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ON

CELLULAR ASPECTS OF IMMUNITY

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is also phagocytosis of inert particles. We know that these inert particles are probably not influenced at all by the presence of complement, so there may be two kinds of process: a fundamental process of phagocytosis similar to this of inert particles where complement is not involved, and an immunological process where complement is certainly implicated.

Robineaux: I tried to demonstrate some morphologically different processes of phagocytosis. I cannot say whether they are fundamentally different, that is to say whether each of them involves a different mechanism at a molecular level or not. I think, as you do, that we could give all these phenomena the name of phagocytosis.

As for the second point, it is generally admitted that complement is not necessary for phagocytosis to occur; however, it enhances the phagocytosis of particles which have previously fixed a specific antibody. The phagocytosis of inert particles does not require complement but it is known that some of the particles adsorb proteins which can thus modify their surface properties so that they are made adherent.

Humphrey: The liver seems to be able to distinguish quite sharply between native and thermally altered proteins. We found evidence, both in livers perfused *in vitro* and in living rats, that there must be more than one mechanism involved in the uptake and subsequent catabolism of native and denatured material (Freeman, T., Gordon, A. H., and Humphrey, J. H. [1958]. *Brit. J. exp. Path.*, 39, 459).

Dr. Robineaux, did you see labelled material in the nucleus of the cells, or was it entirely outside the nucleus?

Robineaux: We never observed fluorescent material in the nuclei. Labelled material has been seen localized in the Golgi zone, in the same place as vacuoles are localized in the process of pinocytosis.

Smith: What information have you about the differentiation cells may make between homologous and heterologous proteins, or between degraded products of homologous and autologous proteins?

Robineaux: It is possible that there is no difference in the uptake of homologous and heterologous proteins and that pinocytosis is simply a physiological function of the cell.

Lederberg: Can you give any quantitative estimate of the rate of pinocytosis ?

Robineaux: Holter has studied this in the amoeba and I am hoping to undertake similar experiments. But so far I am unable to answer you.

Lederberg: Have you any morphological evidence of the converse of pinocytosis, a mechanism for ejecting large volumes of fluid, functionally comparable to a contractile vacuole ?

Robineaux: It seems that the cell takes up water, but there must

be some mechanism of excretion, otherwise it would swell considerably. There is no evidence of this mechanism of excretion, but by means of interference microscopy it is possible to demonstrate a process of concentration in the vacuoles. When a vacuole disappears, it proves that the refraction index of its contents has become the same as that of the entire cytoplasm. For this to occur, water has to go out of the vacuole. We show in our paper some evidence of this phenomenon.

Dixon: Are the studies on homologous and heterologous proteins both done with labelled protein ?

Robineaux: No. I intend to study the penetration into these cells of homologous and heterologous proteins labelled with different substances—for example with sulphofluorescein which gives yellow-green fluorescence, and sulphorhodamine which gives orange fluorescence—in order to find whether there is a competition between the two types of protein or not.

Voisin: This question of penetration of autologous, homologous and heterologous proteins is very important from the theoretical point of view, but since we cannot see the proteins without labelling them—that is without denaturing them to some extent—it seems very likely that there will not be any difference in the morphological studies between autologous, homologous and heterologous proteins. This therefore remains a theoretical consideration of great interest but one which cannot be solved by this method. The best approach to this question would be to study the penetration of unlabelled autologous, homologous and heterologous proteins by the Coons technique, utilizing specific fluorescent antibodies to the phagocytized proteins.

Lederberg: Dr. Robineaux, how could pinocytosis, which involves a mass of fluid, discriminate between autologous and heterologous solutes ? One could safely assume that there is at least one molecule of autologous protein and at least one molecule of heterologous protein in every such droplet.

Robineaux: It is possible, as has been shown in amoeba, that proteins get fixed on the cell surface so that they induce the formation of pinocytosis droplets; so far, this has not been demonstrated with macrophages. In amoeba, glucose can penetrate into the cell by pinocytosis only if proteins are present in the medium. Brandt recently showed that these proteins were first fixed on the surface of amoeba, then they penetrated into the cell by pinocytosis, and finally they were laid out on the vacuole wall. There might be a selective pinocytosis process in as far as there might be a selective adsorption onto the cellular surface of proteins which induce the pinocytosis. This would be an interesting problem to investigate.

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DISCUSSION

Kunkel: Have you tried to separate the antibodies concerned in the different antigen fractions?

Lapresle: Yes, but so far I have not succeeded. I tried by electrophoresis on a column of agar gel but the mobilities were so close that it was not possible to separate them. We have also tried by chromatography on a cellulose column. It might be possible to separate them in this way but there was not enough antibody to be detected, so we shall have to try again.

