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Symposium on In Vitro Studies of the Immune Response

I. Variations in the Immune Response to a Simple Determinant[1]

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INTRODUCTION

The complex mixture of antibody molecules formed against diverse protein antigens generally appears increasingly avid the longer the interval between immunization and bleeding. That is, the antibodies produced late in the immune response form particularly stable complexes with the corresponding antigen (1, 11, 12, 15, 16, 21). (For references to older observations of increasing avidity with time, see K. Landsteiner, *The Specificity of Serological Reactions*, Harvard University Press, Cambridge, Mass., rev. ed., 1945, p. 144–145.) In parallel with these changes, it has been found that the anti-dinitrophenyl antibodies isolated from serum at various intervals after immunization have increasing affinity for simple haptens. Thus, the average association constant for the reversible binding of ε-2,4-dinitrophenyl-L-lysine increases about 1,000-fold between 2 and 8 weeks after antibody formation is initiated (9).

Two alternatives were considered in seeking an explanation for the variations in affinity of serum antibodies (9). One possibility is that the nature of the antibodies synthesized and secreted by the lymph node cells changes with time and that this change is directly responsible for the changing properties of the serum antibodies. Alternatively, it seemed possible that the spectrum of binding constants of antibodies synthesized by the lymph nodes is invariant in time, the changes noted in the serum reflecting merely the preferential combination of certain antibody molecules with antigen remaining in the serum and extracellular space, followed by elimination of these specific complexes. Thus, in the early period after immunization, when the amount of antigen is relatively large, antibody molecules of high affinity combine with antigen and are eliminated, leaving behind in the serum molecules of lower average affinity than those secreted. As the concentration of antigen diminishes with time, more of the secreted molecules persist in the circulation and the average affinity of the serum antibody increases, approaching that of the antibody secreted.

Accordingly, the present study was undertaken primarily to determine whether the antibody molecules produced by lymph node cells are of constant average affinity or whether they vary in affinity with time after immunization.

Lymph node cells removed from rabbits immunized with dinitrophenylated proteins continue to synthesize and to secrete antibody when they are suspended in an artificial medium (14). The secreted proteins can be made highly radioactive by providing the cells with radioactively labeled amino acids. If the nodes are taken from hyperimmunized rabbits, over half of the protein secreted may be anti-dinitrophenyl antibody (14). Nevertheless, the amount of antibody obtained from lymph node cell suspensions is too small for standard binding studies. An assay was therefore developed which exploits the radioactive labeling of the secreted antibodies. With this assay it has

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been possible to show that, after injection of dinitrophenylated bovine γ globulin, the antibodies synthesized and secreted by isolated lymph node cells gradually increase in their affinity for the dinitrophenyl determinant. This change accompanies and is apparently responsible for the similar change in serum antibody. The same assay has been used to study the heterogeneity of anti-dinitrophenyl molecules and also their affinity when produced in the secondary response.

Assay for Relative Affinity of Trace Amounts of Labeled Antibody

The assay for relative affinity of labeled antibodies is based on the theory for the binding of univalent ligands by macromolecules. The reversible combination of ligand with binding sites on the antibody molecules proceeds according to the reaction:

$$\text{antibody site} + \text{ligand} \rightleftharpoons \text{antibody site-ligand complex} \quad (1)$$

If there are two independent classes of antibody sites, each with a characteristic binding constant, the fraction of sites of each kind bound by ligand is given by the equations:

$$\frac{\theta}{1-\theta} = K(x - \theta B - \theta' B')$$
$$\frac{\theta'}{1-\theta'} = K'(x - \theta B - \theta' B') \quad (2)$$

where x is the total ligand concentration, B is the concentration of antibody sites of the first class with association constant K, and θ is the fraction of sites of that class occupied by ligand. B', K', and θ' are the analogous quantities for the antibody sites of the second class. If B' is much less than B, these equations simplify to:

$$\frac{\theta}{1-\theta} = K(x - \theta B) \quad (3A)$$

$$\frac{\theta'}{1-\theta'} = K'(x - \theta B) \quad (3B)$$

Typical binding curves based on equations 3A and 3B are shown in Fig. 1.

