contaminants can alter immune function, the mechanisms of action have yet to be determined. This paper reviews the effects of hydrocarbon environmental contaminants on lymphocyte function and presents an approach which may serve to delineate the mechanisms of action. The approach is based on the use of the developmental phases of an immune response and assays which can be used for their functional assessment. Possible interactions between environmental contaminants and lymphocyte function and factors which must be considered in the evaluation of immune status are discussed. In addition, a study on the influence of the chronic exposure to two polyhalogenated hydrocarbons, PCB and HCB, on several parameters of lymphocyte function in mice is presented.

Introduction

It has been established by several investigators that environmental contaminants can alter the immune response (*1*, *2*). Since lymphocyte function is integral in the development of an immune response, it is appropriate to delineate its role in environmental contaminant-induced immune dysfunction. It is the purpose of this presentation to explore the possible interactions between the lymphocyte and commercially valuable hydrocarbons and their impurities which have become environmental contaminants (EC) based on an approach which may provide insight into the mechanisms by which they cause immune dysfunction. Factors which should be considered in the assessment of chemical-induced immunotoxicity will be discussed and data from a study on the influence of two halogenated hydrocarbons, polychlorinated biphenyl (PCB Aroclor 1016) and hexachlorobenzene (HCB), on cell-mediated immune function will be presented.

To assess the functional status of the lymphocyte following exposure to EC it is necessary to understand its role in the development of an immune response. The primary function of the immune system is the recognition and elimination of cellular and noncellular entities which are non-self, (bacteria, viruses, protozoa, etc.), or altered-self (transformed cells), which would otherwise compromise the health of the host. This requires a mechanism which is capable of specific recognition of the antigen at the molecular level and a complex network of functional immunocompetent cells. It has been well established that the B lymphocyte is required for the development of an antibody response and that the T lymphocyte is required for the development of a cell-mediated immune response. Although the role of the macrophage as an accessory cell in the immune response must be carefully considered in the interpretation of EC-induced immunotoxicity

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bon EC-induced alterations of lymphocyte function.

Assessment of Environmental Contaminant-Induced Immune Dysfunction

The development of an immune response depends upon the proper function of three basic phases: (a) initial recognition of the antigen, (b) activation, which includes proliferation and differentiation of reactive clones of lymphocytes and (c) the expression of immunity (Fig. 1). This concept can be applied to the assessment of EC-induced lymphocyte toxicity.

Depending on the nature of the foreign substance, initial recognition of the antigen may occur through a combination of direct and/or indirect interactions with receptors on the lymphocyte surface membrane. Some antigens, such as lipopolysaccharide (LPS), polymerized flagellin (POL), and polyvinylpyrrolidone (PVP), which are T-independent antigens, can interact directly with specific immunoglobulin receptors on B lymphocytes. Cellular antigens such as sheep erythrocytes (SRBC) or serum protein antigens such as bovine serum albumin (BSA) or bovine gamma globulin (BGG) require development of humoral immune responses and are T dependent antigens. Responses to histocompatibility antigens, however, are primarily dependent on interactions with T lymphocytes.

Since surface membrane components of immunocompetent cells play such an integral role in the recognition phase, alteration of either the number of these surface structures or changes in their physicochemical properties may lead to a modified immune response perhaps due to alterations in cell-cell interactions which are necessary for the second phase of an immune response, activation. The importance of the integrity of cell surface structures and their possible alteration by xenobiotics can be understood.

Lymphocyte activation follows initial recognition of antigen and results in proliferation and differentiation of responding clones of lymphocytes. Many of the events of activation have been investigated following stimulation with polyclonal mitogens such as phytohemagglutinin (PHA) and gram negative bacterial lipopolysaccharide (LPS). Increased turnover and synthesis of phospholipids occurs within the first hour following exposure to PHA (*4*, *5*). An increased turnover of the fatty acid moiety of lecithin is also observed within this time interval (*6*). An increase in the level of cAMP is noted within

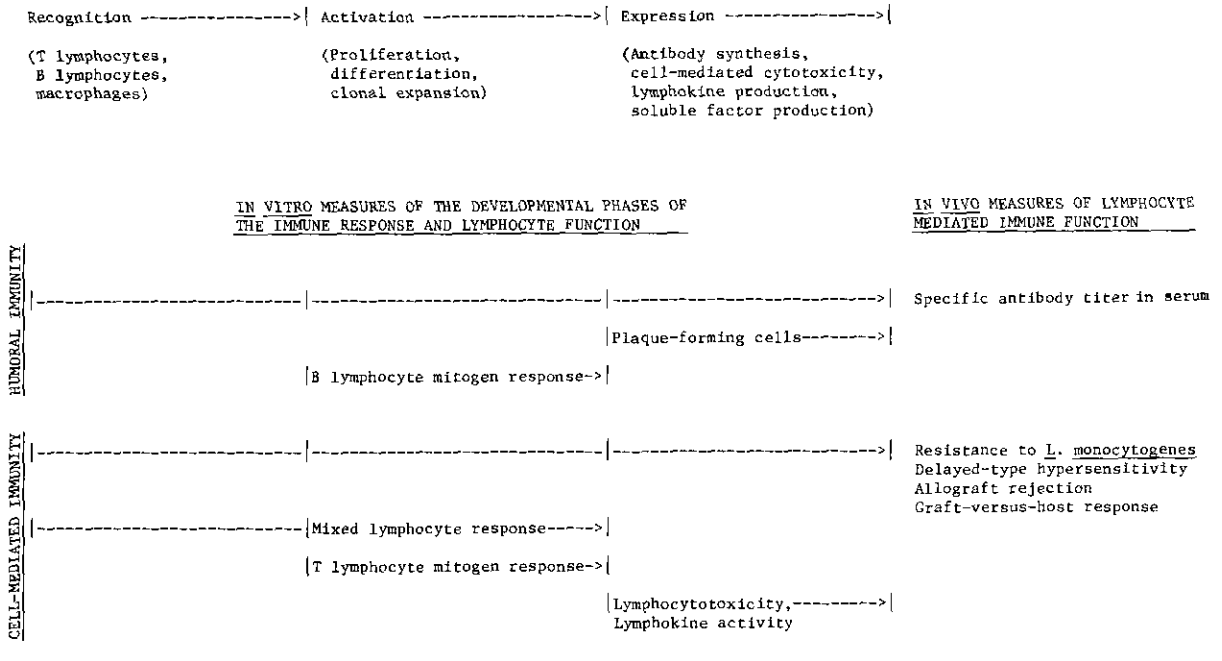

FIGURE 1. Developmental phases of an immune response and assays which can be used for their functional assessment.

decline to below control levels by 6 hr (*7*). Nuclear changes include the increased phosphorylation of nuclear proteins (*8*). PHA stimulates RNA synthesis within 30 minutes and protein synthesis within 4 hr followed by DNA synthesis within 24 hr which reaches a peak between 48 and 72 hr (*9*). Enzymes which display increased activity following activation include acid phosphatase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, DNA polymerase and DNAase (*10-14*). Morphological and cytochemical changes also occur following activation and include lymphocyte enlargement and basophilia. Exposure to alloantigens results in similar responses.

The expression of humoral immunity is the production of specific antibody and it has been well established that the B lymphocyte-derived plasma cell is the effector cell. The expression of cell-mediated immunity is represented by tissue rejection responses such as allograft rejection or graft-versus-host activity in which the effector cell is the cytolytic T lymphocyte (*15*). Also, lymphokine production by T lymphocytes plays a major role in cell-mediated immune responses such as the resistance to *Listeria monocytogenes* or the development of delayed-type hypersensitivity responses (*16*, *17*).

Available *in vivo* and *in vitro* techniques allow the examination of the developing immune response and perhaps clarification of the mechanisms of EC-induced immunotoxicity (Fig. 1).

Specific Antibody Titer in Serum

The inoculation of test animals with certain antigens results in the development of a humoral immune response. The concentration of specific antibody in serum can be used as a measure of the functional status of all three developmental phases of the humoral immune response. Specific antibody can be detected by several methods of different sensitivities which include antigen-antibody immunoprecipitation, passive hemagglutination/hemolysis, hemagglutination/hemolysis and enzyme-linked immunosorbent assays. The antibody response to T dependent antigens such as proteins, erythrocytes, bacterial exotoxins and viruses requires cooperation among B lymphocytes, T lymphocytes and macrophages (*18*) whereas the antibody response to the T independent antigen, LPS, requires B lymphocytes and macrophages but not T lymphocytes. Antibody responses to T dependent versus T independent antigens have, therefore, been used by some investigators (*1*, *19*, *20*) to determine the role mechanisms of immunotoxic compounds.

Plaque-Forming Cells

When spleen cells are removed from mice which have been immunized against SRBC and incubated in a semisolid gel containing the SRBC antigen, the subsequent addition of excess complement causes the formation of clear areas (plaques) where antibody- and complement-mediated erythrocyte lysis has occurred (*21*). Single antibody-producing cells are responsible for each plaque and the number of plaque-forming cells (direct PFC) is a measure of the number of spleen cells which are producing specific IgM antibody at the time of the assay. The addition to the test system of anti-mouse IgG immunoglobulin measures the number of cells which are secreting specific IgG antibody (indirect PFC). Since the IgG response to SRBC seems to be dependent on T lymphocyte regulation (*15*), the direct versus indirect PFC count is used by some investigators (*22*) to detect alterations in the T helper lymphocyte population.

Mitogen Responsiveness

Polyclonal mitogens such as PHA and LPS are thought to activate lymphocytes by binding to glycoprotein receptors on the cell surface (*23*). Although these mitogens seem to bind only certain glycoproteins which may be present on either T lymphocyte subpopulations, B lymphocytes, or accessory cells such as macrophages, their effects are independent of antigen binding specificity since polyclonal activation by mitogens results in B lymphocyte differentiation to cells producing immunoglobulins of many idiotypes (*15*) or results in T lymphocyte differentiation into cells which express non-specific cytotoxicity. The B lymphocyte mitogen, LPS, activates the B lymphocyte non-specifically at sites other than Ig receptors and the activation is direct (*24*). The mechanism of T lymphocyte activation by PHA is controversial.

The measurement of mitogen-induced (^{3}H)-thymidine incorporation in lymphocytes is an assessment of the activation phase of the immune response with the advantage of by-passing specific antigen initial recognition (*15*). The interpretation of such an experimental design must include the consideration of the role of the macrophage in mitogen-induced T lymphocyte activation since it has been suggested that there are macrophage-dependent and macro-independent PHA-responsive T lymphocyte subpopulations (*25*). The use of the B lymphocyte mitogen, LPS, and the T lymphocyte

the chemical-induced lesion, if any, by exposing a tendency to alter lymphocyte activation in general, or, T or B lymphocyte activation in particular.

Resistance to *Listeria monocytogenes*

The inoculation of mice with *Listeria monocytogenes* results in an immune response which is mediated by T lymphocytes and monocytes (*26*). Since immunity cannot be transferred by immune serum and treatment of immune spleen cells with anti-theta serum abrogates their ability to confer resistance (*15*), the resistance to *L. monocytogenes* infection, which is measured by mortality rate or the number of viable bacteria present in the spleen, liver or serum of infected animals, is regarded as a measure of cellular immunity. However, since the monocyte is the effector cell it is not possible to implicate either T lymphocyte or monocyte dysfunction in EC-induced impaired resistance using this study.