Fagraeus: We did some transfer experiments, in Prof. Grabar's department, with spleen cells from rabbits injected with antigen from 6 to 48 hours before the transfer to fresh rabbits, and they did not keep the antigenicity for very long. Have you injected these products into animals and then taken the spleen out after a certain time to see if you could find the same products as you found *in vitro* ?

Lapresle: I have not done that.

Lederberg: Do you think that an enzyme which operates at pH 3·5 should have some physiological significance in normal metabolism; or is it involved in the solubilization of proteins in necrotic foci where the pH can drop very low ?

Lapresle: This pH does not conflict with a possible physiological activity, since Rous showed (1925, *loc. cit.*) that when the phagocytic cells are active their intracellular pH is about 3·5 (see text).

Humphrey: In the whole spleen does the degradation of the albumin stop at the stage you described, or is most of it broken down much further ?

Lapresle: I do not know, but when the degradation is pursued further you get products which do not precipitate but which retain the ability to combine with the antibodies.

Medawar: Do these enzymes occur in blood leucocytes, and if so, do they occur in the polymorph or lymphocyte fraction or in both ?

Lapresle: They were demonstrated in leucocytes by Barnes (1940, *loc. cit.*) in a lymphocyte fraction as well as in a polymorph fraction.

Robineaux: Could it be that there is a correlation between the enzymic activity and the fixation of the protein on the mitochondria?

Lapresle: I do not know about that.

Halpern: What is the molecular weight of the sub-products ?

Lapresle: I do not know. I studied this with Slizewicz (1958, *loc. cit.*) and we found a family of products which were not homogeneous and which had a mean sedimentation coefficient less than that of the original molecule.

Lederberg: The apparent heterogeneity of your enzyme may be due to its complex formation with other proteins as quasi-substrates. At pH 6 or 7 your protease should be inactive but perhaps still capable of making such complexes with other components in the extracts.

Harris: Have you tried to use these enzymes at pH 7 ?

Lapresle: Yes, but it does not work.

Miles: Does this enzymic extract attack the rabbit albumin as well ?

Lapresle: I have not tried that yet.

Humphrey: I think you know, Dr. Lapresle, that Dr. E. M. Press is working with Dr. R. R. Porter on spleen enzymes which are perhaps the same as those whose action you have studied. Their pH optimum is 3·5, when tested on haemoglobin as substrate, though of course haemoglobin is broken down much further. The interesting thing is that in beef spleens they find quite a large number of well-defined active peaks by ion-exchange chromatography, all with apparently identical enzymic activity. Each spleen showed a different distribution pattern for these enzymes, and the only two which were indistinguishable came from identical twin cattle. I do not think they would put too much emphasis on this, but it may be significant.

Grabar: I discussed that with Dr. Porter very recently. In general he has used a stepwise increase or modification of the pH and ionic strength and each time he got a new peak. Thus he made the same observation as Dr. Lapresle, but when Drs. Lapresle and Webb had a gradual modification of their buffer there were less pronounced peaks. So it is possible that for each of these substances there is stepwise liberation. It is also possible, as Prof. Lederberg pointed out, that in every extract the enzyme may be more or less in combination with one of the other proteins present in the tissue. So when we undertake fractionation of such an extract we may also have some of the activity bound in some way with other proteins present in the extract; this would explain this heterogeneity. Dr. Lapresle wanted only the degradation of serum albumin, and therefore it was not of primary importance for him to study this particular case.

extracts; in the extracts both γ-globulins and many specific antibodies were found. On the other hand lymphocytes were extracted from the thoracic duct and, although γ-globulin was found in the extracts, it was not possible to demonstrate that the γ-globulin contained specifically anti-ovalbumin antibodies.

Seligmann: Has anyone seen a transfer of soluble material coming from any other cell to a plasmocyte or a precursor of a plasmocyte ?

Thiéry: I have not observed a direct transfer.

Seligmann: In connexion with what Dr. Bernhard said, I may add that five years ago in our work on leucocytic antigens with Prof. Grabar and Jean Bernard, we controlled by immunochemical means the washing of the different types of cells which we took from human blood. In the extracts of normal or leukaemic well-washed lymphocytes isolated from circulating blood we were never able to detect any substance having the immunological specificity of serum γ-globulins. So it seems difficult to think that these circulating lymphocytes, normal or leukaemic, could produce any globulin having the antigenic structure of γ-globulins.

