Failure of Limiting Antigen Doses to Selectively Stimulate High-Avidity Memory Cells

Abstract. *Mouse spleen cells secondarily stimulated with a small dose of antigen, which elicited few antibody-forming cells, produced antibodies of avidity no greater than that of antibodies synthesized by cells stimulated with an optimal antigen dose. This result is in conflict with the hypothesis that maturation of the immune response is based on competition for limiting amounts of antigen among cells with receptors of varying avidity.*

The affinity (*1*) of antibodies for their antigenic determinant increases with time after immunization, and the rate of change is influenced by the size of the initial dose of antigen (*2*). This phenomenon, which 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 (*3*).

In agreement with the clonal selection hypothesis of antibody formation (*4*), it is assumed that cells become committed to the production of a specific antibody by an antigen-independent process of somatic mutation and that such committed cells bear on their surface receptor molecules identical to the antibodies that they or their progeny will synthesize following antigenic stimulation. Early in the immune response there would be sufficient 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 low. Later, as the antigen concentration decreases through catabolism and complexing with newly formed humoral antibodies, the available antigen would be captured preferentially by cells with high-affinity receptors. Selective stimulation of these cells would lead to the production of antibodies with progressively higher average affinity. The time-related change in binding properties of antibodies should be especially evident when the dose of antigen administered is not excessive, so that a limiting antigen concentration can play its selective role; when a high dose of antigen is used there should be little increase in antibody affinity because cells with high-affinity receptors would have little selective advantage over cells with low-affinity receptors. There is experimental evidence consistent with this prediction (*2, 5, 5a*).

We have sought to test the hypothesis just outlined by subjecting equal portions of a cell suspension derived from the spleen of a single, antigen-primed mouse to secondary stimulation with widely different doses of antigen, and by measuring the avidity (*1*) of the antibodies produced by the cells. We found that, contrary to the prediction of the hypothesis, the avidity of the antibodies induced by a limiting antigen concentration was no greater than that of the antibodies induced by the optimal antigen concentration.

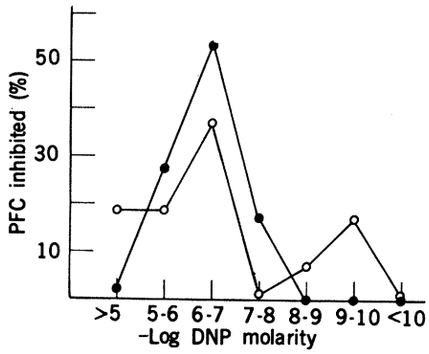

Fig. 1. Frequency distribution of avidities of anti-DNP PFC obtained from spleen cells of two mice secondarily stimulated with an optimal dose of DNP-KLH and propagated in Millipore diffusion chambers. Donor mice were immunized by intraperitoneal injection of 100 μg of DNP-KLH adsorbed on bentonite. Six weeks later, 2×10^7 donor spleen cells were exposed in vitro to 50 ng of fluid DNP-KLH, enclosed in diffusion chambers, and implanted in the peritoneal cavity of irradiated recipient mice. One week later the cells were plated for enumeration of PFC with and without varying concentrations of DNP-BSA as a plaque inhibitor. Hapten concentration was measured spectrophotometrically at 360-nm wavelength, assuming that the molar absorbancy of DNP-lysyl residues is the same as that of ϵ-DNP-lysine (17,530). The percentage of PFC inhibited by each increment of inhibitor concentration was the difference between the percent inhibition found at a given inhibitor concentration minus the percent inhibition at the next lower concentration; PFC not inhibited at the highest DNP concentration used and those still inhibited at the lowest DNP concentration used were scored in the two avidity classes at the extreme left and extreme right of the distribution profile, respectively. The numbers of uninhibited PFC per million cells harvested from the chambers are indicated for each mouse. (Open circles) Host No. 760, 50 ng of antigen dose, 32,260 PFC per million cells. (Closed circles) Host No. 743, 50 ng of antigen dose, 13,333 PFC per million cells.

