

## ANTIGEN MODULATION OF THE IMMUNE RESPONSE

### THE EFFECT OF DELAYED CHALLENGE ON THE AFFINITY OF ANTI-DINITROPHENYLATED BOVINE GAMMA GLOBULIN ANTIBODY PRODUCED IN ADOPTIVE RECIPIENTS

BY T. L. FELDBUSH,\* PH.D., AND J. L. GOWANS, M.B.

*(From the Medical Research Council, Cellular Immunology Unit, Sir  
William Dunn School of Pathology, Oxford University, Oxford, England)*

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The average intrinsic association constant of anti-hapten antibody increases with time after immunization with hapten-protein complexes, and the rate of change is influenced by the size of the initial dose of antigen (1-7). Steiner and Eisen (3) suggested that as the concentration of antigen falls after immunization, new populations of cells are selected which produce antibody of progressively increasing affinity. Selection by antigen is assumed to depend on the presence of receptors upon a lymphocyte which correspond in affinity to the antibody synthesized by that cell after interaction with antigen (6, 8). Thus, only those cells having high affinity receptors would be stimulated by the small amount of antigen remaining some weeks after immunization, while both high and low affinity cells would be stimulated initially.

The cells which generate the secondary response after boosting with antigen (memory cells) are also thought to be a population whose properties are determined by the prevailing concentration of antigen. Thus, Steiner and Eisen (4) and Paul et al. (9) have reported that after secondary challenge with homologous hapten-protein complexes, the amount of antibody produced is usually increased but not the affinity. This indicates that the population of cells producing antibody after boosting is qualitatively similar to that present immediately before boosting. Similarly, Hamaoka et al. (10) have shown by adoptive transfer that donor cells taken at various times after immunization and challenged in a new host produced antibody with the same  $\gamma^1:\gamma^2$  ratio as that seen in the donor.

These observations on the secondary response provide an opportunity to test the notion that changes in the affinity of antibody are due to selection of cells by antigen. Thoracic duct lymphocytes, which are rich in memory cells (11-13), were taken from rats immunized 2 wk earlier with a hapten-protein conjugate and injected into heavily irradiated hosts. The affinity of the antibody produced in the recipients after immediate challenge was compared with that produced in other hosts challenged 6 wk after cell transfer. The theory of Steiner and Eisen (3) predicts that little change of affinity should be observed between the two groups because the transferred memory cells had resided in an antigen-free environment.

\* Present address: Department of Microbiology, College of Medicine, University of Iowa, Iowa City, Iowa 52240.

In both mice and rabbits it has been claimed that the production of anti-hapten antibody in response to immunization with hapten-protein conjugates depends upon the collaboration of lymphocytes from the bone marrow and thymus (14-16). Since it is also claimed that most of the lymphocytes which emerge from the thoracic duct are derived from the thymus, some observations were made on the effect of lymphocyte depletion (by chronic drainage from the thoracic duct) upon both the amount and affinity of secondary antibody produced in rats to a hapten-protein conjugate.

#### *Materials and Methods*

*Reagents.*—Dinitrophenylated bovine gamma globulin (DNP-BGG)<sup>1</sup> and dinitrophenylated human serum albumin (DNP-HSA) were prepared according to the method of Little and Eisen (17) and contained, respectively, 39 and 26 moles DNP/mole of protein. DNP-lysine-<sup>3</sup>H was prepared according to Eisen, Simms, and Potter (18) and kindly supplied by Prof. R. R. Porter.

*Animals.*—All experiments employed either male or female F<sub>1</sub> hybrids of the highly inbred strains AO (Albino) and HO (Hooded).

*Immunization.*—Rats were immunized intraperitoneally with 0.1 ml of a mixture, each 1.0 ml of which contained 10 mg of alum-precipitated DNP-BGG (19) and  $2 \times 10^{10}$  killed pertussis organisms (pertussis vaccine, Wellcome Ltd., Beckenham, England). Primarily immunized rats were challenged intravenously with 1 mg of DNP-BGG in saline.