Delayed-Type Hypersensitivity

The subcutaneous administration of antigens such as PPD, DNFB, and DNCB to pre-sensitized animals results in delayed-type hypersensitivity response. This response is characterized by an erythematous lesion at the site of antigen injection and is due, in part, to the infiltration of monocytes in response to lymphokine production by T lymphocytes. The severity of the response, as determined by the degree of swelling or the amount of leakage of intravenously administered radiolabelled compounds into the site, is considered to be an *in vivo* measure of all three developmental phases of a cell-mediated immune response.

Allograft Rejection

Allograft rejection results when a graft such as a skin graft, which carries histoincompatible antigens, is grafted onto an immunocompetent recipient. A tissue rejection response mediated by T lymphocytes is initiated and cytolytic T lymphocytes, which are capable of destroying the grafted tissue, develop. Since a normal rejection response occurs in the absense of B lymphocytes, the allograft rejection response may be considered to be an *in vivo* measure of all three developmental phases of a cell-mediated immune response.

Graft-Versus-Host Response

Injection of immunocompetent cells into an immunoincompetent animal expressing histoincompatible antigens results in a graft-versus-host response (GVHR). It has been firmly established that the GVHR is an expression of cell-mediated immunity (*27*) and that the response is mediated by the T cell (*15, 28, 29*). The GVHR has three prerequisites. First, the recipient must be unable to react against the donor cell. Experimentally this may be accomplished by the injection of immunocompetent parental strain cells injected into young F_1 hybrids. Second, the donor and recipient cells must be histoincompatible. The injection of parental strain cells into F_1 hybrid recipients satisfies this requirement. Third, the donor cells must be immunocompetent. The severity of the graft-versus-host response induced by immunocompetent cells from control and treated animals can serve as an assessment of the functional status of the recognition, activation, and expression phases of the immune response as indicated by the development of a completed response, i.e., splenomegaly (*30*) or the increase in weight of the draining lymph node (*31*), and reflects the functional ability of the lymphocytes which are involved (*32*).

Mixed Lymphocyte Responsiveness

The mixed lymphocyte response (MLR) is generally accepted to represent the recognitive and proliferative phases of the cell-mediated immune reaction (*33-35*). It is accepted that differences in the antigens which are coded for by the I region of the H-2 locus, Ia antigens, are the predominant stimuli in the mixed lymphocyte response (MLR) and GVHR (*15*) and are present on T and B lymphocytes and macrophages but are not found on erythrocytes, brain, liver and kidney tissue or on fibroblasts (*36*). In addition to the antigens coded for by the H-2 locus, the antigens coded for by the Mls locus, which is outside the H-2 complex, are important stimuli in the MLR (*37*) and these antigens are present only on B cells (38).

There is extensive evidence which indicates that the cells which respond to Ia differences are T lymphocytes (*39-41*) and it is known that the receptor is antigen specific, is on or near the cell surface and can be readily synthesized (*42, 43*). In addition, the receptor may be controlled by the Ir gene (*44*) which may also regulate T cell, B cell and macrophage interaction (*15*). Therefore, the presentation of alloantigen of irradiated stimulator cells to responder cells *in vitro* results in a proliferative response by the responder cells which can be quantitated by (^{3}H)-thymidine incorporation and can be used as an evaluation of the recognition and proliferation phases of EC-exposed responder cells.

Lymphocyte-Mediated Cytotoxicity

T lymphocyte-mediated cytotoxicity is recognized as the final effector phase of cell-mediated immunity (*15*). Although Ia differences are sufficient for the development of a MLR, H-2K or H-2D region differences are also required for optimal *in vitro* cell-mediated lymphocytotoxicity (CML) (*45*). Antigens coded for by the H-2D and H-2K region have been found on all the cells which have been tested (*15*). Direct cytotoxicity is primarily mediated by the T lymphocyte (*46, 47*) and evidence indicates that one population of T lymphocytes must recognize the Ia differences and interact with the cytotoxic T lymphocyte (CTL), which directs its activity against H-2K and H-2D differences. In addition, separate populations of CTLs are directed against either H-2D or H-2K antigens (*48*).

Target cell lysis begins with brief contact of CTLs with target cells and results in target cell death within 3-24 hr (*49*). Since this response can be inhibited by either a millipore barrier between the target cells and the lymphocytes, antisera against the target cell or trypsinization of the cytotoxic lymphocyte, T cell recognition and contact with the target cell surface appears to be necessary for target cell destruction (*50, 51*).

Cell-mediated lymphocytotoxicity (CML) can be assayed by several *in vitro* techniques including short-term ^{51}chromium release from ^{51}chromium-labeled target cells and is an assessment of the effector phase of the expression of cell-mediated immunity (*15, 52*). Although the recognition and activation phases of the response to alloantigens may be intact as indicated by comparable values in MLR (^{3}H)-thymidine incorporation between cells from control and chemical-treated animals, the cytotoxic mechanisms may, nevertheless, be functionally impaired.

Lymphokine Production

Lymphokines are products of activated T lymphocytes and play an important role in local immune responses such as the delayed-type hypersensitivity response. The activity of lymphokines such as migration inhibitory factor (MIF) or macrophage activating factor (MAF) produced by isolated lymphocytes from EC-exposed animals which have been activated *in vitro* by stimulated macrophages isolated from normal animals can be measured *in vitro* with macrophages from normal animals. The activity of lymphokines, therefore, can be used as a measure of T lymphocyte function which may serve to dissect EC-induced lymphocyte impairment from monocyte impairment in cell-mediated immune dysfunction and deserves more attention.

Influence of Environmental Contaminants on Immune Function

Possible mechanisms by which EC may directly or indirectly influence the development of an immune response are shown in Figure 2. Molecular

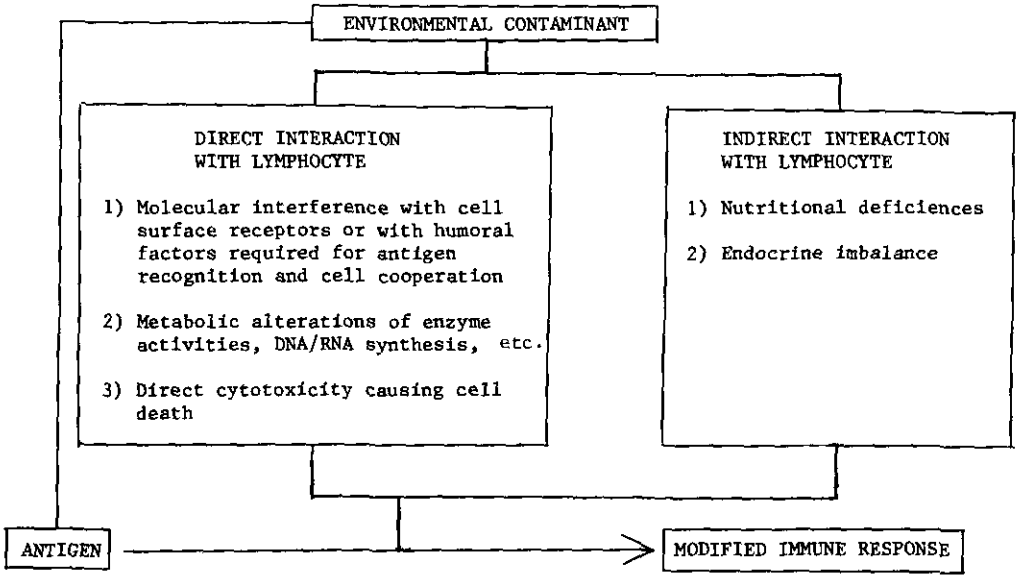

FIGURE 2. Influence of environmental contaminants on immune function.

interference with the production or function of cell surface receptors could result in a partial or complete loss of lymphocyte function or in changes in receptor specificity or affinity for antigen in addition to impairment of the cooperation among lymphocytes and accessory cells. Dandliker et al. (*53*) have demonstrated, however, that although post-immunization administration of pesticides, such as Dinoseb and Parathion, reduced the serum ovalbumin antibody concentrations in hamsters, there were no alterations in antibody binding affinity. TCDD (2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin)-induced alteration of lymphocyte cell membranes has been suggested by Faith (*54*) as a possible mechanism of toxicity. Metabolic interference with lymphocyte enzyme activities or DNA/RNA synthesis in lymphocytes in mitosis would result in inadequate numbers of reactive cells. Compounds such as azothioprine, methotrexate, and cyclophosphamide act in this way to cause therapeutic immunosuppression. EC may also be directly cytotoxic to stem cells in bone marrow or thymus and could cause selective depletion of chemical-sensitive clones of precursor or mature lymphocytes such as suppressor or effector T lymphocytes, B lymphocytes, or memory cells.

EC may also indirectly influence immune responsiveness by altering the nutritional status of the host (*55-57*). Chemical-induced alteration of endocrine function which would result in elevated glucocorticoid levels and steroid imbalance has also been suggested as a cause of immune dysfunction, however, studies with polybrominated biphenyl (PBB) and TCDD, which cause depletion of the hydrocortisone-sensitive cortical thymocytes, have not supported this hypothesis (*22, 58, 59*).

An EC may also directly interact with an antigen to alter its recognition by the immune system or alter sensitivity to immune attack.

Any of the above alterations of lymphocyte function may result in a modified immune response due to: (1) an over- or under-regulated response, (2) altered antigen recognition, (3) the inability of reactive clones to expand in number, (4) an insufficient number of reactive precursor cells, or (5) altered affinity or specificity for the antigen.

Lymphoid Processing of Environmental Contaminants

The influence of the lymphocyte on the disposition of the environmental contaminant must also be considered (Fig. 3). The compound, for example,

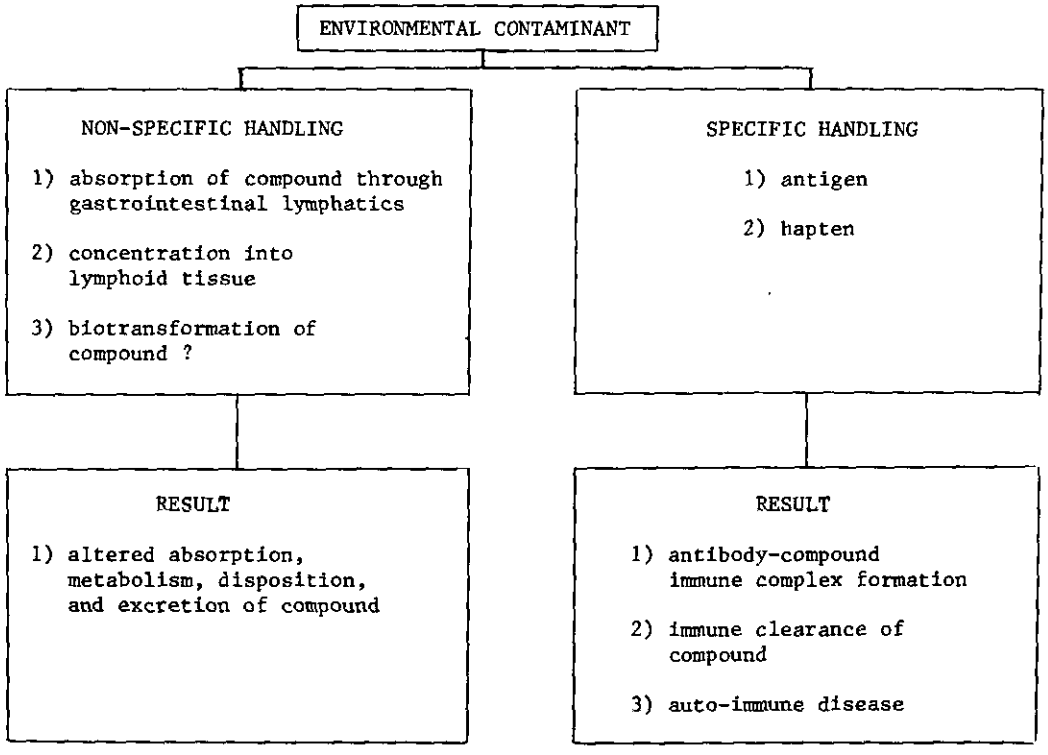

FIGURE 3. Lymphoid processing of environmental contaminants.

immune response directed against the compound. Antibodies to DDT, malathion and dieldrin have been demonstrated (*60*, *61*). Alternatively, the compound may complex with protein (*62*) and act as a hapten (*63-65*) and result in antibody directed against the hapten-protein conjugate which may lead to the formation of immune complexes. This mechanism for the pathogenesis of vinyl chloride disease has been suggested by Ward et al. (*66*).

