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PROPAGATION OF ANTIBODY FORMING CELLS IN DIFFUSION CHAMBERS.(U)
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Millipore diffusion chambers, implanted into the peritoneal cavity of irradiated recipient mice, were used to culture immunologically competent mouse cells undergoing a secondary response to the hapten DNP. This technique permitted *in vitro* manipulations to be combined with physiological *in vivo* maintenance of the cells. Several parameters of the immune response were studied utilizing diffusion chambers, including the maturation of the immune response, genetic control of the magnitude and of the heterogeneity of the immune

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response and the age-related decline of immune potential. Termination of immunologic tolerance and the induction of tolerance in young-adult and old mice were also studied utilizing in vivo techniques.

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U. S. Army Contract DADA 17-73-C-3069

Propagation of Antibody Forming Cells in Diffusion Chambers

Final Report (1 March 1973 - 31 March 1977)

1. Introduction

Analysis of cell interactions in the immune response requires techniques that permit experimental manipulations of cell populations *in vitro* at least at some point during the course of the immune response. Generally, this has been accomplished by performing the experiments entirely *in vitro* (e.g. 1, 2) or by transferring cells to irradiated recipients (e.g., 3, 4). While much information has been acquired by the use of these procedures, each has certain disadvantages. Cells held in culture may be under less than optimal physiological conditions and may also be removed from the effects of regulatory mechanisms that might be present in the animal but absent *in vitro*. In transfer experiments the magnitude of the immune response is often estimated by enumeration of antibody forming cells in the spleen of recipient animals. Since only a small portion (about 10%) of the injected cells "home" to the spleen of the recipient, the response may be assessed on a selected population which may not be representative of the entire population of donor cells. For example, suppressor T-lymphocytes preferentially "home" to the spleen (5).

For several years we have used a procedure in which spleen cells from mice primed with dinitrophenylated keyhole limpet hemocyanin (DNP-KLH) undergo a secondary response in Millipore diffusion chambers (6). The secondary response is then measured by enumerating the DNP-specific plaque forming cells (PFC) recovered from the chambers 7 to 9 days after implantation (7). This procedure appears ideally suited for investigations of cell interactions, for the following reasons: (a) just prior to implantation, cell suspensions can be subjected to *in vitro* manipulations, such as antigenic stimulation, removal of T or B lymphocytes, addition of normal or immune syngeneic cells, etc.; (b) the diffusion chamber represents a closed system, in which the implanted cells may proliferate, differentiate or die, but cannot escape; (c) the cells are propagated in a physiological environment, since they have access to nutrients and other soluble factors of the host; (d) the use of a hapten-carrier conjugate as the immunogen allows flexibility of experimentation, since antibody-producing B cells have hapten specificity, while helper T cells are specific for the carrier (8).

This report deals with investigations of a number of basic immunologic problems that were carried out utilizing the diffusion chamber procedure.

2. Maturation of the Immune Response

It has been known for some time that the affinity of antibodies for their antigenic determinant increases with time after immunization, and that the rate of change is influenced by the size of the initial dose of antigen (9). This phenomenon, that has been termed maturation of the immune response, has been explained as an expression of competition for limited amounts of antigen on the part of antigen-binding cells bearing surface

receptors specific for the antigenic determinant, but having varying affinities for it (9). Early in the immune response there would be sufficent antigen to bind and stimulate cells with low affinity receptors as well as cells with high affinity receptors. The average affinity of the antibodies produced at this stage would be relatively low. Later, as the antigen concentration decreases through catabolism and complexing with newly formed antibody, the available antigen would be captured preferentially by cells with high affinity receptors. Selective stimulation of these cells would lead to production of antibodies with progressively higher average affinity.

We sought to put this hypothesis to a direct test. Swiss mice were immunized by intraperitoneal injection of 100 µg of DNP-KLH adsorbed on bentonite. Two to five weeks later the mice were killed and their spleens were dispersed into single cell suspensions. The spleen cells from individual mice were divided into equal portions and each portion was stimulated *in vitro* with a different dose of fluid DNP-KLH. Each portion was cultured separately in a diffusion chamber implanted into the peritoneal cavity of an irradiated recipient mouse. Seven days later, the cells were recovered from the diffusion chamber and tested for numbers of anti-DNP PFC (6). At the same time, plaque inhibition experiments (7) were carried out by incorporating varying concentrations of DNP-BSA into the agar layer of replicate plates. The avidity of the anti-DNP antibodies released by the PFC is inversely related to the concentration of DNP required for plaque inhibition (7). By plotting the percentage of the total PFC that was inhibited by each increment of DNP concentration, we obtained a frequency distribution profile that reflected the heterogeneity of avidity of PFC as well as their relative frequency in each avidity class.