*Cell Transfer.*—Lymphocytes were obtained from the thoracic duct by the technique of Bollman, Cain, and Grindlay (20) at various times after primary immunization. Thoracic duct lymphocytes (TDL) were collected only during the first 14 hr after cannulation, washed once, and injected intravenously into syngeneic recipients which had received, 24 hr earlier, 850 rads of whole body  $\gamma$ -radiation from a <sup>60</sup>Co source. Recipients were challenged either immediately, or after a delay of 6 wk, with 1 mg of DNP-BGG in saline. Immunized cell donors were challenged intravenously with 1 mg of DNP-BGG in saline after lymph had been drained from the thoracic duct for 5 days in order to deplete the animals of their pool or recirculating lymphocytes (21). Serum for the assay of antibody was accordingly obtained from the tail blood of three groups of challenged rats: normal, actively immunized; lymphocyte depleted, actively immunized; irradiated, adoptively immunized with TDL.

*Antigen-Binding Capacity (ABC).*—Antibody to DNP-lysine was determined by the 50% ammonium sulfate method originally described by Minden and Farr (22) and modified by Brownstone, Mitchison, and Pitt-Rivers (23) and by Stupp, Yoshida, and Paul (24). Whole rat serum was diluted 1:10 in borate saline, pH 8.2 (25), and 1:50 and 1:250 in 10% normal rat serum:borate saline, the latter being added to insure a constant amount of gamma globulin. 0.1 ml of each dilution was added to duplicate tubes and 0.1 ml of  $2.2 \times 10^{-7}$  M DNP-lysine-<sup>3</sup>H added and the tubes left overnight at 4°C. 2 vol of 75% saturated ammonium sulfate was added, the tubes incubated 30 min at 4°C, and then mixed with 1.0 ml 50% saturated ammonium sulfate. The tubes were centrifuged at 2300 rpm for 30 min, the supernatant discarded, and the tubes allowed to drain for 2 hr. Controls consisting of normal serum and radioactive hapten were also processed to determine both total radioactivity and nonspecifically bound DNP-lysine-<sup>3</sup>H. All precipitates were dissolved in 1.0 ml of distilled water and counted in

<sup>1</sup> *Abbreviations used in paper:* ABC, antigen-binding capacity; DNP-BGG, dinitrophenylated bovine gamma globulin; DNP-HSA, dinitrophenylated human serum albumin; TDL, thoracic duct lymphocytes.

10-ml triton-toluene scintillation cocktail (26). Calculation of the ABC was made according to Brownstone et al. (23) and expressed as micrograms of DNP-lysine bound by 1 ml of whole serum. Relative antibody affinity was determined as described by Minden and Farr (22) by comparing the ABC at  $2.2 \times 10^{-7}$  M to the ABC at  $2.2 \times 10^{-8}$  M.

*Quantitative Precipitin.*—Total antibody to DNP was determined as described by Eisen, Carsten, and Belman (27). 0.1 ml of serum was mixed with 0.1 ml of DNP-HSA diluted to appropriate concentrations in borate saline, pH 8.2. After incubation at 4°C for 60 hr and washing, the precipitates were dissolved in 0.1 M NaOH and antibody content calculated from the optical density at 360 and 278  $\mu$ .

*Antibody Affinity.*—The affinity of antibody for DNP-lysine was determined by micro-equilibrium dialysis essentially as described by Eisen, Simms, and Potter (18). 0.075 ml of whole serum, diluted to contain 20–30  $\mu$ g antibody/ml was dialyzed for 40 hr in lucite chambers against five concentrations of DNP-lysine- $^3$ H ranging from  $8.25 \times 10^{-8}$  to  $3.2 \times 10^{-6}$  M. The average intrinsic association constant ( $K_0$ ) was then calculated by the Sips plot and the double reciprocal plot (28).

In preliminary experiments we observed that the antibody produced to DNP-BGG in rats was sensitive to heat and to repeated ammonium sulfate precipitation. Accordingly, frozen whole serum was used for all the determinations. Extensive controls showed that the measurements were not influenced by nonspecific binding of DNP-lysine by components of normal rat serum.