The lymphocyte may also be involved in the nonspecific handling of the EC. Since it has been demonstrated that some compounds are absorbed by the lacteals and carried through the thoracic duct before they enter the general circulation (*67*), it is possible that the T lymphocyte, which is the predominant lymphocyte in the thoracic duct, passively adsorbs the compound and transports it to lymphoid tissue where it is released down a concentration gradient. This hypothesis would partially explain the ability of lymphoid tissue to concentrate xenobiotics to concentrations above serum levels (Results section).

Environmental Contaminants

Table 1 lists the results of several studies on the effects of environmental contaminants on different functional parameters of both humoral and cell-mediated immune responses. Although these studies demonstrate that exposure to environmental contaminants results in immune dysfunction, comparison of these studies for the purpose of defining a mechanism of immunotoxicity is difficult since only one parameter of the immune response is assessed in each study. Furthermore, there is great variability, from one study to another, in the age and species of the test animal, in the concentration and formulation of the compound and in the route and duration of exposure to the compound.

Several recent studies, however, have attempted to determine possible mechanisms of immunotoxicity by testing more than one immune parameter in animals on the same experimental protocol (Table 2). For example, Vos et al. (*19*) fed 0, 500, 1000, and 2000 mg HCB/kg to weanling rats for 3 weeks and

Table 1. Compounds tested for immunotoxicity using a single parameter of immune function.

Compound	Test animal	Immune parameter tested	Antigen	Effect	References
		Humoral immunity			
DDT	Chicken	Serum antibody	BSA (bovine serum albumin)	Decrease	(*68*)
DDT	Chicken	" "	BSA	No effect	(*69*)
DDT	Chicken	" "	*Salmonella pullorum*	No effect	(*69*)
DDT	Human	" "	Diptheria	No effect	(*70*)
DDT	Rabbit	" "	*Salmonella typhi*	Decrease	(*71*)
DDT	Rat	" "	Diptheria	Decrease	(*72*)
DDT	Rat	" "	Ovalbumin	Decrease	(*73*)
HBB	Guinea pig	" "	Tetanus toxoid	Decrease	(*74*)
Mirex	Chicken	" "	BSA	No effect	(*68*)
PCB Aroclor 1221	Rabbit	" "	PRV (Pseudorabies virus)	Decrease	(*75*)
PCB Aroclor 1242	Rabbit	" "	PRV	Decrease	(*75*)
PCB Aroclor 1254	Rabbit	" "	PRV	Decrease	(*75*)
Ametryne	Mouse	Number of plaque-forming cells	SRBC (sheep erythrocytes)	Decrease	(*76*)
Carbaryl	Mouse	" "	SRBC	Decrease	(*76*)
Chlordimeform	Mouse	" "	SRBC	Decrease	(*76*)
DDT	Chicken	" "	SRBC	No effect	(*68*)
DDT	Mouse	" "	SRBC	Decrease	(*76*)
DES	Mouse	" "	SRBC	No effect	(*77*)
DES	Mouse	" "	LPS (lipopolysaccharide)	Decrease	(*77*)
HCB	Mouse	" "	SRBC	Decrease	(*80*)
Mirex	Chicken	" "	SRBC	Decrease	(*68*)
Orthophenylphenol	Mouse	" "	DNP-Ficoll	Decrease	(*78*)
Orthophenylphenol	Mouse	" "	SRBC	Decrease	(*78*)
Parathion	Mouse	" "	SRBC	Decrease	(*76*)
PBB	Mouse	" "	SRBC	Decrease	(*79*)
PCB Aroclor 1242	Mouse	" "	SRBC	Decrease	(*80*)
		Cell-mediated immunity			
TCDD	Mouse	Allograft rejection time	Transplantation antigens	Increase	(*81*)
TCDD	Mouse	Graft-vs-host response	Transplantation antigens	Decrease	(*82*)
TCDD	Rat	Delayed-type hypersensitivity	Tuberculin	No effect	(*82*)
TCDD	Mouse	T-lymphocyte mitogen	PHA (phytohemagglutinin)	Decrease	(*83*)
Vinyl chloride	Mouse	T-lymphocyte mitogen	PHA	Increase	(*84*)

Table 2. Compounds tested for immunotoxicity using multiple parameters of immune function.[a]

			Humoral immunity				Cell-mediated immunity								
			Serum antibody titer		No. of plaque-forming cells to SRBC	LPS mitogen response	Resistance to *L. monocytogenes*	Allograft rejection time	Delayed-type hypersensitivity					Lymphokine activity (MIF)	T cell mitogen response	
Compound	Test animal	Reference	Antigen	Effect					Antigen	Effect	GVHR	MLR	CML			
Carbaryl	Rabbit	(*85*)	SRBC	NE	—	—	—	—	Tuber	NE	—	—	—	—	—	—
Carbofuran	Rabbit	(*85*)	SRBC	NE	—	—	—	—	Tuber	DEC	—	—	—	—	—	—
DDT	Rabbit	(*85*)	SRBC	NE	—	—	—	—	Tuber	DEC	—	—	—	—	—	—
DDT	Rat	(*86*)	BSA	INC	—	—	—	—	BSA	INC	—	—	—	—	—	—
HCB	Mouse	–	–	–	–	NE	—	—	—	—	DEC	NE	DEC	—	PHA	NE
HCB	Rat	(*87*)	LPS	NE	—	NE	DEC	NE	—	—	—	—	—	—	ConA	NE
			Tet tox	INC											PHA	NE
			T spir	INC												
HCB	Rat	(*19*)	LPS	NE	—	INC	NE	NE	PPD	NE	—	—	—	—	PHA	NE
			Tet tox	INC												
Methylparathion	Rabbit	(*85*)	SRBC	NE	—	—	—	—	Tuber	DEC	—	—	—	—	—	—
Mirex	Chicken	(*68*)	BSA	NE	NE	—	—	—	—	—	—	—	—	—	—	—
PBB	Human	(*88, 89*)	—	—	—	—	—	—	—	—	—	DEC	—	—	—	—
PBB	Mouse	(*22*)	—	—	DEC	—	—	—	DNFB	NE	—	—	—	—	—	—
PBB	Mouse	(*90*)	—	—	NE	DEC	—	—	—	—	—	—	—	—	ConA	DEC
															PHA	DEC
PCB Clophen A60	Guinea pig	(*91*)	Tet tox	DEC	—	—	—	—	FCA	DEC	—	DEC	—	—	—	—
PCB Aroclor 1016	Mouse	b	—	—	—	NE	—	—	—	—	NE	NE	NE	—	PHA	NE
PCB Aroclor 1254	Rabbit	(*85*)	SRBC	NE	—	—	—	—	Tuber	DEC	—	—	—	—	—	—
TCDD	Guinea pig	(*81*)	Tet tox	DEC	—	—	—	—	Tuber	DEC	—	—	—	—	—	—
TCDD	Mouse	(*92*)	SRBC	NE	DEC	NE	NE	—	DNFB	DEC	—	—	—	—	ConA	NE
TCDD	Mouse	(*81*)	—	—	—	—	—	—	—	—	DEC	—	—	—	PHA	DEC
TCDD	Rat	(*81*)	—	—	—	—	—	DEC	—	—	DEC	—	—	—	ConA	NE
															PHA	DEC
TCDD	Rat	(*53, 58*)	BGG	NE	—	—	—	—	Oxa-	DEC	—	—	—	—	ConA	DEC
		(*93*)							zalone						PHA	DEC
TCDD	Rat	(*59*)	BGG	NE	—	—	—	—	PPD	DEC	—	—	—	—	ConA	DEC
															PHA	DEC
TCDF	Guinea pig	(*59, 94*)	BGG	NE	—	DEC	—	—	PPD	DEC	—	—	—	DEC	ConA	NE
															PHA	DEC

[a]Abbreviations: BGG = bovine gamma globulin; BSA = bovine serum albumin; CML = cell-mediated lymphocytotoxicity; ConA = concan avalin A; DEC = decrease; DNFB = dinitrofluorobenzene; FCA = Freund's complete adjuvant; GVHR = graft-vs-host response; INC = increase; LPS = lipopolysaccharide; MIF = migration inhibitory factor; MLR = mixed lymphocyte response; NE = no effect; PHA = phytohemagglutinin; PPD = purified protein derivative; SRBC = sheep erythrocyte; Tet tox = tetanus toxoid; T spir = *T. spiralis*; Tuber = tuberculin; g pig = guinea pig.
[b]See Results, this paper.

antibody to tetanus toxoid, a T dependent antigen, was significantly increased, the titer of serum antibody to LPS, a T-independent antigen, was not significantly different from control values. Also, HCB administration had no significant effect on cell-mediated immune functions such as the resistance to *Listeria monocytogenes* infection, skin transplant rejection times or delayed-type hypersensitivity reactions. Therefore, the author concluded that although postnatal exposure to HCB does not significantly influence cell-mediated immunity, it has a stimulative influence on humoral immunity and suggested that T helper cell function may be enhanced by HCB. However, since spleen cell responsiveness to a B mitogen was also significantly increased, the sensitivity of the B lymphocyte to immunologic alteration following HCB exposure cannot be ignored and it is possible that the B lymphocyte may be more responsive to both humoral factors, produced during an immune response by T helper cells, and mitogens. This suggests, perhaps, an HCB-induced increase in receptor affinity or avidity for regulating factors.