It was found (10) that stimulation of primed cells with an optimal dose of DNP-KLH (50 ng) gave rise to large numbers of PFC which, in some of the mice, were divided into two distinct subpopulations, one of high and the other of low avidity. The same subpopulations of PFC were present after stimulation of the same cells with a suboptimal dose of DNP-KLH (50 pg), but the number of PFC was about one-tenth that obtained with the optimal dose. This last finding clearly indicates that there was not enough antigen to stimulate all of the memory cells present among the primed cells. These results question the validity of the model of maturation of the immune response (9) which postulates that, in the presence of limiting quantities of antigen, only cells bearing high affinity receptors should be capable of capturing the antigen and of being stimulated by it to produce high avidity antibody.

In the work reported above it was found that approximately one half of the random bred mice whose spleen cells were undergoing a secondary response to DNP in diffusion chambers exhibited a bimodal frequency distribution with respect to the avidity of their PFC. The other half of the mice had a unimodal frequency distribution. We therefore decided to examine a number of inbred mouse strains for both magnitude of the secondary PFC response and avidity of PFC. It was thought that this study would give an indication of the dependence of various parameters of the immune response on genetic factors.

We found (11) that the eight inbred strains and three F_1 hybrid mice could be divided into three groups whose responses were significantly different from each other in terms of magnitude. There was no correlation between magnitude of the immune response and H-2 genotype or gamma G_{2a} allotypes. The marked differences in magnitude of the immune response among different mouse strains suggest genetic control of this characteristic. Moreover, high responsiveness appeared to be inherited as a dominant trait, since all three F_1 hybrids tested responded like their high-responder parent and not like the low- or intermediate-responder parent. The strains and F_1 hybrids examined could also be divided into three groups according to the average avidities of the antibody produced. There was no correlation between average avidity and magnitude of the immune response, H-2 genotypes or gamma G_{2a} allotypes. Similarly, no correlation was found between average avidity of the parent strains and that of the F_1 hybrids. Thus, it appears that the avidity of anti-DNP antibodies is, at least in part, under genetic control, but it was not possible to distinguish clearly between genetic and environmental factors that contribute to the determination of antibody avidity. The antibodies of individual mice of a given strain were highly heterogeneous in terms of frequency distribution of avidities. The intra-strain variation was as marked as the interstrain variation. No conclusion as to the possible genetic control of this parameter could be made from the experiments.

3. Termination of Immunologic Tolerance with Antigen-Antibody Complexes

We have reported previously that immunologic tolerance in mice can be terminated by stimulation of spleen cells with antigen complexed to rabbit origin antibody, but not with antigen alone (12,13). These results imply that the spleen of tolerant animals contains cells capable of responding to the tolerated antigen, provided it is presented in an appropriate immunogenic form. A similar conclusion was reached by Chiller et al. (14) who found that, under certain circumstances, B-cells from mice tolerant to human IgG were capable of responding to that antigen, whereas T-cells were not. Accordingly, it was possible that our success in breaking tolerance with antigen-antibody complexes (12,13) was due to stimulation by the rabbit origin antibody in the complex of T-cells specific for rabbit immunoglobulin in mice which had carrier-tolerant T-cells, but hapten-responsive B-cells. We therefore attempted to terminate immunologic tolerance with antigen complexed with antibody elicited in mice syngeneic with the tolerant host.

Balb/C mice were made tolerant to chicken egg white lysozyme or to pneumococcal capsular polysaccharide type III (S-III). The mice were splenectomized and their spleens made into single cell suspensions. Spleen cells from the tolerant mice were incubated in vitro with the tolerated antigen mixed with varying concentrations of immune serum or normal serum from Balb/C mice, and injected intraperitoneally into the tolerant mice. One and two weeks later the mice were bled and their serum was examined for the presence of antibodies.

The percentage of mice which mounted an immune response was significantly greater among mice whose spleen cells were exposed to antigen-antibody

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complexes than among mice whose spleen cells were exposed to antigen mixed with normal serum. Thus, tolerance was terminated by treatment of spleen cells with antigen complexed with syngeneic antibody, under conditions in which specific interaction between T-cells and the antibody portion of the complex was precluded. In addition, tolerance to S-III was also terminated by antigen-antibody complexes. Since S-III is regarded as a thymus-independent antigen in mice, tolerance limited to T-cells cannot be invoked in this case (15).

Further support for the view that the ability of antigen-antibody complexes to terminate tolerance was not due to interactions of rabbit origin antibody with T-cells specific for the heterologous immunoglobulin was obtained by analysis of the cells responsible for tolerance to lysozyme. Balb/C recipient mice were lethally irradiated (900 r) and injected intravenously with mixtures of bone marrow cells and thymocytes from normal or lysozyme-tolerant syngeneic mice. At the same time, the recipient mice received lysozyme intraperitoneally. One week later the mice were again challenged with lysozyme. The mice were then assayed for anti-lysozyme antibodies. Recipients of both bone marrow and thymus cells obtained from normal donors mounted an anti-lysozyme response, while no response was found when either bone marrow cells or thymocytes or both cells were obtained from tolerant donors. Therefore, tolerance in the donors was not confined to T-cells, but was a property of both T-cells and B-cells (15).