## RESULTS

*Transfer of Secondary Responsiveness to DNP by Lymphocytes from Immune Donors.*—Fig. 1 shows that irradiated rats receiving  $1.6 \times 10^8$  or  $4 \times 10^7$  TDL from syngeneic donors, which had been immunized 1 month earlier with 1.0 mg DNP-BGG, produce significant amounts of antibody to DNP when challenged immediately with DNP-BGG in saline. The peak of the adoptive response was delayed by about 4 days in comparison with that of the normal secondary response. A tenfold reduction in the dose of antigen used for challenge reduced the peak titer of the normal response by about twofold, but had little effect on the adoptive response generated by a transfer of  $1.6 \times 10^8$  TDL; some reduction was observed with the lower cell dose. No antibody could be detected in irradiated rats given immune TDL without challenge or in normal rats given DNP-BGG in saline intravenously. These results are in agreement with those of Gowans and Uhr (11) and Ellis, Gowans, and Howard (13) in showing the presence in rat thoracic duct lymph of cells carrying immunological memory.

Celada et al. (29) found that the affinity of antibody produced in adoptive recipients receiving cells from animals immunized 2–4 months earlier, depended upon the number of cells transferred and/or on the dose of antigen used for challenge. Since the ABC for any serum is the product of both the amount of antibody and its affinity, it is not clear which of these factors is responsible for the effect of antigen and cell dose on ABC shown in Fig. 1. Accordingly, the ratio of ABC with  $2.2 \times 10^{-8}$  M and  $2.2 \times 10^{-7}$  M DNP-lysine- $^3$ H was calculated in order to obtain a relative measure of antibody affinity (22). No