In order to test the sensitivity of the developing immune system in young animals to the effects of HCB, Vos et al. (*87*) fed pregnant rats a diet containing 50 or 150 mg HCB/kg from days 1-3 of pregnancy through weaning and continued the exposure of the pups to the same diets until 5 weeks of age when several parameters of humoral and cell-mediated immunity were assessed. Cellular immune resistance to *Listeria monocytogenes* was significantly decreased although allograft rejection time and PHA and Con A mitogen responsiveness were unaltered. Serum antibody titers to the T dependent antigens, tetanus toxoid and *Trichinella spiralis*, however, were significantly increased and the author concluded, based also on a previous study (*19*), that HCB suppresses cellular immunity and enhances humoral immunity and that the developing immune system is more vulnerable to chemical insult than the established immune system of the adult. Faith and Luster (*54*) demonstrated with rats that either pre- or postnatal short-term exposure to TCDD during the developmental period of the immune system caused a decreased T mitogen responsiveness and delayed-type hypersensitivity responsiveness which recovered by 270 days of age and, therefore, showed that the effects are reversible. There was no alteration of the antibody response to BGG and it was suggested that T helper function was not altered by TCDD exposure but that TCDD effects a distinct subpopulation of T cells which do not have helper immune responses (*58*, *93*).

A study by Luster et al. (*90*) indicated that although short-term exposure to 0.03-30 mg PBB/kg could reduce spleen cell responsiveness to the B lymphocyte mitogen, LPS, in mice and could reduce the spleen cell responsiveness to the T cell mitogens, PHA and Con A, in both mice and rats, the antibody response to SRBC was not significantly impaired in either mice or rats. In contrast, however, Fraker (*22*) reported that short-term exposure of mice to 10, 100, and 1000 ppm PBB in their diet reduced both the direct and indirect plaque antibody response to SRBC but that the delayed-type hypersensitivity response was unimpaired and suggested that PBB affected B cells and helper T cells. The influence of PBB on the immune response remains unclear.

Since the involvement of the immune system in the expression of environmental chemical toxicity is evident following short-term exposure, it is appropriate to determine the sensitivity of lymphocyte function to modification following chronic exposure to xenobiotics, to delineate the mechanism of functional alteration of the lymphocyte and to simultaneously develop a test system for the assessment of the potential toxicity of new chemicals. Most studies, however, which have evaluated the influence of EC on lymphocyte function have followed an experimental design in which the test compound is administered to the test animal either daily or weekly for a period of up to several weeks at which time different parameters of the immune system are challenged with the appropriate antigens and the response is measured on one day only. This approach does not take into account several characteristics of either the immune response or exposure patterns to EC.

The immune response is characterized by an antigen specific temporal response which depends on the ability of the lymphoid system to react quickly and support rapid amplification of the number of reactive cells. Protocols for the assessment of EC alteration of immune responsiveness should, therefore, examine alterations in the specificity of the response and its amplitude and temporal distribution. In addition, since exposure to EC is characterized by chronic exposure to compounds at low concentrations, during which immune responsiveness could vary considerably due to: (a) the continuous replenishment of new reactive cells to the lymphocyte pool, (b) changes in the pattern of biohandling of the compound and its metabolites, and (c) changes in the local environment of the lymphocyte in lymphoid tissue, such as

compound, the effects of long-term exposure should be examined at appropriate intervals.

Therefore a study was designed to assess the influence of the chronic exposure of two polychlorinated aromatics of environmental interest, PCB (Aroclor 1016) and HCB, on the three developmental phases of the immune response in mice. Lymphocyte function was assessed at intervals during the chronic exposure period and only one form of antigen was used (the transplantation antigens) in addition to the lymphocyte mitogens LPS and PHA. When possible, the lymphocyte response was evaluated during the entire period of the primary response. In addition, the concentration of each compound in spleen, thymus and liver tissues, spleen cells and serum was determined at each exposure interval for comparison of lymphocyte function with the compound concentration.

Since much is known about the major histocompatibility complex (MHC) of the mouse (H-2) and many of the antigens, receptors and mechanisms of cell-cell interactions have been well-documented (*15, 27, 33*), the histocompatibility complex was employed as the experimental antigen and antigen-responsive system.

Many immune responses are controlled by the H-2 complex (*95*), for example, the production of transplantation antigens, cell-mediated lympholysis of target antigens, the mixed lymphocyte reaction, the graft-versus-host response, the production of Ia antigens and lymphocyte interactions. Immunotoxic compounds could interfere with the production, expression or function of the H-2 controlled antigens and, thereby, result in a decrease in antigen recognition during the initial encounter or during the final effector stage as well as a modification of the normal metabolic and interactive processes of an immune response. Also, since the H-2 complex controls immune responses to several antigens (*96*), it is possible that the H-2 complex may be involved in the response to toxic compounds.

Materials and Methods

Animals

Male C57BL/6Tex mice with a histocompatibility gene complex denoted as H-2^b, male B6D2F1 (H-$2^{b,d}$) mice (BDF1), pregnant C57BL/6Tex mice which had been mated with D6B2A/2 (H-2^d) mice (DBA) and male DBA mice were supplied by Timco Texas. Male AKR mice (H-2^k) were supplied by Jackson Laboratories, Bar Harbor, Maine. Mice were purchased at an initial weight of 18-20g. Albany Medical College. Control animals were fed Wayne mash. Experimental animals were fed a diet containing either 167 ppm Aroclor 1016 (PCB) or 167 ppm hexachlorobenzene (HCB) in Wayne mash. Food and water were provided *ad libitum*. Body and organ weights were determined for each experimental period and the relative organ weights were calculated. An analysis of variance and the Student's *t*-test were used to determine statistical significance of differences between control groups and experimental groups at $p \leq 0.05$.

Chemicals

The test chemicals used were the polychlorinated biphenyl mixture Aroclor 1016 (Monsanto) and hexachlorobenzene (C_6Cl_6) (HCB) (Eastman practical grade) which was purified by passage through activated charcoal and then recrystallized two times from boiling benzene. Both compounds were dissolved in acetone for incorporation into Wayne mash and mixed for at least 2 hr. Liquid-gas chromatographic analyses were conducted to assure the proper concentration of the test chemicals in the diets.

Tumor Cells

The methylcholanthrene-induced DBA/2 lymphoma, P388, benzopyrene-induced C57BL/6 lymphoma, EL-4, and the spontaneous DBA/2-derived mastocytoma, P815, were maintained in ascites form by weekly intraperitoneal (IP) inoculation and serial passage.

Spleen Cell Isolation Procedure

Mice were killed by cervical dislocation and weighed. A midline ventral incision into the peritoneal and thoracic cavities exposed the major visceral organs for gross examination *in situ* and allowed a blood sample to be taken from the abdominal vein. Spleens were removed aseptically and weighed in a sterile tissue culture dish (Falcon 3001, 35 × 10 mm, Oxnard, Calif.). The thymus, lung and liver were then removed and weighed. Approximately 10-60 mg samples of each tissue, in addition to 100-200 μl of the final spleen cell suspension (0.5-1 × 10^6 cells) and 100-200 μl serum, were frozen in glass vials for later liquid-gas chromatographic analysis for PCB or HCB content. Only those groups of mice used in mixed lymphocyte cultures were used for body and organ weight data (n = 5-6 per group). The spleen was then teased across sterile #60 mesh stainless steel into cold

ferred into polypropylene tubes (Falcon 2059, 17 × 100 mm, Oxnard, Calif.). Cells which remained in suspension after a 5-min settling period were used, thereby discarding unwanted debris and cell aggregates. Cell yield and viability were assessed immediately using trypan blue (0.4% Trypan Blue in saline (GIBCO)) mixed 1:10 with the cell suspension and appropriately diluted with 3% acetic acid and counted using a hemacytometer. The cells were then washed three times in cold HBSS and spun at 180 *g* and suspended to a concentration of 5×10^6/ml with RPMI 1640 media (GIBCO) and 10% heat-inactivated (57°C, 30 min) fetal bovine serum (FBS) (GIBCO). Cell suspensions were kept on ice and recounted just before they were dispensed into cultures to assure proper concentrations. In addition, a portion of the isolated spleen cells was diluted to a concentration of 5×10^5/ml with media and 200 µl of this suspension was pelleted onto a glass slide using a Cytospin (1000 *rpm* × 5 *min*) (Shandon Elliott, Camberley, Surrey, England). The cells were stained with Wright's stain and counted by differential cell count.

Graft-Versus-Host Response (GVHR)

A GVHR was induced in neonatal (< 24 hr) BDF1 mice by the IP injection of 1×10^7 spleen cells isolated from either control or chemical-treated C57BL/6 mice following 3, 6, 13, or 37 weeks of dietary administration of the test chemical. Spleen cells from 4-10 donor C57BL/6 mice from each diet group were used for the GVHR assay. The degree of splenomegaly was determined for each of the 4-18 neonatal recipient BDF1 mice which were used for each diet group after each diet treatment interval. The inoculum was administered in a volume of 0.5 ml RPMI 1640. Injection of spleen cells isolated from control BDF1 mice (isogeneic) served as a negative control for the GVHR. The split-litter procedure (*97*) was used to obviate experimental error due to variation between litters and to allow comparison of test chemicals within litters. Spleen and body weights of the neonates were determined on the ninth day of maternal rearing following inoculation with spleen cells. Results are expressed as the spleen index which was calculated by dividing the relative spleen weight of neonates inoculated with cells from control or chemical treated donors by the mean relative spleen weight of non-injected littermates. A spleen index of greater than 1.3 was considered to be a positive GVHR (*98*). Analysis of variance and the Student's *t*-test were used to determine statistical significance between positive GVHR responses.

One-way MLR assays were conducted using a modification of the procedure described by Rich and Rich (*99*). Responder splenocytes from either control or chemical-treated adult male C57BL/6 mice (4-6 mice per group) were either cultured alone, for the determination of the rate of background DNA synthesis, or co-cultured with equal numbers (5×10^5 cells) of allogeneic stimulator splenocytes from control DBA mice. Cultures were contained in a final volume of 0.2 ml RPMI 1640 (GIBCO) supplemented with a 2 *mM L*-glutamine (GIBCO), 10% FBS and 50-100 units penicillin (GIBCO) and 50-100 µg streptomycin (GIBCO) per ml (P-S) in 96-well flat bottom microtiter plates (Falcon 3040, Micro-test II with lids) in a humidified atmosphere of 5% CO_2 at 37°C (National Appliance, model 3341, Portland, Ore.) for 1-6 days. Viability and cell number of microcultures were determined daily. Splenocytes used as the stimulator cells were irradiated (2000 rads, Gammator Cs-137 Irradiator, Model M-38-1, Isomedix Inc., Parsippany, N.J.), washed three times in cold HBSS, and suspended to the proper concentration in media.

DNA synthesis was assayed by the addition to each culture of 1.0 µCi tritiated thymidine (NET-027, methyl-(^{3}H)-thymidine, specific activity 6.7 Ci/mmole, New England Nuclear Corp., Boston, Mass.) for the last 18 hr of culture. Cells were harvested by aspiration onto glass fiber filter strips by using an automated sample harvester (Skatron, Flow Laboratories, Rockville, Md.). The filter discs were dried, and the radioactivity of triplicate cultures was measured in 3 ml universal LSC cocktail (Aquasol-2, New England Nuclear Corp.) by using an automatic liquid scintillation spectrometer (Packard Tri Carb Model 3390, Downers Grove, Ill.) (counting period, 2-5 min, (^{3}H) channel, 50% gain, 50-1000 window). Data are presented as the arithmetic mean ± standard error of the counts per minute (cpm) of triplicate cultures. An analysis of variance and the Student's *t*-test were performed to determine the statistical significance of the differences between the means of control groups and experimental groups at $p \leq 0.05$.