Female Swiss Webster mice of the NIH strain were immunized by intraperitoneal injection of 100 μg of dinitrophenylated keyhole limpet hemocyanin (DNP-KLH) (*6*) adsorbed on bentonite (*7*). Two to 7 weeks later the mice were killed and their spleens were made into single cell suspensions. Portions of each suspension, each containing approximately 2×10^7 spleen cells, were mixed with 50 μg, 50 ng, or 50 pg of DNP-KLH and placed in Millipore diffusion chambers which were implanted into the peritoneal cavity of irradiated (600 r) NIH recipient mice (*8*). One week later the spleen cells were recovered from the chambers and plated in agar containing sheep erythrocytes coated with dinitrophenylated rabbit anti-sheep erythrocyte antibodies of the immunoglobulin G (IgG) class for enumeration of anti-DNP hemolytic plaques (*8*). All plaques detected in this system are of the indirect type and are presumably formed by antibodies of the IgG class (*8, 9*). Replicate plates were prepared with and without varying concentrations of dinitrophenylated rabbit IgG (DNP-IgG) (*6*) or dinitrophenylated bovine serum albumin (DNP-BSA) (*6*) added to the agar as plaque inhibitors (*9*). Plaque inhibition has been used to measure avidity or relative affinity of antibodies released by single cells, and has been shown to correlate with conventional measurement of serum antibody affinity (*5a, 9, 10*).

It was determined in earlier experiments (*8*) that a dose of 50 ng of DNP-KLH induced the largest number of plaque-forming cells (PFC). This dose was therefore considered to stimulate the greatest number of DNP-specific memory cells, and was included in each experiment as a standard of reference with which we compared the avidity of the PFC elicited by larger (50 μg) or smaller (50 pg) doses of DNP-KLH in the same population of memory cells.

The numbers of PFC obtained with each concentration of inhibitor were compared with the numbers of PFC obtained in the absence of inhibitor. The data were plotted as the percent of total PFC inhibited by each increment of inhibitor concentration. A frequency distribution profile was thus obtained in which PFC were divided in

classes of increasing avidity with decreasing concentrations of inhibitor required for their inhibition. In the examples illustrated in Fig. 1, DNP concentrations of 10^{-10} to $10^{-5}M$, in log increments, were incorporated into replicate plates. This allowed us to divide the PFC into seven classes, namely, those not inhibited by $10^{-5}M$ DNP (which we assumed would be inhibited at higher DNP concentrations), those inhibited by DNP concentrations between 10^{-5} and $10^{-6}M$, and so forth, ending with the PFC still inhibited by $10^{-10}M$ DNP (which we assumed would not be inhibited by lower DNP concentrations). Two patterns of distribution were encountered in cells exposed to an optimal dose of antigen (50 ng of DNP-KLH): a highly heterogeneous distribution, in which two (or more) distinct populations of PFC with different avidities could be discerned (mouse 760, Fig. 1), and a moderately heterogeneous, approximately normal distribution, in which most of the PFC fell into one class (mouse 743, Fig. 1). The pattern of distribution appeared to be a characteristic of the donor mouse, since cells from single donors propagated in replicate chambers yielded similar distribution profiles. One pattern of avidity distribution was found with one half of the donor mice, while the other half yielded the second pattern. There was no difference in magnitude of immune response between highly heterogeneous [mean ± standard error (S.E.) of eight donors: 40,855 ± 8,640 PFC per million cells] and moderately heterogeneous cell populations (mean ± S.E. of eight donors: 29,180 ± 8,024 PFC per million cells). Whether the pattern of avidity of the PFC is genetically determined requires further investigation.