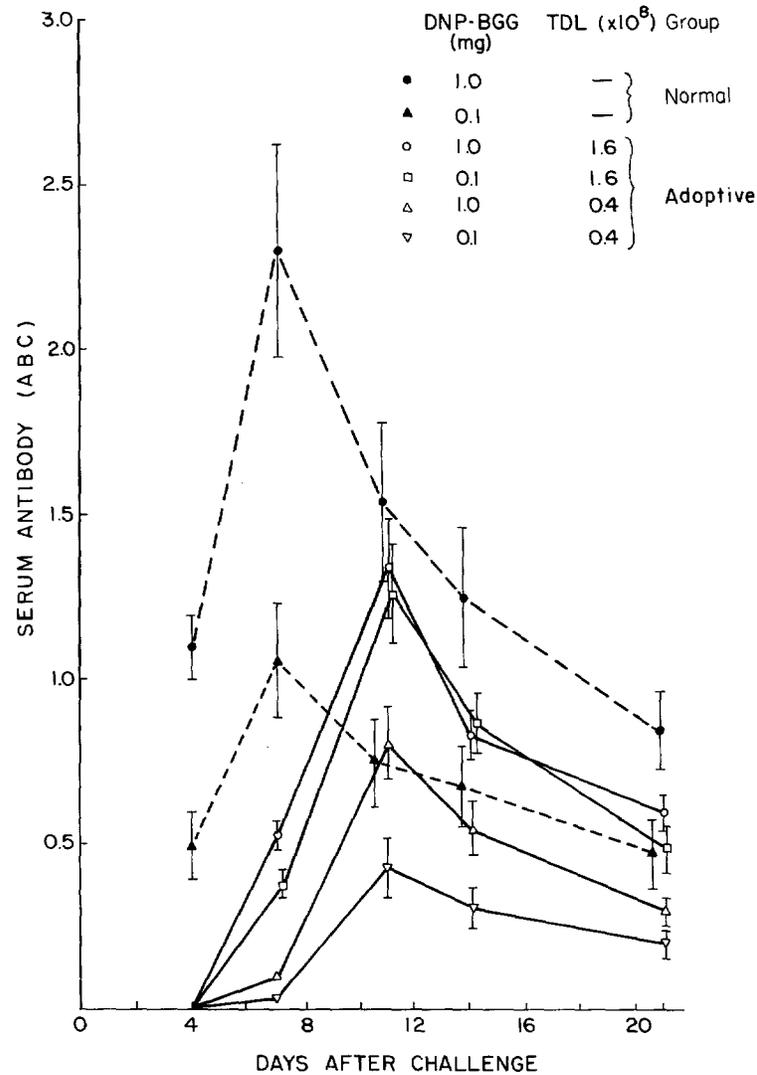


FIG. 1. Anti-DNP responses in normal immunized rats and in irradiated rats given thoracic duct lymphocytes from immunized syngeneic donors; both groups challenged intravenously with either 1.0 or 0.1 mg of DNP-BGG in saline. Antibody measured by antigen binding capacity (ABC) of sera using  $2.2 \times 10^{-7}$  M DNP-lysine- $^3\text{H}$ . Each point is mean value from six rats  $\pm$ SD. Normal immune rats challenged with 1 mg (●---●) or 0.1 mg (▲---▲) DNP-BGG. Irradiated rats given 1 mg of DNP-BGG and either  $1.6 \times 10^8$  (○-○) or  $0.4 \times 10^8$  (□-□) TDL. Irradiated rats given 0.1 mg of DNP-BGG and either  $1.6 \times 10^8$  (△-△) or  $0.4 \times 10^8$  (▽-▽) TDL.

significant differences in the ratios were found between the various groups of animals (Table I) and it can be concluded that, in these experiments, ABC is measuring antibody concentration.

*Secondary Anti-DNP Response in Normal and Lymphocyte-Depleted Rats.—*

*Lymphocyte depletion before secondary challenge:* Secondary responses in rats to sheep erythrocytes and tetanus toxoid (21) and to bacteriophage  $\phi\chi$  174 (11) are not affected by drainage of lymph and lymphocytes from the thoracic duct for 5 days before antigenic challenge. Table II compares the secondary anti-DNP response of normal immunized rats with that of immunized rats

TABLE I  
*Relative Affinity of Secondary Anti-DNP Antibody in Normal Immunized Rats and in Irradiated Rats Given Antigen together with Thoracic Duct Lymphocytes (TDL) from Immunized Donors\**

Secondary response	i.v. dose of TDL ( $\times 10^8$ )	i.v. challenge by DNP-BGG	No. rats	Relative affinity anti-DNP antibody† Days after challenge				
				4	7	11	14	21
		<i>mg</i>						
Adoptive	1.6	1.0	6	0	0.50	0.32	0.37	0.36
	1.6	0.1	6	0	0.49	0.33	0.41	0.44
	0.4	1.0	6	0	0.42	0.46	0.37	0.40
	0.4	0.1	6	0	0.51	0.44	0.47	0.59
Normal	—	1.0	6	0.50	0.52	0.52	0.46	0.42
	—	0.1	6	0.40	0.36	0.41	0.38	0.47

\* See Fig. 1 for values of ABC in this experiment assayed with  $2.2 \times 10^{-7}$  M DNP-lysine- $^3\text{H}$ .

† Average ratio of ABC for six sera assayed at two concentrations of DNP-lysine- $^3\text{H}$ :  $2.2 \times 10^{-8}$  M:  $2.2 \times 10^{-7}$  M.

subjected to drainage from a thoracic duct fistula before secondary challenge. It is clear that lymphocyte depletion lowered the response, especially on day 7 and with 1.0 mg DNP-BGG challenge. At 11 and 14 days after challenge the differences were not so great.

*Affinity of Secondary Antibody in Normal and Lymphocyte-Depleted Rats, and in Adoptively Immunized Recipients.—*Secondary responsiveness to DNP can be transferred by TDL from immunized donors (Fig. 1), yet immunized rats depleted of lymphocytes by chronic drainage from the thoracic duct still mount a secondary response when challenged (Table II). It is not known whether the memory cells which remain in the animal after drainage are simply residual members of the same population as appear in thoracic duct or whether they are a different population of sessile cells established in lymphoid tissue as a result

of primary immunization (11). Information on this point was sought by determining the average intrinsic association constant ( $K_0$ ) of secondary antibody in these two situations by equilibrium dialysis. Table III shows that there was little difference in the affinity of antibody at the peak of the secondary response in the adoptively immunized rats and in the lymphocyte-depleted cell donors. Antibody of similar affinity was also produced by normal immunized rats challenged at the same time.