Mitogen-Induced Blast Transformation

Mitogen responsiveness assays were conducted using the same method as employed in mixed-lymphocyte cultures; however, either 40 µg/ml phytohemagglutinin (PHA-M, B grade, Calbiochem, La Jolla, Calif.) or 10 µg/ml gram negative bacterial lipopolysaccharide (LPS, *Salmonella typhosa*, Westphal, Difco, Detroit, Mich.) were added to the

when the cultures were first established.

Cell-Mediated Lymphocytotoxicity

Sensitized lymphocytes from C57BL/6 mice immunized with the DBA/2 tumor, P815, were tested for their ability to recognize and kill cells which express alloantigen (H-2^d). The DBA/2 tumor, P388, was used as target cells to obviate anti-tumor antigen activity directed against P815 tumor antigen. The PHA-induced AKR blast splenocyte (H-2^k) was used as a target cell to detect chemical-induced nonspecific killing and the C57BL/6 tumor, EL-4, (H-2^b) was used to detect chemical-induced alteration of recognition of self.

Immunizations. Control and chemical-treated male C57BL/6 mice (4-6 mice per group) used in cytotoxicity assays were inoculated IP with 3×10^7 live P815 mastocytoma cells in 0.5 ml HBSS 10 days prior to spleen cell isolation. Tumor cells used for the immunization were harvested from DBA mice inoculated ip 7 days earlier with 1×10^6 P815 mastocytoma cells.

Labeling of Target Cells. P388 and EL-4 tumor cells were obtained by peritoneal lavage with a 22 gauge needle by using 5 ml HBSS from isogeneic

earlier. PHA-induced blast lymphocytes were prepared by culturing spleen cells from adult male AKR mice for 2 days (1×10^6/ml) with 40 μg PHA-M/ml. Prior to labeling, target cells were washed two times in HBSS and suspended to 2-20 $\times 10^6$/ml in media without serum. The cells were labeled by incubating 0.5 ml cell suspension with 100-200 μCi ^{51}Cr (0.1-0.2 ml, NEZ-030S, sodium chromate in saline solution, specific activity 200-500 Ci/g, New England Nuclear Corp.) in a 50 ml conical test tube on an aliquot shaker in a humidified atmosphere of 5% CO_2 at 37°C for 45-60 min. The labeled cells were then washed five times with 20 ml HBSS supplemented with 10% FBS, counted and suspended to a concentration of 2×10^5/ml.

Cytotoxicity Assay. Spleen cells from control and chemical-treated nonimmunized C57BL/6 mice immunized with P815 mastocytoma cells 10 days earlier were suspended in media RPMI 1640 supplemented with 2 *mM*-glutamine and 10% FBS and dispensed, in duplicate, into 96-well, flat bottom microtiter trays in 100 μl volumes. Spleen cells were not pooled. Chromium-labeled target cells (P388, AKR blast, and EL-4) were then added in 100 μl volumes of the same media to the appropriate wells. The effector cell:target cell ratio was 100:1 in all cultures, i.e., 2×10^6 effector cells:2×10^4 target cells per well. In addition, C57BL/6 ratios of 30:1 and 10:1 for later construction of titration curves for the determination of the ED50 for specific lysis (*100*). Spontaneous release of the label was determined by incubating target cells in media only. The microtiter plates were spun at 25 *g* for 2 min to increase cell-cell contact and minimize reaction time and then incubated without rocking in a humidified atmosphere of 5% CO_2 at 37°C. To examine the kinetics of the cytotoxic response the specific lysis was determined after 3 and 5 hr of incubation, at which times the plates were spun at 500 *g* for 10 min at 7°C. A 100 μl portion of the supernatant of each well was transferred into a polystyrene gamma counting tube (15.6 × 125 mm, Amershan, Arlington Heights, Ill.) and counted for 1 min in an automatic gamma counting system (Searle, Model 1185 Series, Searle Analytical Inc., Waltham, Mass.) with the window centered at 3221 KeV and with a width of 100 KeV.

The experimental release (ER) of label was determined by calculating the mean counts per min (cpm) of the supernatant samples of duplicate cultures; less background counts, of each culture containing effector cells and labeled target cells and multiplying by 2 to account for the volume of supernatant actually counted. The spontaneous release (SR) for each target cell type was determined by calculating the mean cpm of quadruplicate cultures of labeled target cells, less background, and multiplying by 2 to account for the fraction of the total volume of supernatant actually counted. The maximal release (MR) of label was determined by mixing 100 μl labeled target cells with 300 μl distilled water in polypropylene tubes (Falcon, 2058) which were passed through four freeze-thaw cycles. The mean cpm, less background counts, was then calculated and multiplied by 4 to account for the volume actually counted. The total incorporated label was determined by calculating the mean cpm, less background counts, of eight 100 μl samples of labeled target cells. The percent specific ^{51}Cr release was then calculated as follows: % specific ^{51}Cr release = (ER - SR)/(MR - SR) × 100. Data are presented as the arithmetic mean ± standard error of the percent specific ^{51}Cr release calculated for individual mice (n = 3-6) of the control and experimental groups. An analysis of variance and the Student's t-test were used to determine statistical significance of the difference between the means of control groups and experimental groups at $p \leq 0.05$.

Organochlorine Residue Analysis

Liquid-gas chromatographic analysis of Aroclor 1016 and HCB was conducted using a modification

Holden and Marsden (*101*).

Results

Graft-Versus-Host Response

The inoculation of neonatal BDF1 mice with 10^7 spleen cells from C57BL/6 mice which were fed either a control diet or a diet containing 167 ppm PCB for 3, 6, 13, or 37 weeks or a diet containing 167 ppm HCB for 3, 6, or 13 weeks resulted in a positive graft-versus-host response in all groups. No significant effect of chemical exposure of the donors on the GVH response was demonstrated (Table 3). However, exposure to HCB for 37 weeks resulted in a significant reduction of 20% in the graft-versus-host activity of HCB-treated cells. Spleen cells from normal BDF1 mice did not produce a GVHR in neonatal BDF1 mice.

Spleen Cell *in Vitro* Background DNA Synthesis, Viability, and Differential Counts

Spleen cells isolated from mice which were fed PCB for 3, 6, 13, 24, and 40 weeks and cultured in media for 1-6 days did not show any alterations in DNA synthesis during the culture periods as compared to control cultures (Fig. 4). In addition, the number of viable cells per culture for each day of culture, which was determined only in the 3 and 40 week groups, was comparable to control values at both diet duration intervals tested (data not presented). These results indicate that PCB did not alter culture viability. Also, there were no shifts from control values in the population densities of small or large lymphocytes, polymorphonuclear leukocytes or macrophages in either the 3 or 40 week groups (data not presented).

HCB for 3 weeks (Fig. 4) and cultured in media for 1-6 days, demonstrated no alterations in DNA synthesis as compared to control values, for the culture period. However, spleen cells from mice which were fed HCB for 6, 13, 24, and 40 weeks demonstrated a pattern of increased DNA synthesis during the first day of culture. It was first observed in the 6-week group (205% increase). In the 13-week group, DNA synthesis was elevated 279% during the first day of culture and fell to below the control value by the third day. The increase in DNA synthesis during the first day of culture in the 24 week group was significantly above (1431%) control values and remained above control values through the second day of culture. In the 40 week group, DNA synthesis was 628% above the control value on the first day of culture but was comparable to the control values thereafter. There were no differences from control values in the number of viable cells throughout the culture period in the 3- or 40-week groups (data not presented). In addition, in the 3- and 40-week group there were no alterations from control values in the cell population densities of small or large lymphocytes, polymorphonuclear leukocytes, and macrophages (data not presented).

Mixed Lymphocyte Response (MLR)

Spleen cells isolated from C57BL/6 mice which were fed either a control diet or a diet containing 167 ppm PCB 1016 or HCB for 3, 6, 13, 24, or 40 weeks responded with a transient increase in DNA synthesis when stimulated in a one-way mixed lymphocyte culture with equal numbers of irradiated spleen cells from DBA mice (Fig. 4). Peak DNA synthesis by C57BL/6 spleen cells from control, PCB- and HCB-treated groups stimulated by DBA cells occurred on the sixth day of culture in the 3-week group, on the fifth day of culture for spleen cells from PCB- and HCB-treated mice, but on the

Table 3. Graft–host activity of spleen cells from C57BL/6 mice fed either a control diet, PCB 1016, or HCB for 3-37 weeks and injected into neonatal BDF1 mice.[a]

		Spleen index[a]			
Donor cell strain	Diet	3 weeks	6 weeks	13 weeks	37 weeks
C57BL/6	Control	2.78 ± 0.21	2.07 ± 0.28	2.21 ± 0.12	2.24 ± 0.12
C57BL/6	PCB	2.77 ± 0.25	2.57 ± 0.34	2.13 ± 0.11	2.63 ± 0.20
C57BL/6	HCB	2.38 ± 0.28	2.26 ± 0.24	2.25 ± 0.12	1.80 ± 0.09*
BDF1	Control	1.10 ± 0.10	0.91 ± 0.02	0.93 ± 0.04	—

[a]Data presented as mean spleen index ± standard error. See Material and Methods section for experimental details. There were 4-10 donor mice and 4-18 recipient mice for each diet group and each diet interval. Asterisk indicates statistical significance from control value at $p \leq 0.01$.