Lederberg: Is anything known of the origin of the ergastoplasmic vesicles and lamellae ? Could they be remnants of membranes which are infolded during pinocytosis—by analogy with the origin of the myelin sheath from the Schwann cell membrane ?

Bernhard: Several authors, among them Palade, admit that in macrophages and perhaps also in other cells this phenomenon of invagination does exist. Membranes coming from outside, which are originally cellular membranes, can afterwards become ergastoplasmic membranes. Personally, I believe that this phenomenon exists, but it would be erroneous to admit that *all* intracytoplasmic membranes are of external origin. What we need in all cases are lipoprotein layers which may secondarily be transformed into a specific support for RNP granules.

Simonsen: I think you rightly stressed the impossibility of deciding, by morphological methods alone, whether there is any such thing as a transition between the plasmocytic and lymphocytic series. Dr. M. Holub reported some findings which I think are very pertinent (1960, *In* Mechanisms of Antibody Formation, Proceedings of Symposium, Prague). He put thoracic duct lymph into a diffusion chamber which was then implanted intraperitoneally into newborn rabbits. He added to these lymphocytes a heterologous antigen and found not only that antibody formation occurred but also that, when the cellular content of the tissue chamber was examined histologically, there was very slight mitotic activity but strong evidence of development of pyroninophilic cells. He concluded that most of these had developed from the lymphocytes. Could these

in the spleen and also in the thymus, which I believe you examined too. I thought I could see in the microscope that the centre of this necrosis very often started as an accumulation of polymorphs, which I suppose to have been derived from the host, and that the reaction around it was perhaps rather the reaction of the *grafted* cells, which is exactly the opposite of your interpretation. Irrespective of which interpretation is right, I think we both agree that what brings about this necrosis, as well as the spleen enlargement, is the graft versus host reaction. My answer to your final remark lies in the experiment I described in my paper, in which grafting from adult F_1 hybrids to newborns of the parental strain failed to produce splenomegaly; I think it should have done so if your interpretation was true.

Burnet: I was concerned rather with the continual passage from one chicken to another. If Isaacson's interpretation was correct, the possibility would be that your immunologically competent cells in the next passage were derived from the recipient rather than from the donor cells which had multiplied in the recipient.

Medawar: Some of Loutit's experiments on radiation chimeras clarify an essentially analogous situation. The cell which is responsible for secondary radiation sickness (which is generally interpreted as a graft versus host reaction) can also be serially passaged, and there is no ambiguity about the provenance of the cell concerned because it is identified by Ford's chromosomal marker. So I do not think we need doubt Dr. Simonsen's *prima facie* belief that he is serially propagating the descendants of the cell which he originally inoculated into the first embryo.

Gorer: By putting an antigenic tag on the different cells one can show fairly easily, by a cytotoxic test, which sort of cell is being propagated, at least in mice; and there again it is a donor cell.

Mitchison: Another unambiguous reaction takes place when cells of adult turkey origin are transplanted into chick embryos. The cells react against their host, and can continue to do so in serial passage. The cells of the graft are certainly responsible for the reaction, since turkey globulin can be detected on the surface of the host erythrocytes.

Lederberg: You indicated that a dose as small as 30,000 lymphocytes could give selective proliferation. Were those lymphocytes from unsensitized adult donors ?

Simonsen: Yes.

Lederberg: If you use sensitized donors or passage material, will smaller numbers of cells then set off the reaction ? And what would the limit be in that case ?

Simonsen: I have not pinned that down in chickens yet, but there is strong evidence on this point from experiments on mice. If F_1

are summarized in Table I. They appear to divide the unresponsive states into two clearly distinct classes: the "tolerant" states, states of *essential non-reactivity*, in which there has been a central failure of the immunological response, namely: (A), (B), and (C); and the states represented by (D) and (E) in which there is some reason to believe that immunological activity has been thwarted rather than suppressed. However, we are not yet in a position to classify (D) and (E) with certainty. The case for separating (D) from (E) seems to rest mainly upon the interpretation of the "non-immune" rate of decay of antigen in protein-overloading paralysis, but if it turned out that some of this antigen was in the form of a circulating antigen-antibody complex the distinction of principle would disappear.

Possible Theories of Immunological Tolerance

In this section an attempt will be made to answer the question: what kinds of theories of immunological tolerance is it now possible to devise? The answer clearly depends upon the choice we make between various alternative possibilities that are at present entirely open. To begin with, however, we shall make two assumptions, (A) and (B).

(A) *The maintenance of the tolerant state depends upon the persistence of antigen: tolerance disappears when, or shortly, after, antigen disappears.* The evidence that justifies this assumption has been given above. For reasons that will be given later, it is important to notice that the "tolerance" it refers to is tolerance conceived as a property of the whole organism and not as a property of a cell or cell-lineage.