*Affinity of Anti-DNP Antibody in Rats Given Immune Thoracic Duct Lymphocytes and Challenged either Immediately or 6 Wk after Cell Transfer.*—The final

TABLE II  
*Secondary Anti-DNP Antibody Response in Normal and Lymphocyte-Depleted\* Rats*

Experiment No.	Interval, primary immunization to challenge	Challenge dose of DNP-BGG	After challenge	Mean ABC†		P value
				Normal rats	Lymphocyte-depleted rats*	
	<i>wk</i>	<i>mg</i>	<i>days</i>			
1	8	1.0	7	4.01 (4)	2.97 (3)	≤0.3
		0.1	7	5.08 (4)	0.84 (2)	≤0.1
2	2	1.0	7	2.08 (6)	1.27 (7)	≤0.05
3	4	1.0	7	2.3 (6)	0.75 (3)	≤0.05
		0.1	7	1.23 (7)	0.70 (3)	≤0.01
		1.0	11	1.54 (6)	0.86 (3)	≤0.05
		0.1	11	0.75 (6)	0.64 (3)	≤0.05
		1.0	14	1.25 (6)	0.78 (3)	≤0.5
		0.1	14	0.68 (7)	0.67 (3)	≤0.2

\* Drained of lymph and lymphocytes from a thoracic duct fistula for 5 days before challenge.

† Assayed with  $2.2 \times 10^{-7}$  M DNP-lysine- $^3$ H. Number of rats in parenthesis.

group of experiments aimed to test the idea that the increasing affinity of antibody with time after immunization is due to the selective pressure exerted by a falling concentration of antigen (3). The test consisted of placing memory cells in an antigen-free environment for a period before antigenic challenge; the prediction was that secondary antibody generated at the beginning and end of this period would not differ in affinity.

The design of the experiment is illustrated in Fig. 2. Two groups of irradiated rats (B and C) were given thoracic duct lymphocytes from donors immunized with DNP-BGG 2 wk earlier. One group was challenged with DNP-BGG immediately (B) along with normal immunized controls (A); the other group was challenged 6 wk later (C). Further controls consisted of normal immunized

rats challenged at 8 wk (E), and irradiated rats receiving antigen together with thoracic duct cells from donors immunized 8 wk previously (D).

The results, set out in Table IV, showed that the capacity of the adoptively immunized rats to give a secondary response waned during the interval of 6 wk between cell transfer and challenge; the antigen-binding capacity of sera from group C is lower than that from group B. However, the affinity of the antibody was approximately the same in the two groups. On the other hand, thoracic duct cells which were not collected from the donors until 8 wk after

TABLE III  
*Affinity of Secondary Anti-DNP Antibody in Normal, Lymphocyte-Depleted, and Adoptively Immunized Rats*

Group	Status of rat before secondary challenge§	Rat No.	i.v. TDL* ( $\times 10^6$ )	K <sub>a</sub> (L/M $\times 10^{-7}$ ) Days after challenge	
				7	11
A	Immunized‡	1	—	2.8	—
		2	—	5.3	—
	Normal	3	—	1.2	—
B	Immunized‡	4	—	4.0	—
		5	—	5.8	—
	Lymphocyte depleted	6	—	3.4	—
C	Irradiated Adoptively immunized	7	1.6	—	1.6
		8	1.6	—	4.2
		9	1.6	—	4.9
		10	1.6	—	7.5
		11	1.6	—	2.7

\* Group C irradiated rats given thoracic duct lymphocytes from group B rats. Drainage of lymphocytes from group B continued for 5 days before challenge.

‡ Rats primarily immunized 8 wk before challenge.

§ All rats challenged intravenously with 1 mg of DNP-BGG in saline.

primary immunization were able to generate an adoptive immunity after immediate challenge (D) equal in magnitude to that observed in normal immunized controls (E); and the affinity of the antibodies (D and E) was higher than that from the other three groups.