Suppose that one class of sites is on radioactively labeled antibody molecules and the other on unlabeled molecules. If B', the concentration of labeled sites, is relatively very small, the concentration of unlabeled sites is nearly the same as the concentration of total sites. The binding constants K and K' can then be determined by measuring the fraction of total and the fraction of labeled antibody sites which are occupied by ligand. These measurements could be made if it were possible to separate the bound from the free antibody sites. However, for ligands which are small in size relative to the antibody molecule, such separation has not been accomplished. In contrast, if the ligands are large and multivalent (e.g., dinitrophenylated human serum albumin), their combination with antibody results in the formation of complex aggregates which often precipitate, especially if the complexes are formed in the region of antibody excess. If it is assumed that unprecipitated antibody molecules have neither of their sites occupied by ligand, whereas precipitated molecules have both of their sites occupied, then the process of precipitation results in the desired separation of bound from free sites. By measuring the fraction of labeled and the fraction of total antibody in the precipitate, θ' and θ can be determined.

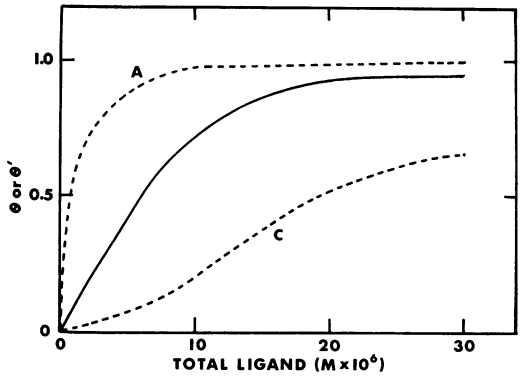

FIG. 1. *Theoretical binding curves based on equations 3A and 3B in text. Solid line, binding of ligand to antibody sites of the first class: $B = 10^{-5}$ M, $K = 10^{6}$ M^{-1}. Dashed line, binding of ligand to antibody sites of the second class: B' is much less than B, $K' = 10^{7}$ M^{-1} for sites A and 10^{5} M^{-1} for sites C. θ is the fraction of sites of the first class occupied by ligand, and θ' is the analogous fraction for sites of the second class.*

The procedure for carrying out the assay is, therefore, a modification of the standard precipitin analysis. Samples of labeled antibody are mixed with a reference antiserum containing unlabeled antibody of the same specificity. Various amounts of antigen are added, and the protein content and radioactivity of the resulting precipitates are determined. For each precipitate, the results are expressed as the fraction of the maximal precipitable protein or radioactivity. This fraction (θ or θ') is plotted against the amount of antigen added to form the precipitate. Figure 2 shows the precipitation patterns when two different labeled antibodies were compared, one at a time, with the same reference antiserum. It is evident that labeled antibody A precipitates more readily than the antibodies in the reference serum. In contrast, labeled antibody C precipitates less readily than the reference antiserum.

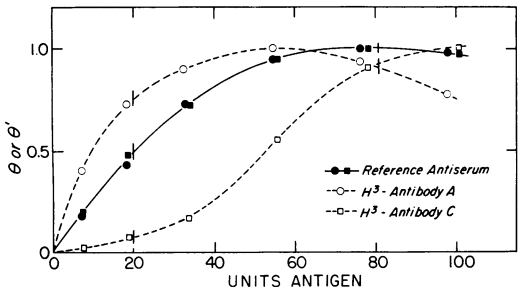

FIG. 2. *Precipitin curves for H^3-labeled antibodies in the presence of reference antiserum. Extracellular medium containing H^3-labeled antidinitrophenyl antibodies was obtained from the lymph node cell suspension of a rabbit (A) 9 weeks and (C) 8 days after immunization with 2 mg of dinitrophenyl-bovine γ globulin. The reference serum was pooled antiserum obtained 5 weeks after immunization with dinitrophenyl-hemocyanin. The precipitating antigen was dinitrophenyl-human serum albumin and is expressed as absorbance units (360 mμ), normalized with respect to the maximal amount of antibody precipitated. The vertical bars indicate the two points used to determine the relative affinity; θ and θ' are the fractions of total and radioactive antibody precipitated.*