4. Decline of Humoral Immune Response in Aged Mice

It has long been known that the activity of the immune system declines in an age-related fashion, after reaching a peak in young-adult animals (16). We investigated the secondary response of mice to DNP-KLH in terms of numbers of DNP-specific PFC produced in diffusion chambers.

We found (17) that the shapes of the dose response curves obtained with young and old cells were very similar. Fifty nanograms of DNP-KLH provided the greatest antigenic stimulation for both young and old cells, with greater and lesser doses resulting in progressively lower responses. Since selection of memory cells for proliferation and antibody production is thought to depend on the avidity of their receptors for the antigen and on the concentration of the antigene (9), the results suggest that the average avidity and distribution of avidities of receptors were similar in cells of both age groups. The kinetics of the secondary PFC response revealed no difference between young and old cells in the time of initial and peak response. We concluded that there was no age-related difference in the time required for antigen-induced cell differentiation and for cell duplication. However, the peak response of old cells was much smaller than that of young cells.

There was a negative correlation between the number of nucleated cells/spleen and the log of the number of PFC in old mice. This suggested that an excess of cells capable of suppressing the immune response may be present in the spleen of old, immunodeficient mice. This hypothesis was confirmed in experiments in which young and old secondarily stimulated spleen cells were co-cultured in diffusion chambers (18). In all experiments fewer PFC were found in chambers containing mixtures of old and young

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spleen cells than in chambers containing young cells alone. Pretreatment of old cells with anti-Θ serum and complement abolished the ability of the cells to suppress the response of young cells. Thus, the effect was attributed to an excess of suppressor T cells in the spleen of old immunodeficient mice.

We then attempted to analyze the function of suppressor T cells, helper T cells and B cells in old mice (19). Spleen cells from aged mice primed with DNP-KLH were treated with anti-Θ serum and complement in order to remove suppressor T cells. Since this treatment would be expected to deplete spleen cell suspension of helper T cells as well, spleen cells from young mice immunized with the carrier, KLH, were added to the treated old cells. The cells were then boosted with DNP-KLH, cultured in diffusion chambers and assayed for DNP-specific PFC. Control cultures included old cells treated with normal serum and complement to which young KLH-primed cells had been added, and old cells treated with either anti-Θ serum or normal serum and complement which were cultured alone, without the addition of young KLH-primed cells. From the numbers of PFC obtained in the various experimental groups we were able to make inferences as to the function of the various lymphocyte types in aged mice. We concluded that an excessive production of suppressor T cells is the first immunologic lesion of aging. This occurs at a time when both B cell and helper T cell functions were often intact. The increased suppressor activity was followed by a decreased helper activity and finally by loss of B cell function.

We also investigated the susceptibility to tolerance induction in young and old mice (20). In order to distinguish between tolerance induction in T cells and B cells, we used two different tolerogens: deaggregated human gamma globulin (DHGG) for carrier-specific T cell tolerance (21) and dinitrophenylated syngeneic mouse gamma globulin (DNP-MGG) for hapten-specific B cell tolerance (22). The tolerogens were injected over a wide range of concentrations. One week after tolerance induction the mice were challenged with an immunogenic dose of DNP-HGG adsorbed on bentonite. One week later the response of the mice was assessed in terms of DNP-specific PFC.

Two major findings emerged from this work. First, ten times more DNP-MGG was required to induce hapten-specific tolerance in old mice than in young mice. In contrast, there was no difference in the dose of DHGG required to induce carrier-specific tolerance in young and in old mice. The 10-fold greater minimal tolerizing dose of DNP-MGG was attributed to an age-related decrease in average affinity of B cell receptors for the ligand DNP. Although it is not clear by what processes such a decrease in receptor affinity might take place, the phenomenon itself has been reported (23).

The second noteworthy findings concerns the pattern of carrier-specific T cell tolerance induction in young mice. Tolerance occurred in two distinct zones of tolerogen dosage, separated by a zone in which there was no demonstrable tolerance. While high and low zone tolerance have been described at the level of the intact animal (24), this is the first report of the existence of two zones of tolerance as a property of T cells. It was found later (McIntosh and Segre, unpublished) that suppressor T cells are induced in high zone, but not in low zone, tolerance.

5. Conclusions

We have utilized the culture of immunologically competent cells in Millipore diffusion chambers to investigate a number of phenomena of immunological relevance. This procedure, coupled with the enumeration of plaque forming cells, has permitted the study of the mechanisms responsible for the maturation of the immune response, the genetic control of the magnitude and the heterogeneity of the humoral response to a hapten, the analysis of the lymphocyte function in aging, the role of antibody in the regulation of immunologic tolerance. The diffusion chamber technique allows in vitro experimental manipulations of lymphoid cell population while maintaining the advantages of a physiologic in vivo environment for the cells under study.

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