#### DISCUSSION

Secondary immune responsiveness to bacteriophage  $\phi\chi$  174 (11) and to tetanus toxoid (13) can be transferred to heavily irradiated rats by means of small lymphocytes from the thoracic duct of immunized syngeneic donors. Antibody is not formed after cell transfer if no antigen is given; the lymphocytes are not secreting antibody at the time of transfer but are carrying the property

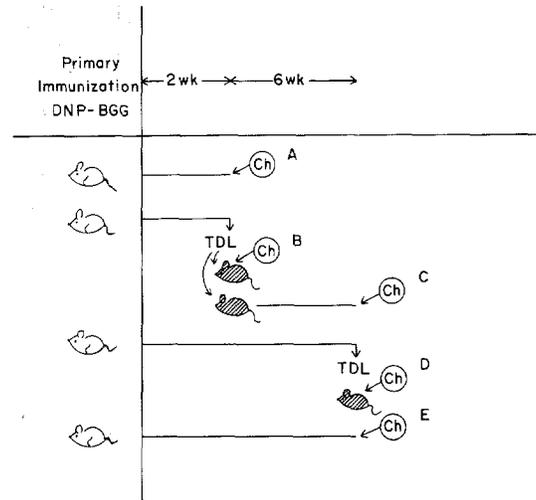


FIG. 2. Design of experiment to compare affinity of secondary anti-DNP antibody in normal immune rats (groups A and E) and in irradiated rats given TDL from primarily immunized donors (groups B, C, D). Irradiated recipients challenged either immediately after cell transfer (groups B and D) or after a delay of 6 wk (group C) to determine influence of antigen-free environment on affinity of adoptive secondary antibody.

Ch: challenge intravenously with 1 mg of DNP-BGG in saline. A-E: experimental groups. Results in Table IV.

TABLE IV  
*Affinity of Secondary Anti-DNP Antibody in Normal and Adoptively Immunized Rats\**

Group‡	No. rats per group	Adoptive immunization		Active immunization	Day of assay after challenge	ABC¶	K <sub>0</sub> (L/M × 10 <sup>7</sup> )	
		Donor	Recipient§				Mean	Range
		Interval, primary immunization to cannulation of thoracic duct	Interval, cell transfer to challenge	Interval, primary immunization to challenge				
		wk	wk	wk	day			
A	6	—	—	2	7	1.98	0.7	0.5–0.9
B	8	2	0**	—	11	1.40	0.8	0.5–0.6
C	8	2	6	—	11	0.60	0.9	0.7–1.0
D	8	8	0**	—	11	2.86	5.6	3.0–8.0
E	6	—	—	8	7	2.31	3.8	2.0–5.0

\* See Fig. 2 for experimental design.

‡ See Fig. 2.

§ Each recipient given  $1.6 \times 10^8$  thoracic duct lymphocytes 24 hr after radiation.

|| All rats challenged with 1 mg of DNP-BGG in saline.

¶ Antigen-binding capacity assayed with  $10^{-7}$  M DNP-lysine-<sup>3</sup>H.

\*\* 0 = cell transfer and challenge, simultaneously.

of immunological memory. Similarly, in the present experiments, thoracic duct lymphocytes from rats immunized with DNP-BGG enabled irradiated recipients to produce anti-DNP antibody when they were challenged with the immunizing conjugate.

Although lymphocytes from the thoracic duct of rats immunized with  $\phi\chi$  174 or tetanus toxoid are very effective at transferring immunological memory, animals depleted of lymphocytes by chronic drainage from a thoracic duct fistula immediately before challenge can still mount secondary responses of normal magnitude. In contrast, the secondary anti-DNP response was always depressed about twofold by lymphocyte depletion in the present experiments. A modest depression of this kind would probably have passed unnoticed in the antibody assays employed in the earlier work. On the basis of experiments in mice and rabbits (14–16), the depression of the anti-DNP response in the present study could be explained by assuming a preferential loss from the thoracic duct of thymus-derived, carrier-specific lymphocytes, the presence of which normally ensures the efficient generation of anti-hapten antibody by marrow-derived lymphocytes. These assumptions have not yet been put to the test in rats.