In these studies the precipitating antigen selected for the assay is a dinitrophenylated protein, usually human serum albumin, different from the immunizing antigen, usually dinitrophenylated bovine γ globulin. Since the dinitrophenyl-lysyl residue is the only group common to both proteins, the differences in precipitation might be expected to reflect variations in the affinity for this determinant. Nevertheless, the equations for the binding of simple ligands cannot simply be assumed to apply to multivalent ligands or to precipitation. The formation of a precipitate involves both a change in phase (from soluble to insoluble complexes), as well as an undetermined number and variety of nonspecific reactions between antibody and antigen molecules, i.e., intermolecular interactions that do not involve the active sites. Despite these limitations, the precipitin curves in Fig. 2 resemble quite closely in the region of antibody excess the theoretical binding curves in Fig. 1. Evidently, antigen multivalence and the complications of precipitation do not entirely exclude the application of the binding theory to this system.

For univalent ligands it would be possible to determine K', the binding constant for sites on radioactive antibody molecules, directly from equation 3B. However, with multivalent antigens the relevant ligand concentration cannot be determined. It is nonetheless possible to eliminate the ligand term by combining equations 3A and 3B, thus:

$$K' = \frac{\theta'}{1-\theta'} \Big/ \frac{\theta}{1-\theta} K$$

Now K' is expressed in terms of K, the association constant for the reference antibody which can be obtained by an independent method such as equilibrium dialysis (6) or fluorescence quenching (7, 9, 24).

The method of calculation of K' is illustrated by the following example, based on the precipitation curves in Fig. 2. For labeled antibody A: when θ, the fraction of total antibody precipitated, is 0.5, θ', the fraction of radioactive antibody precipitated, is 0.75. Therefore, K' is equal to 3 K. Similarly, for labeled antibody C: when θ is 0.5, θ' is 0.08; K' is equal to 0.087 K. By the method of fluorescence quenching, anti-dinitrophenyl antibody isolated from the reference antiserum was found to have an average association constant, K, of approximately 10^7 M^{-1}. Therefore, the derived association constants for labeled antibodies A and C are 3×10^7 and 8.7×10^5 M^{-1}, respectively.

This procedure for determining binding constants of labeled antibodies can be checked by another method that is more direct and involves no assumptions about the relation of the theoretical binding curves to the precipitin data. For

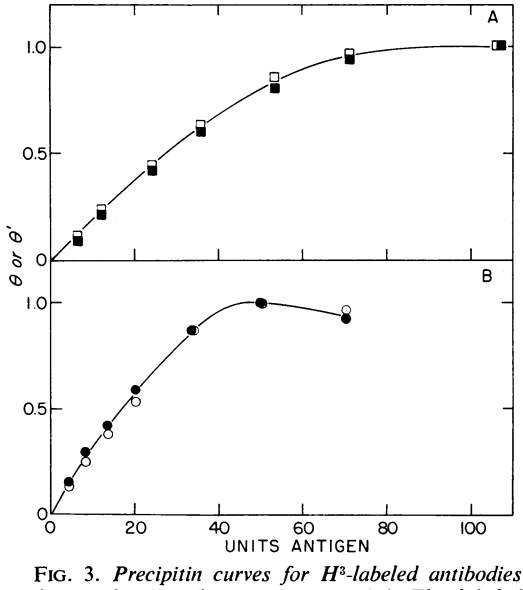

FIG. 3. *Precipitin curves for H^3-labeled antibodies and "matching" reference antisera. (A) The labeled antibody (□) was the same as antibody C in Fig. 2; the reference antiserum (■) was pooled serum obtained 10 days after immunization with dinitrophenyl-bovine γ globulin. (B) The antibody (○) was the same as antibody A in Fig. 2; the reference antiserum (●) was pooled serum obtained 12 weeks after immunization. The antigen units, θ and θ', are the same as in Fig. 2.*