Stimulation of primed spleen cells with 50 μg of DNP-KLH resulted in a much smaller number of PFC (average 2,827 PFC per million cells harvested) than stimulation of the same cells with 50 ng of DNP-KLH (average 41,988 PFC per million cells). Inspection of the data plotted as in Fig. 2 indicated that the avidity of the PFC resulting from stimulation with the larger antigen dose was lower than that of PFC elicited by the optimal dose. When PFC elicited by 50 ng of DNP-KLH exhibited a bimodal distribution of avidities, the subpopulation of PFC of high avidity was absent from the cells stimulated with 50 μg of DNP-KLH (Fig. 2). A rank correlation test (*11*) of the hypothesis that PFC elicited

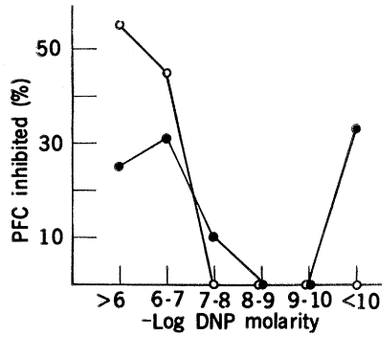

Fig. 2. Avidity distribution of anti-DNP PFC found in two samples of spleen cells from a single donor mouse exposed to 50 μg and 50 ng of DNP-KLH, respectively, 5 weeks after priming, and propagated in separate diffusion chambers. (Open circles) Host No. 697, 50 μg of antigen dose, 1720 PFC per million cells. (Closed circles) Host No. 699, 50 ng of antigen dose, 35,200 PFC per million cells. See legend of Fig. 1 for additional details.

by the two antigen doses belonged to different populations revealed that they did, within 95 percent confidence limits, in five of six cases (coefficients of correlation were − .6, − .3, .1, .3, .375 and 1.0). In the one case where there was correlation, there were no high-avidity PFC elicited by either dose and the distribution pattern was of moderate heterogeneity.

Primed spleen cells stimulated with 50 pg of DNP-KLH yielded fewer PFC (average 5,283 PFC per million cells) than did the same cells stimulated with 50 ng of DNP-KLH (average 45,942 PFC per million cells). Contrary to expectation, the avidity of the PFC elicited by the smaller antigen dose was no greater than the avidity

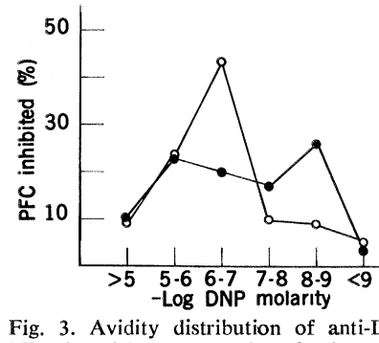

Fig. 3. Avidity distribution of anti-DNP PFC found in two samples of spleen cells from a single donor mouse exposed to 50 ng and 50 pg of DNP-KLH, respectively, 7 weeks after priming, and propagated in separate diffusion chambers. (Closed circles) Host No. 723, 50 ng of antigen dose, 83,809 PFC per million cells. (Open circles) Host No. 725, 50 pg of antigen dose, 4200 PFC per million cells. See legend of Fig. 1 for additional details.

of the PFC elicited by the optimal dose. The rank correlation test indicated identical distributions of avidity between PFC elicited by the optimal and suboptimal doses in six of eight cases (coefficients of correlation were .46, .6, .68, .73, .9, .9, 1.0, and 1.0). The smallest correlation coefficient was found for the paired series illustrated in Fig. 3, where a greater percentage of the PFC was in the low-avidity classes after stimulation with 50 pg of DNP-KLH than after stimulation with the optimal dose of 50 ng. In the other case in which the correlation was not significant, 45 percent of the PFC elicited by 50 pg of DNP-KLH were of comparatively low avidity, requiring between 10^{-6} and $10^{-8}M$ DNP for inhibition.

The finding that PFC induced by a supraoptimal dose of antigen (50 μg) had low avidity can be reconciled with the hypothesis of cell selection by antigen (*3*) by assuming that memory cells with high-avidity receptors were able to capture larger amounts of antigen than cells with low-avidity receptors and were preferentially tolerized. Theis and Siskind (*12*) reported that rabbits which had been rendered partially tolerant to dinitrophenylated horse serum albumin produced small amounts of low-affinity anti-DNP antibodies upon subsequent immunization with the same hapten-protein. They theorized that the low-affinity antibodies were produced by the progeny of cells that had escaped induction of tolerance because their low-avidity receptors had not captured sufficient antigen for immunologic paralysis.