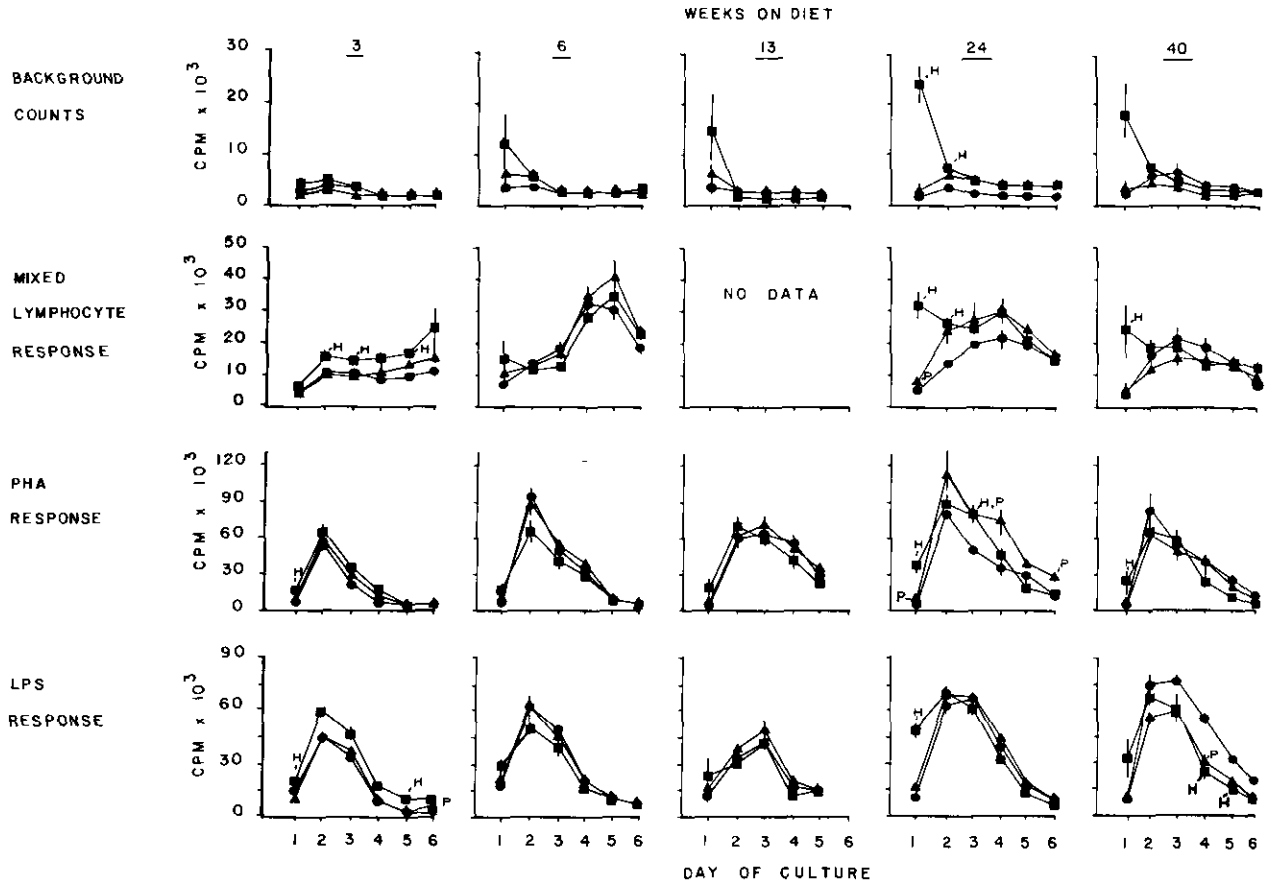

FIGURE 4. Mean (^{3}H)-thymidine incorporation in CPM ± standard error in cultures of spleen cells from mice fed either a control diet, or a diet containing Aroclor 1016 (PCB) or hexachlorobenzene (HCB) for 3-40 weeks and cultured alone (background counts), with alloantigen (mixed lymphocyte response), or with mitogens (PHA and LPS responses): (●) control; (▽) PCB; (□) HCB. See Materials and Methods section for experimental details. n = 4-6 for all points. P (PCB) or H (HCB) indicates statistical significance from control values at $p \leq 0.05$.

fourth day for spleen cells from control mice in the 6-week group, on the fourth day of culture in the 24-week group, and on the third day of culture in the 40-week group. There were no significant chemical-induced shifts of the time or magnitude of the peak response.

Alloantigen-induced DNA synthesis in spleen cells from C57BL/6 mice which were fed HCB for 3 weeks was significantly greater than control values for days 1, 2, 3, and 5 of culture when stimulated with DBA alloantigen. Although DNA synthesis in mixed lymphocyte cultures of C57BL/6 spleen cells with DBA cells is significantly greater than the control values on the first two days of culture after exposure to HCB 24 weeks and only on the first day of culture after exposure to HCB for 40 weeks, the increase is due to the elevated background synthesis observed at the initiation of cultures as previously noted and does not reflect an alteration of alloantigen reactivity.

Mitogen Responsiveness

Figure 4 also shows the mitogen-induced DNA synthesis of cultured spleen cells from C57BL/6 mice which were fed a diet containing 167 ppm PCB or HCB for 3-40 weeks. A transient increase in tritiated thymidine ([^{3}H] - TdR) incorporation which followed the addition of 40 μg/ml PHA-M (a T cell mitogen) at the initiation of culture and which generally peaked on day 2, but peaked on day 3 in control and PCB-treated cultures in the 13-week group, was observed in all control and experimental

control values in the time or amplitude of the peak response as determined by (^{3}H)-TdR incorporation in cultures of splenocytes from mice which were fed PCB for 3, 6, 13, or 40 weeks. However, following 24 weeks of dietary exposure to PCB, a significant increase in DNA synthesis was observed in PHA-stimulated splenocytes on days 1, 3, and 6 of cultures and was above control values, but not statistically significant, on days 2, 4, and 5 of culture. There were no significant shifts in the time or magnitude of the peak response of PHA-induced DNA synthesis in splenocytes from mice which were fed HCB although the response was significantly greater than control values on day 1 of culture after 3 weeks exposure to HCB, significantly above control values on days 1 and 3 of culture in the 24-week group and above control values on the first day of culture in the 40-week group. There were no alterations in the 6- and 13-week groups. The increase in DNA synthesis on day 1 of culture in the 24-week group and on day 1 in the 40-week group are associated with the high background (^{3}H)-TdR incorporation rate previously mentioned and, therefore, do not represent chemical-induced alterations of the response to PHA.

The addition of 10 μg/ml LPS (a B cell mitogen) at the initiation of cultures of spleen cells from control mice or mice fed either PCB or HCB for 3-40 weeks resulted in an increase in the (^{3}H)-TdR incorporation rate, which generally peaked on days 2 or 3, in all control and experimental cultures and the peak responses in experimental cultures were comparable to control values. However, LPS-induced DNA synthesis was significantly greater than control values on the sixth day of culture of splenocytes from mice which were fed PCB for 3 weeks but there were no chemical-induced alterations observed in mice which were fed PCB for 6, 13, or 24 weeks. However, exposure to PCB for 40 weeks resulted in a profound decrease in LPS-induced DNA synthesis on day 4 of culture.

LPS-induced DNA synthesis by spleen cells from mice which were fed HCB for 3 weeks was above control values on days 1 and 5 of culture but there were no alterations observed in mice which were fed HCB for 6 or 13 weeks. An increase in DNA synthesis was observed on the first day of culture of cells from mice which were fed HCB for 24 weeks, however, the increase is associated with the elevated background (^{3}H)-TdR incorporation rate previously mentioned and, therefore, does not represent chemical-induced alteration of the response to LPS. However, exposure to HCB for 40 weeks resulted in a significant decrease in LPS-induced DNA synthesis on days 4 and 5 of culture.

Sensitized spleen cells from C57BL/6 (H-2^b) mice fed a control diet for 3-40 weeks and immunized with 2×10^7 live P815 (H-2^d) tumor cells 10 days prior to CML assays exhibited specific lysis of ^{51}Cr-labeled cells which expressed the H-2^d alloantigen, i.e., P388 tumor (H-2^d) (Fig. 5). There was no lysis of allogeneic AKR (H-2^k) PHA-blast cells against which they were not immunized, which indicated that lysis was specific, and there was no lysis of syngeneic EL-4 (H-2^b) tumor cells which indicated the absence of any alteration of cytotoxicity directed against self. Only data for the lysis of the P388 tumor are presented here; see Silkworth (*102*) for complete data. Also, specific lysis of labeled target cells by nonsensitized effector cells was less than 5% in all trials.

The mean spontaneous release of label over the 5-hr incubation period for all intervals was 13% for the P388 tumor, 10% for the EL-4 tumor, and 44% for the AKR PHA-blast cell. Maximum freeze-thaw release of label was 83-88% for all labeled cells.

When the effector cells were from immunized mice which were fed a control diet for 3, 6, and 13 weeks (Fig. 5), an effector cell:target cell ratio of 100:1 resulted in the progressive specific release of ^{51}Cr from labeled P388 tumor cells of approximately 43% and 69% after 3 and 5 hr incubation, respectively. However, effector cells from immunized mice which were fed a control diet for 24 and 40 weeks caused the progressive specific ^{51}Cr release from P388 tumor cells of approximately 26% and 57% after 3 and 5 hr incubation, respectively, in the 24-week group and 16% and 36% after 3 and 5 hr incubation, respectively, in the 40-week group. These results may indicate an age-related decrease in specific cytotoxicity in the control population which began after 13 weeks of experimental housing and approximately 18 weeks of age. In addition, an effector cell dose-response was observed when effector cell:P388 target cell ratios of 30:1 and 10:1 were used.

There were no significant alterations from control values of the specific lysis of labeled target cells by spleen cells from immunized C57BL/6 mice which were fed 167 ppm PCB 1016 for 3 to 40 weeks (Fig. 5). There were no significant alterations from control values of the specific lysis of labeled target cells by spleen cells of immunized C57BL/6 mice which were fed 167 ppm HCB for 3, 13, 24, or 40 weeks (Fig. 5). However, the incubation of sensitized spleen cells from mice exposed to HCB for 6 weeks with labeled P388 target cells at an effector: target cell ratio of 100:1 resulted in the significantly decreased specific lysis of only 9% and 17% (20%

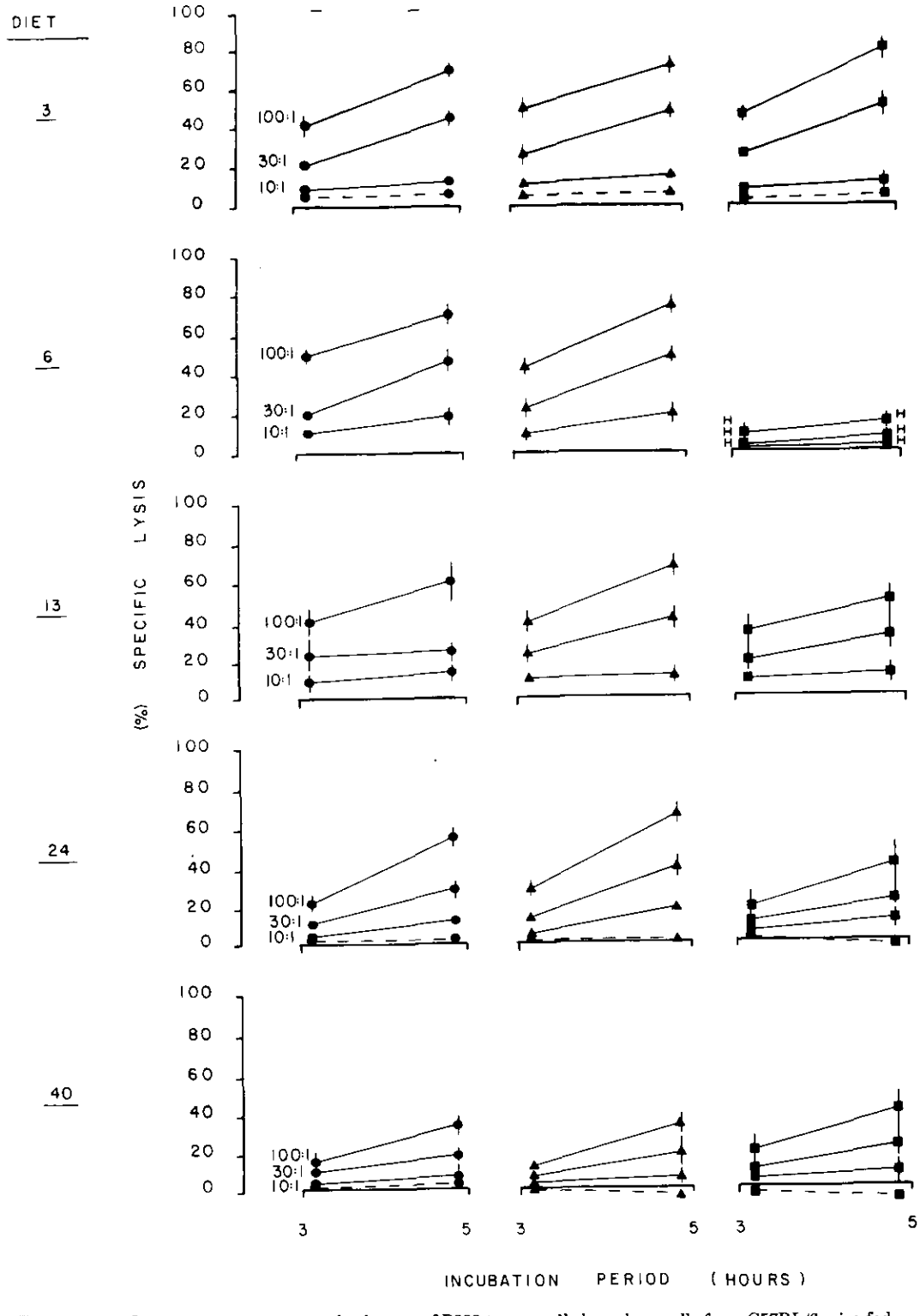

FIGURE 5. Mean specific lysis ± standard error of P388 tumor cells by spleen cells from C57BL/6 mice fed either a control diet, or a diet containing PCB or HCB for 3,6,13,24 and 40 weeks and either (—) immunized with P815 tumor cells or (–) not immunized: (●) control; (▽) PCB; (□) HCB. See Materials and Methods section for experimental details. n = 3-6 for immunized, n = 1 for nonimmunized lines. H indicates statistical significance from control values at $p \leq 0.05$.