any labeled antibody preparation, it is possible to find an antiserum which, when precipitated together with the labeled antibody, gives a coincident precipitin curve. The average binding constant of the antibodies in this serum is then assumed to be the same as that of the labeled antibody preparation. Thus, in Fig. 3A, labeled antibody C is matched by the chosen antiserum, as indicated by the overlapping precipitin curves; antibodies isolated from this reference antiserum have an affinity for ε-2,4-dinitrophenyl-L-lysine of 9.3×10^5 M^{-1}. Similarly, in Fig. 3B, labeled antibody A is matched by an antiserum whose isolated anti-dinitrophenyl molecules bind ε-2,4-dinitrophenyl-L-lysine with an average association constant of 10^8 M^{-1} or more. These, then, are the approximate association constants of the labeled antibody preparations.

The agreement between this direct procedure and the method of calculation based on equations 3A and 3B is relatively good for labeled antibody C, and not as good for labeled antibody A. However, with respect to the determination of association constants of high-affinity antibodies, there is some uncertainty in the precise value of the binding constant when K lies above 5×10^7 M^{-1}.

If one wishes to determine the relative binding strengths of a number of labeled antibody preparations, it is most convenient to compare them all to the same reference antiserum. For each labeled antibody, the results can be expressed numerically as the ratio of specific activities at two points along the precipitin curves. In Fig. 2, for example, at point 1, 50% of the reference antibody and 75% of labeled antibody A is precipitated. The specific activity of this precipitate in arbitrary units is 1.5. At point 2, 100% of the reference antibody and 90% of this labeled antibody is precipitated. The specific activity of this precipitate is 0.9. The ratio of these specific activities is called the *relative affinity* of the labeled antibody: relative affinity = specific activity at point 1/specific activity at point 2 = 1.5/0.9 = 1.7. Similar calculations indicate that the relative affinity of labeled antibody C with respect to the same antiserum is 0.17. If the precipitin curves of the labeled antibody and reference antiserum coincide (constant specific activity of the precipitates), then the relative affinity of the labeled antibody with respect to that reference serum is 1.0 (Fig. 3). If the precipitin curve for the labeled antibody lies above the curve for the reference serum (Fig. 2, H^3-antibody A), its relative affinity with respect to that serum is greater than 1.0. Conversely, if the precipitin curve for the labeled antibody lies below that of the reference serum (Fig. 2, H^3-antibody C), its relative affinity is less than 1.0. In routine assays, the entire precipitin curves, such as those in Fig. 2, are not obtained. Only the specific activities of two precipitates are determined; these are so chosen that the amount of reference antibody precipitated at point 1 is 40 to 50% of the maximum, and the amount at point 2 is 90 to 100% of the maximum.

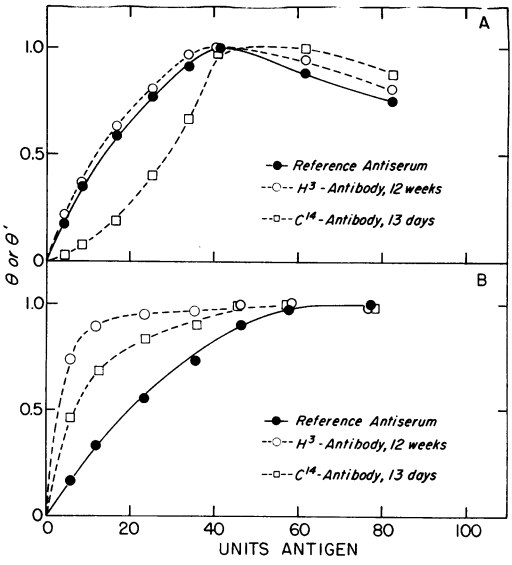

FIG. 4. *Precipitin curves for secreted antibodies in the presence of reference antisera. (A) The reference serum was obtained 12 weeks after immunization with 2 mg of dinitrophenyl-bovine γ globulin, at the same time and from the same rabbit that furnished the cells that formed the H^3-antibody. (B) The reference serum was obtained 13 days after immunization, at the same time and from the same rabbit that furnished the cells that formed the C^{14}-antibody. The antigen units, θ and θ', are the same as in Fig. 2.*

When several labeled antibody preparations are compared to the same reference antiserum, they can be ordered according to their relative affinities. For many purposes, such comparative ordering of labeled antibodies is all that is required. If one wishes to obtain the approximate binding constants for the labeled antibody preparations, one can use either of the two methods described above. Regardless of its precise quantitative interpretation, the assay is a sensitive method for *comparing* the binding strength of labeled antibodies. Therefore, it can be used to answer the question posed in the Introduction: whether, during the interval after immunization, lymph node cells change with respect to the affinities of the antibody molecules they synthesize.