In contrast, our finding that the avidity of the PFC elicited by 50 pg of DNP-KLH was similar to that of the PFC elicited by the optimal dose of 50 ng cannot be reconciled with the hypothesis of cell selection by antigen. Since the number of PFC elicited by 50 pg of DNP-KLH was only one-tenth of that elicited by 50 ng, it is apparent that the lower antigen dose was not sufficient to stimulate all the memory cells present among the spleen cells from primed mice. That 50 pg of DNP-KLH did stimulate a portion of the memory cells is indicated by the finding that spleen cells placed in diffusion chambers 2 weeks after priming with DNP-KLH, but not subjected to secondary stimulation in vitro, contained only 30 PFC per million cells when harvested 7 days later (*8*). Similar results were obtained with primed cells receiving only 0.5 pg of DNP-KLH as

secondary stimulation. Furthermore, cells secondarily stimulated with an optimal antigen dose 2, 5, and 11 weeks after priming contained less than 100 PFC per million cells for the first 3 days of culture in diffusion chambers (*8*). Finally, primary stimulation in vitro followed by culture in diffusion chambers gave rise to only 80 direct PFC per million cells and no indirect PFC, indicating that there was no accidental priming by cross-reacting environmental antigens in our system.

If complexing of antigen with cell surface receptors is the initial obligatory step for the induction of memory cells, and if induced cells and their progeny are restricted to the production of antibodies which are identical to their receptors, a limiting dose of antigen should have induced only cells with high-avidity receptors whose progeny cells should have produced only high-avidity antibodies. Since this was not the case, we conclude that one or both of the above premises are incorrect; complexing of antigen with cell receptors may not be the only critical determinant step in induction of memory cells and/or such cells and their progeny may synthesize antibodies which are at least functionally different from precursor cell receptors.

Others have reported findings which are in conflict with the hypothesis of cell selection by antigen. Harel *et al.* (*13*) found that antigenic competition reduced both the amount and the affinity of anti-DNP antibodies in guinea pigs. They suggested that antibody affinity may increase during the differentiation of a single clone, and that antigenic competition may block the differentiation of the antibody-producing cells at an early stage. A similar suggestion was made by Macario *et al.* (*14*), who found that small fragments of lymph nodes from primed rabbits, believed to contain one or very few clones of memory cells, elaborate antibodies of progressively higher affinity during a 40-day period of cultivation in vitro. Werblin and Siskind (*15*) found that in rabbits showing a progressive increase in average antibody affinity, low-affinity antibodies persisted in approximately constant amounts from 7 days to 1 year after immunization. The persistence of low-affinity antibodies for such a long time is in conflict with the hypothesis of cell selection by antigen, which postulates that maturation of the immune response results from preferential stimulation of high-affinity cells, and lack of stimulation of low-affinity cells, by decreasing amounts of administered antigen.

We have presented here the results of a direct experimental test of the hypothesis of cell selection by antigen. We find that the experimental evidence does not support the hypothesis.

DIEGO SEGRE
MARIANGELA SEGRE
College of Veterinary Medicine, University of Illinois, Urbana 61801