The affinity of secondary anti-DNP antibody, measured by equilibrium dialysis, was not significantly affected by depleting the animals of lymphocytes immediately before antigenic challenge, and antibody of very similar affinity was produced in irradiated rats after cell transfer and challenge. Two points emerge from these findings. First, the failure of lymphocyte depletion to affect the affinity of secondary antibody suggests that collaboration by thymus-derived lymphocytes in this response may not involve “antigen focusing” (14), assuming that collaboration occurs at all. Second, the affinity measurements suggest that the performance of lymphocytes in the thoracic duct of immunized rats provides an accurate measure of the immunological history of memory cells both in the intact animal and in the animal after lymphocyte depletion. Since all three groups yielded antibody of the same affinity, it is very likely that the cells responsible for initiating the production of secondary antibody in the three situations (presumably marrow-derived lymphocytes) were subjected to the same selective pressures by antigen and responded to these pressures in a similar way. If they are functionally a uniform population it is not clear why some memory cells are difficult to withdraw from the animal by thoracic duct drainage while others are rapidly recirculating.

The main aim of the present experiments was to test the suggestion of Steiner and Eisen (3) that the increase in the affinity of antibodies with time after immunization is, in fact, due to the selection of new populations of cells by the progressively waning concentration of antigen. This assumes that the precursors of cells capable of producing antibody of high affinity will be preferentially stimulated by low concentrations of antigen. The test consisted of transferring lymphocytes from the thoracic duct of immunized rats to irradiated recipients

which were either challenged immediately or after a delay of 6 wk. It was predicted that residence for 6 wk in an antigen-free environment would not result in any change in the affinity of secondary antibody generated by the memory cells when groups of animals were challenged at the beginning and end of this 6 wk period. This prediction was borne out by the results of the experiments shown in Table IV. Lymphocytes taken from rats immunized 2 wk previously with DNP-BGG produced more anti-DNP antibody when challenged immediately in irradiated hosts than when antigenic challenge was delayed for 6 wk. This waning of immunological memory is similar to that observed by Celeda (30) in adoptive transfer experiments in mice. Although residence in an antigen-free environment predictably resulted in no increase in the affinity of secondary antibody, some reservation must be expressed about the extent to which affinity did rise in the control animals and in the irradiated recipients of thoracic duct lymphocytes when antigen was given 8 wk after immunization. In these control groups the affinity had risen four- to fivefold between 2 and 8 wk after immunization, the maximum recorded rise being about tenfold. This rise between 2 and 8 wk after immunization is much less dramatic than that reported by Eisen and Siskind (1) in rabbits, but the overall results were sufficiently consistent to point to the importance of residual antigen in determining the amount and affinity of the antibody which can be generated in immunized animals by subsequent secondary antigenic challenge.

If the residual antigen in immunized animals selects new populations of cells carrying receptors of progressively higher affinity, then it seems most reasonable to suppose that the population of memory cells upon which selection operates is dividing continuously. There is no direct experimental evidence on this point although it should not be difficult to determine whether the small lymphocytes which carry immunological memory in rats turn over rapidly or slowly. In the absence of specific antigen they may not divide and may thus appear from labeling experiments to be "long-lived." Whatever proves to be the case, it is already known that the small lymphocytes in the thoracic duct which mediate immunological memory are recirculating cells because they can be recovered from the thoracic duct of intermediate hosts into which they were injected without antigenic challenge 4 wk previously (31). It is also clear that small lymphocytes carrying memory can generate the plasma cells which secrete the secondary antibody (13).

#### SUMMARY

Secondary immune responsiveness to dinitrophenylated bovine gamma globulin (DNP-BGG) was transferred to heavily irradiated rats by means of small lymphocytes from the thoracic duct of immunized syngeneic donors. Affinity of the antibody produced by the adoptive recipients when challenged immediately was the same as that seen in immunized controls and cell donors,

suggesting that the performance of lymphocytes in the thoracic duct of immunized rats provide an accurate measure of the immunologic history of memory cells both in the intact animal and the lymphocyte-depleted donor. The relevance of this to cell cooperation in the production of antibody to hapten-protein complexes is discussed.

Direct evidence on antigen modulation of the immune response was also obtained. When immune thoracic duct lymphocytes were transferred to adoptive recipients and challenged either immediately or after a delay of 6 wk, it was found that, although the amount of antibody decreased with this delay in challenge, the affinity remained constant. Thus it would seem that in control rats, the increase in the affinity of antibody with time after immunization is, in fact, due to the selection of new populations of cells by the progressively waning concentration of antigen.

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