(B) *Any one antibody-forming cell (and its derived lineage) responds to only one antigen at any one time.* In other words the antibody response is singular and not plural—an idea that seems to have arisen out of the beautiful work of A. H. Coons (see Coons, 1958) and which is supported, or at all events not contradicted, by the work of Nossal and Lederberg (1958) and

White (1958). (More recent evidence of E. S. Lennox and M. Cohn suggests that this is not the invariable rule, but I shall make the assumption nevertheless, if only to emphasize that its acceptance does not oblige us to believe in the "predestination" theory that forms the subject of the first of the three pairs of alternative possibilities discussed below.)

Fortified by these two assumptions, one of them dubious, we may now turn to consider three sets of alternative possibilities within and between which a firm choice must be made before there can be any question of propounding a theory of tolerance.

Each antibody-forming cell and its derived lineage responds to only one antigen . . .

(C1) . . . *and this is the only antigen to which it could have responded* ("predestination" theory).

(C2) . . . *but it could have responded to a different antigen if that other antigen had engaged it first* ("pre-emption" theory).

Alternative C1 amounts to the declaration that antibody-forming cells are a heterogeneous population, the individual members of which are in some way predetermined, preadapted or predisposed to react upon some one antigen; its conceptual history has been traced back to Ehrlich, though we associate it more concretely with the names of Jerne (1955), Talmage (1957), Burnet (1957, 1959), and Lederberg (1958). It provides an *a priori* explanation of the "one cell: one antibody" relationship (and, conversely, must be false, or in need of drastic modification, if that relationship does not hold good); it leads to a consistent theory of immunological tolerance (see below), and explains the fact that an organism is not swamped by one antigen to the prejudice of its ability to react upon any other. But how do individual antibody-forming cells come to be allotted their particular capacities to react? Burnet (1959) now believes that pre-adapted variants arise as "part of the general process of differentiation during embryonic and post-natal development". But if that is so, why should we not regard exposure to antigen itself as the specific inductive stimulus which commits an (until then) pluripotent cell to a

perhaps as a result of hypersensitivity to antigen at some stage of its maturation. This possibility has been suggested by Burnet (1957) and by Lederberg (1958).

(E2) *The inception of tolerance involves some change in the immature antibody-forming cell other than its death and elimination.* According to hypothesis E1 there is no such thing as a tolerant *cell*: tolerance is a state that can be enjoyed only by a whole organism. It was Woodruff (1959) who first called our attention to the fact that no-one has yet demonstrated the existence of a tolerant cell. Much evidence superficially suggests that tolerant cells do exist, but on closer enquiry it turns out to be impossible, at present, to distinguish between the presence of a tolerant cell and the absence of a non-tolerant cell.

Before discussing the theories of tolerance that might be arrived at by making various choices between the alternatives set out above, it should be pointed out that only a limited number of combinations is in fact possible. If we accept E1 (that the inception of tolerance involves the destruction of a cell) then we must also accept C1 (the predestination hypothesis), for otherwise all the future antibody-forming cells of an organism would be killed when a foetus is exposed to antigen. Again, the choice of the combination E1, C1 and A makes it virtually obligatory to accept D2; E1, C1 and A jointly declare that persistence of antigen is needed to maintain a state of tolerance that has been brought about by the destruction of certain specifically preadapted immature antibody-forming cells; but why should there be any need for continuous exposure to antigen unless to subvert the newly-differentiating stem cells envisaged by alternative D2? This, as I understand it, is the argument of Lederberg (1958). It should also be noted that if D2 and E2 are true—i.e. if antibody-forming cells mature from a quasi-embryonic reservoir throughout life, and if the inception of tolerance does not involve their destruction—then we simply do not know whether persistence of antigen (A) is needed to *maintain* tolerance in cells and cell lineages which have been made tolerant at some earlier stage

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DISCUSSION

Dixon: You asked whether the antigen could be circulating in the form of a complex. This would have to be a very fortuitous situation in which at all times all complexes would have to be in extreme antigen excess, and even then the rate of elimination of such complexes would be somewhat faster than the rate of elimination of the antigen itself; so that I think we can rule out the idea of the non-immune elimination of the antigen being the result of complex formation.

Good: Gitlin (Gitlin, D., Monckeberg, F., and Craig, J. M. [1958]. *A.M.A. J. Dis. Child.*, **96**, 496) has presented convincing morphological evidence which indicates quite strongly that antibody production is *not* going on in the Felton system. So the classical immunological paralysis may not be as different from immunological unresponsiveness to protein antigen and classical immunological tolerance as now seems to be the case.