References and Notes

1. Affinity refers to the interaction between monovalent ligand and a single antigen-combining site; avidity, to the interaction between multivalent ligand and multiple combining sites on a single antibody molecule.
2. H. N. Eisen and G. W. Siskind, *Biochemistry* **3**, 996 (1964); L. A. Steiner and H. N. Eisen, *Bacteriol. Rev.* **30**, 383 (1966); G. W. Siskind, P. Dunn, J. G. Walker, *J. Exp. Med.* **127**, 55 (1968).
3. G. W. Siskind and B. Benacerraf, *Advan. Immunol.* **10**, 1 (1969).
4. F. M. Burnet, *The Clonal Selection Theory of Acquired Immunity* (Vanderbilt Univ. Press, Nashville, Tenn., 1959).
5. E. A. Goidl, W. E. Paul, G. W. Siskind, B. Benacerraf, *J. Immunol.* **100**, 371 (1968).
5a. B. Andersson, *J. Exp. Med.* **132**, 77 (1970).
6. The average number of DNP groups per molecule of KLH was 1725; rabbit IgG had an average of 33 DNP groups per molecule; and two preparations of DNP-BSA had 14 and 26 DNP groups per molecule, respectively.
7. M. B. Rittenberg and K. L. Pratt, *Proc. Soc. Exp. Biol. Med.* **132**, 575 (1969).
8. M. Segre and D. Segre, *Immunol. Commun.* **1**, 143 (1972).
9. G. W. Miller and D. Segre, *J. Immunol.* **109**, 74 (1972).
10. M. Segre, D. Segre, F. P. Inman, *ibid.* **102**, 1368 (1969); C. Y. Wu and B. Cinader, *J. Immunol. Methods* **1**, 19 (1971); J. M. Davie, W. E. Paul, I. Green, *J. Immunol.* **109**, 193 (1972); L. Claflin, B. Merchant, J. Inman, *ibid.* **110**, 241 (1973).
11. A. D. Rickmers and H. N. Todd, *Statistics: An Introduction* (McGraw-Hill, New York, 1967), pp. 397–399.
12. G. T. Theis and G. W. Siskind, *J. Immunol.* **100**, 138 (1968).
13. S. Harel, S. Ben-Efraim, P. Liacopoulos, *Immunology* **19**, 319 (1970).
14. A. J. L. Macario, E. Conway de Macario, C. Franceschi, F. Celada, *J. Exp. Med.* **136**, 353 (1972).
15. T. P. Werblin and G. W. Siskind, *Immunochemistry* **9**, 987 (1972).
16. Research supported by PHS grant AI-09743, U.S. Army Medical Research and Development Command contract DADA 17-73-C-3069, and, in part, by funds from the Illinois Agricultural Experimental Station.

24 April 1973

Diisopropylfluorophosphate: Suppression of Ionic Conductance of the Cholinergic Receptor

Abstract. *When frog sartorius muscles were exposed to diisopropylfluorophosphate, the amplitude and half-decay time of the end-plate current decreased; the half-decay time became almost potential-independent and the equilibrium potential for the end-plate current was more negative than during control conditions. When the excess reagent was removed by washing so that only the phosphorylated acetylcholinesterase remained, the amplitude of the end-plate current was restored, while its half-decay time was markedly increased. These findings reveal that this organophosphate significantly affects the receptor-ionic conductance modulator complex in addition to its well-known anticholinesterase activity.*

At the vertebrate motor end plate, when an inhibitor of acetylcholinesterase (AChE) such as neostigmine is present (*1, 2*) or when AChE is removed by proteolysis (*3*), the time course of the end-plate potential (EPP) and, even more significantly, of the end-plate current (EPC) (*4, 5*), is prolonged. It has been suggested (*2, 4*) that the effects of the anti-AChE drugs are due to their slowing of conformational change in the acetylcholine (ACh) receptor, rather than to the synaptic persistence of ACh. To study the course of activation by ACh of the receptor complex we have now measured by a conventional voltage-clamp technique (*6*) the EPC of the frog sartorius muscle (*7*) during and after treatment with an irreversible AChE inhibitor, diisopropylfluorophosphate (DFP). Acetylcholinesterase was then quantitatively reactivated by treatment with pyridine-2-aldoxime methiodide (2-PAM) (*8*) to provide a subsequent internal control. The results indicate that DFP, in addition to inhibiting AChE, interacts with the complex of the ACh receptor and the ionic conductance modulator (ICM) (*9*) in a reversible manner.

Contrary to expectation, the half-decay time of the EPC in the presence of DFP (0.9×10^{-3} to $1.1 \times 10^{-3} M$) decreased to 50 percent. The amplitude of the EPC was decreased to 70 percent of the control (Fig. 1A, and Table 1). In contrast to the control, the falling phase of the EPC showed two distinct exponential components (2 in Fig. 2A), a fast phase followed by a slow residual current. Thus these effects of DFP on the EPC are similar to those of local anesthetics (*10*) and different from those of reversible AChE inhibitors (*4, 5*). At a lower concentration of DFP ($2.5 \times 10^{-4} M$), the half-decay time of the EPC was decreased by 20