VARIATION IN RELATIVE AFFINITY OF SECRETED ANTIBODY WITH TIME AND IMMUNIZING DOSE

The relative affinity of antibodies produced by lymph node cells at different intervals after immunization was determined by means of the assay described in the preceding section. In the experiment summarized in Fig. 4, secreted antibodies obtained at 13 days and 12 weeks after immunization with dinitrophenylated bovine γ globulin were distinguished by incubating the lymph node cell suspensions with different radioactive isotopes; cells obtained 13 days after immunization were incubated with C^{14}-leucine, and those obtained after 12 weeks were incubated with H^3-leucine. Each 1 ml of cell suspension contained approximately 2×10^7 cells and either 1.5 μc of C^{14}-labeled L-leucine or 30 μc of H^3-labeled DL-leucine. The extracellular media, containing in the one case C^{14}-anti-dinitrophenyl antibody and in the other H^3-anti-dinitrophenyl antibody, were mixed with reference anti-dinitrophenyl antiserum, and various amounts of dinitrophenylated human serum albumin were added to samples of these mixtures. The resulting precipitates were analyzed for C^{14}, H^3, and total protein. The protein and radioactivity in each precipitate were expressed as the fraction of that precipitable at equivalence, and this fraction was plotted against total antigen added.

In Fig. 4A, the reference antiserum was obtained 12 weeks after immunization, at the same time the lymph nodes were removed from one of the rabbits. In Fig. 4B, the reference serum was obtained 13 days after immunization, at the time the lymph nodes were removed from the other rabbit. Both sets of precipitin curves indicate that the efficiency in combining with, and being precipitated by, antigen is greater for the antibody secreted at 12 weeks than for that secreted at 13 days. The relative affinities are expressed numerically by taking the ratios of specific activities at 50% and at 100% precipitation of the reference antiserum. With respect to the 12-week antiserum, the relative affinities are 0.29 and 1.0 for the C^{14} and H^3-labeled antibodies, respectively. This result demonstrates that an increase in the relative affinity of secreted antibodies takes place between 13 days and 12 weeks after immunization.

With respect to the 13-day antiserum, Fig. 4B shows that the relative affinity of the C^{14}-labeled antibody is 1.6. Thus, the relative affinity of the antibodies secreted by lymph node cells 13 days after immunization is higher than that of the antibodies circulating at the same time in the rabbit's serum. This finding is probably related to the rapid change in affinity of the secreted antibodies in the early period after immunization, compared to the slow turnover of serum antibodies. Since the half-life of γG immunoglobulin in the rabbit is approximately 5 or 6 days (4, 5), at any moment the serum contains antibodies that have accumulated for several days and that are of lower relative affinity than those currently secreted. In contrast, many weeks after immunization, the affinity of the antibodies formed by the lymph nodes approaches a plateau, and there is no longer a discrepancy between the affinity of the secreted and serum antibodies. Thus, as shown in Fig. 4A, the relative affinity of the secreted antibody 12 weeks after immunization is 1.0 with respect to reference antiserum obtained from the same animal at the time the lymph nodes were removed.