Lederberg: Prof. Medawar has referred to the apparent tolerance to homografts which is obtained when adult mice given lethal doses of X-radiation are rescued with homologous bone marrow, as J. M. Main and R. T. Prehn (1955. *J. nat. Cancer Inst.*, **15**, 1023) and J. J. Trentin (1958. *Ann. N.Y. Acad. Sci.*, **73**, 799) have shown. However, these authors have also questioned whether they had induced tolerance in the same sense as is supposed for perinatal inoculations, namely the modification of the host lymphoid system, or whether the damaged lymphoid system of the host has merely been supplanted by that of the graft.

This may be an academic point, but what is the explicit evidence that even prenatally inoculated mice have retained the host's antibody-forming tissues ? One might conceive that the graft had again displaced the host cells in these tissues, rather than modified their reactivity.

Medawar: It is very difficult to answer that question, but one fact that is relevant is that if an animal, before it is irradiated, is sensitized against the cells which are subsequently going to be used to recolonize it, then the bone marrow graft is rejected. That is not very good evidence, but it does suggest that one has not completely eliminated all the antibody-forming cells of the host by radiation—something remains, some residue of the previous sensitization. The whole problem raised by Prof. Lederberg has been discussed by D. W. Barnes and J. F. Loutit (1959. *Proc. roy. Soc. B*, **150**, 131).

Monod: It is very difficult to draw a valid parallel between induction of antibody and induction of enzyme; however, I agree that it might be possible to find something akin to enzyme induction in the induction of a system which destroys a particular kind of antigen. The difficulty there will be the same as one always has in comparing the two phenomena—namely that the number of enzymes which a given cell or organism is capable of making is limited, while the number of antigens against which an organism can be made tolerant is, I understand, just as unlimited as the potential number of antigens.

Turning now to the one cell-one antibody hypothesis, I would like to refer to some recent results of Cohn and Lennox which seem completely to eliminate this possibility in the case they have studied. They studied the synthesis of antibodies to three different phages (which are not cross-reacting) in single cells and plasmocytes obtained from animals which had been immunized with two different phages. The outcome is not only that many cells are capable of making two antibodies against T2 and T5, but actually that the number of cells making more than one antibody is much greater than the combined probability. So that rather than having to conclude that there is mutual exclusion between the synthesis of one and another antibody,

antigen-antibody complexes remain longer in the organism than the antigen alone is the presence of haematoxylin bodies in systemic lupus erythematosus. German, from the Rockefeller Institute, put forward this hypothesis as an explanation for the persistence of these bodies in the tissues of patients suffering from this disease. In normal human tissues, such free nuclear material cannot be detected, so that it looks as if the nuclear catabolism is inhibited by the anti-nuclear antibody.

Lederberg: One point of the hypercatabolism formulations makes me uncomfortable; *every* immunologically competent cell must vigilantly maintain this accentuated catabolism of the corresponding antigen.

Burnet: If every cell is made actively tolerant, haven't you still got to postulate that all those cells can still suffer pre-emption by an antigen ?

Medawar: The difficulty is that if you do inject an animal locally with antigen, it appears that only a few cells, for example in the regional lymph nodes, react. I think we may have to introduce an automatic correction when we interpret that phenomenon. We tend to assume that an animal which has not been deliberately injected with a particular antigen is a non-immune animal. Actually, all normal animals are immune; they are immune to a great many antigens which we do not know about, so we must take it that the so-called normal rabbit is a rabbit which is making antibodies against a great many antigens, though not as the result of any intervention of our own.

Lederberg: I might have thought the same, but Coons persuaded me otherwise. What is the quantitative increase in plasma cells that one can observe in a local lymph node after a local inoculation ?

Medawar: I am sure you know the answer to that question far better than I do !

Humphrey: I have made some attempts to test the last hypothesis you discussed, Prof. Medawar, namely that whether a material acts as an antigen or not depends upon the rate and extent of its intracellular catabolism. Even suppose the hypothesis were correct, the difficulty is to decide in what cells to look for a significant difference in rate of catabolism between that in an immunologically unresponsive animal and a normal, or between that of a homologous and a heterologous protein. At first sight the hypothesis receives some encouragement from the results of Coons's fluorescent antibody technique, applied to localizing the antigen in cells after parenteral administration to mice or rabbits of soluble foreign proteins such as human serum albumin. Coons found antigen in quite a wide variety of cells such as parenchymal and reticuloendothelial cells of liver, reticular