and 17% of the control value) after 3 and 5 hr incubation, respectively. Similarly, effector:target cell ratios of 30:1 resulted in the significantly decreased specific lysis of 4% and 7% (18% and 15% of the control) after 3 and 5 hr incubation, respectively, and at ratios of 10:1, a significantly decreased specific lysis of 1% and 2% (11% and 10% of the control values) after 3 and 5 hr, respectively.

The ED_{50}, that is, the number of cells required to cause 50% lysis as determined by linear regression analysis of the dose-response values for each treatment group of the 6-week exposure period, was calculated for 3 and 5 hr incubation. The control ED_{50} values were 2.25×10^6 and 1.4×10^6 for 3 and 5 hr, respectively. The ED_{50} values for the PCB-treated group were 2.13×10^6 and 0.92×10^6 for 3 and 5 hr, respectively. The ED_{50} values for the HCB-treated group were 11.39×10^6 and 6.01×10^6 for 3 and 5 hr, respectively, and represent 5-fold and 6-fold decreases from control values after 3 and 5 hr incubation in effector cell ability to cause the specific lysis of the target cells. In addition, there was no alteration from the control response when labeled EL-4 tumor cells were the target cells.

Residue Analysis

Electron capture liquid-gas chromatography of serum, tissue and isolated spleen cells revealed that PCB concentrations did not follow the same pattern of disposition in all samples (Table 4). The concentration of PCB in the liver decreased from the 3-week value during the 3-13 week period, after which it increased to a steady state level. The concentration of PCB in the spleen consistently decreased from the 6-week value. The concentration of PCB in the thymus remained within 16% of the median value during 40 weeks of exposure. The concentration of PCB in the serum, although varying significantly from each previous measurement, remained within 26% of the median value throughout the observation period. And the concentration of PCB in spleen cells remained within 22% of the median value throughout the observation period.

HCB concentrations reached maximal levels in liver during the 13 to 24 weeks exposure period. The concentration of HCB in the spleen reached its highest level by 6 weeks, after which it decreased. The HCB concentration in serum and thymus reached its highest value by 24 weeks of exposure. The concentration of HCB in the spleen had reached its highest level by 6 weeks, after which it decreased. The concentration of HCB in spleen cells increased from 3 to 24 weeks, after which it decreased, and the kinetics did not correlate with those of whole spleen concentrations.

The ranges of the ratios of the concentrations of HCB to PCB within the same exposure periods were: liver, 4-11:1; spleen, 4-9:1; thymus, 1-4:1; serum, 20-56:1; and spleen cells, 1:5-15. Neither PCB nor HCB was found in random samples of tissue from mice which were fed a control diet for up to 40 weeks.

Discussion

The influence of the chronic dietary administration of two common environmental contaminants (PCB and HCB) on several parameters of lymphocyte function has been investigated. PCB, at the concentration used in this study, did not alter, in any consistent manner, the graft-versus-host response, the mixed lymphocyte response, the mitogenic

Table 4. PCB and HCB residue analysis.

Diet	Tissue	Mean PCB or HCB concentration, ppm[a]				
		3 weeks	6 weeks	13 weeks	24 weeks	40 weeks
PCB						
	Liver	13 ± 1.1	9.1 ± 2.3	4.6 ± 0.5	19 ± 2.9*	21 ± 6.3
	Spleen	—	12 ± 0.2	6.2 ± 0.9*	4.0 ± 0.8	3.4
	Thymus	20 ± 2.9	19 ± 2.0	19–	19–	25 ± 5.2
	Serum	0.18 ± 0.01	0.14 ± 0.01*	0.23 ± 0.02*	0.14 ± 0.01*	0.22 ± 0.01*
	Spleen cells (ng/10^6 cells)	80 ± 23	95 ± 18	92 ± 10	—	61 ± 8.0
HCB						
	Liver	56 ± 5.6	49 ± 11	187 ± 12*	203 ± 42	115 ± 17
	Spleen	—	59 ± 17	40 ± 1.3	18 ± 3.3*	31 ± 7.1
	Thymus	18 ± 1.7	28 ± 6.0	47 ± 9.6	70 ± 17	58 ± 11
	Serum	3.5 ± 0.2	4.1 ± 0.4	7.9 ± 1.0*	8.0 ± 0.8	4.5 ± 0.6*
	Spleen cells (ng/10^6 cells)	5.4 ± 1.5	6.3 ± 1.4	15 ± 2.4*	27 ± 2.6*	12 ± 3.3*

[a]Data presented as mean concentration of PCB or HCB wet weight ± standard error; See Materials and Methods section for experimental details; $n = 1$-5. Asterisk indicates statistical significance from previous sampling interval at $p \leq 0.05$ by analysis of variance and Student's t-test.

response, or cytotoxic activity of spleen cells isolated from experimental animals.

The time and the magnitude of the peak response of spleen cells from PCB-treated mice in all mixed lymphocyte and mitogen stimulated cultures were comparable to the response of spleen cells from control animals. These results indicate that PCB did not consistently alter the initial recognition of a alloantigen or lymphocyte activation by mitogens, as measured by DNA synthesis during the activation phase of the response. However, transient alterations in PCB-treated lymphocyte function were observed and deserve attention.

The increase in PHA-induced DNA synthesis above control values observed following 24 weeks of dietary exposure to PCB may be the result of either an increase in the number of responsive T cells and/or a PCB-induced impairment of suppressor T cell function, which would permit a greater than normal response. However, both the magnitude and the time of the peak response are comparable to control values. The 37% decrease in LPS-induced spleen cell DNA synthesis which was observed after 40 weeks dietary exposure in the absence of simultaneous impairment of PHA-induced DNA synthesis adds to the findings of Loose et al. (*80*), who reported a 69% decrease in the number of plaque-forming cells per million spleen cells during the primary antibody response to SRBC in mice fed PCB 1242 for 6 weeks. However, since the antibody response to SRBC requires B lymphocytes, T lymphocytes and macrophages (*18*), the cell type which was the target cell for PCB toxicity, if indeed there was only one, could not be defined. Since LPS is known to directly stimulate DNA synthesis in cultures of B lymphocytes depleted of T lymphocytes and macrophages (*103*), PCB-induced impairment of B lymphocyte activation is probably not directly due to either impaired T lymphocyte helper activity or macrophage function. These results implicate the B cell as a possible target cell in PCB-induced humoral immunotoxicity.

In the present study, the impairment of B lymphocyte responsiveness to mitogen, however, was not observed until after 40 weeks of PCB exposure, whereas Loose et al. (*80*) demonstrated a decrease in the number of plaque-forming cells after 6 weeks of PCB exposure. While the temporal differences in these observations may be due to differences in the experimental animal strains used and/or the differences in the isomer contents of the PCBs used, it is also possible that the plaque assay, which detects the presence of plasma cells derived from B lymphocytes responsive to sheep erythrocyte antigens, is more sensitive and, therefore, better able to detect alterations from control responses than the LPS mitogen assay, which detects polyclonal B lymphocyte activation, due to the inherent individual variation of the response of the larger proportion of responding cells in the culture.

Although transient PCB-induced alterations of B lymphocyte mitogen responsiveness were observed in the present study, there were no alterations of cytotoxic activity against cell surface alloantigen by sensitized lymphocytes from PCB-treated mice and there was no increase in nonspecific killing. These results suggest that PCB did not interfere with the effector phase of the cell-mediated immune response.

There were no alterations from control values of body weight or relative spleen, thymus, or lung weights or in the number of spleen cells isolated from PCB-treated mice throughout the entire experiment. The relative liver weight was significantly greater than control values during the first 6 weeks of PCB exposure and is consistent with the findings of other investigators (*91*) and is due to marked proliferation of smooth endoplasmic reticulum (SER).

The findings of the present study extend the previous evidence that PCBs alter humoral immunity reported by Vos and Van Genderen (*74*), Koller and Thigpen (*75*), and Loose et al. (*80*) and suggest that PCBs can express selective toxicity on different portions of the immune system. They also indicate that the target site for PCBs causing the depression of antibody-mediated immunity is a mechanism and/or a cell type which is not shared by the components of cell-mediated immunity. Since Vos and Van Driel-Grootenhuis (*91*) reported that exposure of guinea pigs to PCB resulted in a decrease in the relative thymus weight and a decreased delayed-type hypersensitivity skin reaction, the present findings may also indicate species differences in the susceptibility to PCB-induced immunotoxicity. It is possible that a regulatory cell which normally acts to balance both the cell-mediated and the humoral-mediated arms of the immune system is functionally impaired by PCBs and allows an imbalanced response to occur.

Unlike PCB exposure, HCB exposure resulted in the functional alteration of two of the parameters of immune responsiveness measured in this study, i.e., graft-versus-host activity and cell-mediated lymphocytotoxicity. A 20% reduction of GVH activity was observed after 37 weeks dietary exposure to HCB. This result may indicate that splenomegaly is not sensitive enough to detect a functional alteration of GVH activity even though other aspects of immune responsiveness are modified. For example, Vos and Moore (*104*) reported that four weekly doses of 25 μg/kg TCDD reduced the GVH activity of spleen cells of young mice by 25%,

ıe responsiveness to PHA was reduced 67%. However, it may also indicate that the cells involved in the graft rejection response are resistant to acute chemical-induced functional alteration and that only chronic chemical exposure results in detectable immunotoxicity.

Chemical-induced alteration of immune function may not always result in an impaired lymphoid activity. Instead, enhanced activity of certain aspects of immune reactivity may occur and result in an improper or unbalanced overall response.

A 32-76% increase in alloantigen-induced DNA synthesis was observed in cultures of spleen cells from mice fed HCB for 3 weeks. These results may suggest that a population of nonspecifically primed lymphocytes was present in the cultures of HCB-treated spleen cells and were able to support a greater response to alloantigen than control cultures during three days of the culture period, however, the magnitude and time of the peak response are not significantly different from the control response. Since the kinetics of the response were the same as the control response, it is apparent that the increase in the rate of DNA synthesis during the culture period was due to a stimulus present in the culture, i.e., the allogeneic stimulator cells, and not due to a preexisting stimulus *in vivo*. This does not seem to be the case with the increase in the background rate of DNA synthesis observed following longer periods of exposure to HCB and which will be discussed later. There were no HCB-related alterations in alloantigen-induced DNA synthesis, however, during the 40 week exposure period which followed.

Vos et al. (*87*) reported that HCB did not alter skin rejection times of rats exposed to HCB pre- and postnatally until 5 weeks of age. However, allograft rejection also involves effector cell function and, therefore, evaluates the effector phase, in addition to the recognition and activation phases of the immune response.

A 51% increase in PHA-induced DNA synthesis was observed following 24 weeks exposure to HCB only on day 3 of culture. These results compare with the findings of Vos et al. (*87*) that HCB did not profoundly alter PHA responsiveness of spleen cells from rats following pre- and postnatal dietary exposure to 100 ppm HCB through 5 weeks of age although a slight enhancement of the response was noted. In addition, chemical-induced enhancement of the lymphocyte response to a T cell mitogen may represent an alteration in splenic T/B ratios and lead to an inappropriate response to an antigen. For example, if suppressor T cell function is increased, due to the presence of an excessive number of T lymphocyte precursors, B lymphocyte differentiation into a sufficient number of antibody producing plasma cells may also be impaired. Furthermore, the present study extends the findings of Vos et al. (*87*) by indicating that chronic exposure of experimental animals to toxic compounds may be necessary to detect certain immunological dysfunctions.

A decrease of 50-53% in LPS-induced DNA synthesis was observed after 40 weeks exposure to HCB. However, no alterations from the control response were observed in either the magnitude or the time of the peak response. These results extend the findings of Loose et al. (*80*) that the number of PFCs per 10^6 spleen cells was decreased 53% below control values after 6 weeks exposure to HCB and since plaque forming cells are of the B cell lineage, chemical-induced B lymphocyte dysfunction may be indicated. In the same study, Loose also reported a 24% to 42% decrease in IgG_1, IgA, and IgM in mice fed HCB. These results provide more evidence that there is a disparity in the sensitivity between T and B lymphocytes to HCB and that humoral immune functions may be more sensitive than cell-mediated immune parameters to the toxicity of HCB.

A 75-79% reduction in the specific cytotoxicity directed against cell surface alloantigen by sensitized spleen cells from mice exposed to HCB for 6 weeks is associated with the time when the highest concentration of HCB was detected in the spleen. Impairment of lymphocytotoxicity in the absence of alteration of the recognition and activation phases of the immune response indicates HCB-induced functional alteration of the effector phase. This hypothesis is supported by the decreased GVH activity demonstrated in HCB-treated mice since there is evidence which indicates that the effector cells involved in GVH activity are the same subpopulation of cells as those which are responsible for CTL (*15*). It is possible, however, and difficult to exclude with certainty, that HCB-induced alteration of host resistance (*87*, *105*) to infectious agents, such as bacteria, viruses and protozoa, may result in a delayed response or even dysfunction of the mechanisms of host defense and, in turn, may have interfered with the cytotoxicity assay. In addition, a 97% increase above control values in the number of spleen cells isolated was also observed. The high concentration of HCB in the spleen may have caused impaired differentiation of precursor cytolytic cells into effector cells or the compound may have interfered with the cytolytic mechanism itself.

The 1431% increase in background DNA synthesis observed during the first 24 hr of culture of spleen cells from mice treated with HCB for 24 weeks was consistent with a pattern which began to

develop after 6 weeks of HCB exposure. The increased rate of DNA synthesis was no longer present after 2 days of culture and suggests that the population of cells which were synthesizing DNA may have completed synthesis and entered the mitotic and differentiation phases (although the synchrony seems too great), may no longer be viable in the culture, or, most probably, have lost the stimulant for the initiation of DNA synthesis. There are two possible explanations of enhanced DNA synthesis. First, pathogenic organisms present in the HCB-treated animals, due to a decreased host resistance, but not present in control or PCB-treated animals, would result in a chronic condition of lymphoid activation, but would have, perhaps, been eliminated from the cell preparation during the isolation procedure. The absence of bacteria in the blood and spleens of the HCB-treated animals suggests that DNA synthesis is not due to bacterial infection. Furthermore, there was no increase in the number of splenic PMNs, which would have indicated bacterial infection. Viral infection of the HCB-treated animals is a strong possibility and is indicated by the increase in the relative lung weight and altered pulmonary histology. Second, the enhanced rate of DNA synthesis may be due to a compound with mitogenic properties present *in vivo* which is the result of HCB exposure. The compound could be HCB itself, a metabolite of HCB not present in mitogenic concentrations *in vitro* or an HCB-induced cellular product which is mitogenic. An increase in unstimulated ^{3}H-thymidine uptake has also been demonstrated in spleen cell cultures from mice exposed to TCDD or vinyl chloride (*83*, *84*).

The body weights of mice fed HCB for 13 to 40 weeks were consistently less than control mice. A decrease in the amount of adipose tissue was apparent on gross examination and suggested an alteration in lipid metabolism or food consumption, but could not be attributed to a decrease in food consumption due to the technical imprecision of the feed weigh-back method available. A decrease in the body weight and food consumption by rats fed 1000 mg/kg HCB for 3 weeks was reported by Vos et al. (*87*). An increase in relative spleen weights of mice fed HCB for 24 to 40 weeks could suggest either immune reactivity since the percentage of large lymphocytes present in the spleen cell suspensions from mice treated with HCB for 40 weeks was increased (although the increase was not great enough to be statistically significant) or an increase in hemopoiesis, since erythrocytes were more abundant in the spleen cell preparations from HCB-treated mice than from control mice. A decrease in the relative thymus weights observed after 6, 13, and 24 weeks of exposure to HCB may be associated with the thymo-toxic properties seen with other chlorinated hydrocarbons such as TCDD (*104*). In addition, slight cortical atrophy of the thymus was reported by Vos (*87*) in rats fed 2000 mg/kg HCB. However, no histological alterations of the thymus were observed in this study.

An increase in the relative liver weight observed throughout the study is typical of many chlorinated hydrocarbons such as PCB and reflects marked proliferation of SER. It is difficult to delineate the physiological response from hepatotoxicity (*106*), however, and it is possible that alterations of hepatic function may be directly or indirectly associated with altered lymphoid function.

The greatest tissue concentrations of PCB were in thymus and liver and indicates that the thymus, a primary lymphoid organ, may concentrate PCB to levels above serum levels and may act as a storage depot for PCB. Since Vos and Moore (*104*) found that TCDD had a greater influence on CMI following pre- and postnatal exposure than following adult exposure, in addition to the lack of CMI alteration found in the present study in which adult animals were used, these data may indicate that certain compounds are toxic to CMI function only if exposure occurs during the development of the immune system.

The highest tissue level of HCB was localized in the liver and did not increase significantly after 13 weeks of exposure. The highest concentration of HCB in the spleen was observed following 6 weeks of exposure and was coincident with the observed decrease in lymphocytotoxicity of spleen cells sensitized against alloantigen and with the time when the relative spleen weight was the highest value recorded. After 6 weeks, the spleen concentration had declined to, and remained at, less than one-half its highest value and suggests an alteration in the biohandling of the compound. Both spleen and thymus concentrated HCB to values above serum levels and it is of interest to note that as the spleen concentration of HCB decreased with prolonged exposure, the concentration of thymic HCB increased. Since the relative thymus weight decreased during the 3-24 week period of exposure, while the concentration of HCB in the thymus increased, it is possible that thymic affinity for HCB increased with continued exposure or that the compound has great avidity for a certain component of thymic tissue and as other components are mobilized during thymic weight loss, the HCB is retained. The concept of thymic receptor sites for chlorinated hydrocarbons, such as TCDD, which seems to be

under genetic control, has been suggested by Poland et al. (*107*) and may be related to the altered CMI responses observed in this study.

The influence of the dietary administration of HCB on cell-mediated immune functions, in contrast to those observed with PCB, could reflect the different patterns of absorption of these chemicals. In rats, Iatropoulos et al. (*67*) demonstrated that 48 hr after oral administration of a single dose of either ^{14}C-labeled dichlorobiphenyl (DCB), a chlorinated biphenyl, or HCB, that DCB is transported to the liver by the venous portal system. In contrast, HCB is primarily absorbed by the lymphatic system. This pattern of absorption of HCB results in the direct exposure of thoracic duct lymphocytes to high concentrations of chemical before any detoxification by the liver or dilution in the blood is possible. Thoracic duct lymphocyte adsorption or absorption of HCB may help to explain the high concentration of HCB found in lymphoid tissue and its toxicity.

In the present study, the transient dysfunction of cell-mediated immunological parameters, associated with thymus weight reduction and spleen weight increase, may reflect a thymus-dependent toxic expression. That is, the thymotropic properties of HCB result in high concentrations of HCB in mature thymus tissue where it exerts thymotoxic effects. There is evidence which suggests that the immature thymus may be more susceptible to permanent damage than the mature organ (*104*). Reduction in thymus weight may be due to a reduction of thymocyte development and since nonprimed T lymphocytes are short-lived cells (*108*), splenic T lymphocytes may not be replaced and, therefore, T lymphocyte-mediated splenocyte function may be impaired.

In the assessment of the mechanism of action by which HCB caused the suppression of effector phase function in the development of a cell-mediated response, the data of the present study suggest that the lesion is not due to an impaired ability to recognize specific cell surface antigens. Also, the lesion is probably not due to impairment of the mechanism of lymphocyte activation since T lymphocyte mitogen responsiveness, which is thought to by-pass initial specific recognition is not impaired. Therefore, the HCB-induced lesion probably exists beyond antigen recognition and activation and exists within the effector phase since lymphocytotoxicity and GVH reactivity, which are both measures of effector cell function, were impaired following exposure to HCB in this study.

In summary, the influence of two environmental polyhalogenated aromatic hydrocarbons, PCB and HCB, on the development of cell-mediated immune responses has been investigated, and it is concluded that HCB is a more potent immunomodulator than PCB, neither PCB nor HCB interact in a detectable and deleterious manner with the mechanisms of the initial antigen recognition or activation phases of a cell-mediated immune response, and cellular immune dysfunction is related to exposure time to the chemical. Furthermore, it is suggested that: (1) the mechanism of action of the cellular immunotoxicity of HCB is within the effector phase of the immune response; (2) PCB has a more profound influence on parameters of antibody-mediated immunity than on cell-mediated immunity; (3) environmental chemicals can have specific mechanisms of toxicity and, therefore, can influence antibody-mediated immunity while it has no detectable effect on cell-mediated immunity; (4) a single assay of immune function may not be appropriate to detect chemical-induced immune dysfunction, and (5) due to the temporal characteristics of the immune response, the functional status of the immune system cannot be evaluated on only one day of the response but must be evaluated during the entire response period to detect alterations in the magnitude or the time of the peak response.

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