The changes in relative affinity of secreted antibodies with time were examined in more detail by comparing, one at a time, a number of different H^3-labeled secreted antibodies with the same reference antiserum. In each case, the relative affinity was obtained by taking the ratio of the specific activities of two precipitates, one at approximately 50%, the other at approximately 100% precipitation of antibodies in the reference serum. Table 1 shows the variation in relative affinity when secreted antibodies, obtained at intervals from 6 to 70 days after im-

TABLE 1. *Change in relative affinity of secreted antibodies with time after immunization*

Rabbit no.	Days after immunization*	Relative affinity	
		vs. 5-week antiserum†	vs. 13-day antiserum‡
1	6	0.19	0.92
2	6	—	0.69
3	9	0.20	0.75
4	9	0.09	0.75
5	13	0.53	1.6
6	19	0.59	—
7	19	—	1.8
8	40	1.4	2.0
9	70	1.7	—

* Immunization was with 2 mg of dinitrophenyl-bovine γ globulin in complete Freund's adjuvant.

† Pooled reference antiserum was obtained 5 weeks after immunization with 1 mg of dinitrophenyl-hemocyanin in complete Freund's adjuvant. Antibodies from comparable sera have association constants for ϵ-2,4-dinitrophenyl-L-lysine of approximately 10^7 M^{-1}.

‡ Reference antiserum was obtained from rabbit no. 5 when the lymph nodes were removed 13 days after immunization. Antibodies from comparable sera have association constants for ϵ-2,4-dinitrophenyl-L-lysine of approximately 10^6 M^{-1}.

TABLE 2. *Variation in affinity of secreted antibodies with time and immunizing dose*

Antigen*	Time after immunization	Relative affinity†
mg		
0.5	12 Days	0.4
0.5		0.3
100		0.2
100		0.4
0.5	13 Days	0.6
0.5		0.8
100		0.2
100		0.4
0.5	25 Days	1.4
0.5		1.1
100		0.7
100		0.5
0.5	26 Days	1.2
0.5		1.4
100		0.5
0.5	8 Weeks	1.7
0.5		1.7
100		1.1
100		0.7

* Dinitrophenyl-bovine γ globulin.

† Pooled reference antiserum was obtained 5 weeks after immunization with 2 mg of antigen. The association constant for ϵ-2,4-dinitrophenyl-L-lysine of antibodies isolated from this serum was 9.3×10^6 M^{-1}.

munization, were compared to two different reference antisera. With respect to the 5-week antiserum, the relative affinities of the secreted antibodies range from 0.09 to 1.7; with respect to the 13-day antiserum, the relative affinities of the secreted antibodies range from 0.69 to 2.0. These results show an increase in relative affinity of the antibodies synthesized at increasingly late intervals after immunization.

The amount of antigen used for immunization in the experiments summarized in Table 1 was 2 mg of dinitrophenyl-bovine γ globulin. The effect on relative affinity of varying the amount of antigen administered is shown in Table 2. One group of rabbits was immunized with 0.5 mg of dinitrophenyl-bovine γ globulin, the other with 100 mg. Twelve days after immunization there was no difference in relative affinity of the antibodies secreted by the lymph nodes of the two groups of rabbits. In contrast, by 25 days after immunization there was a clear difference between the two groups. The relative affinities in the group which received the larger dose, although higher than at 12 days, were distinctly lower than those in the group which had received only 0.5 mg of antigen. A similar difference between the two groups was again observed 8 weeks after immunization. Thus, increasing the dose of immunizing antigen delays the change in affinity.

HETEROGENEITY OF SECRETED ANTIBODIES

Heterogeneity with Respect to Affinity

Serum anti-dinitrophenyl antibodies have been shown to be heterogeneous with respect to their affinity for simple dinitrophenyl substances (9). In the preceding section, it was shown that the affinity of antibodies secreted by lymph node cells changes with time after immunization. The possibility was considered, therefore, that the antibody molecules secreted at any moment are actually homogeneous, and that the heterogeneity observed in the serum is the result of the accumulation of antibody molecules formed at different times. Accordingly, an experiment was designed to determine whether the antibody secreted during a relatively short interval is homogeneous or heterogeneous with respect to affinity. The experiment was based on the premise that if the secreted antibody is actually heterogeneous, it should be possible to divide it into several fractions which differ in affinity.

Extracellular medium was obtained from a 5-hr incubation of lymph node cells removed from a rabbit 9 weeks after immunization with 2 mg of dinitrophenyl-bovine γ globulin. The medium was mixed with antiserum obtained from a group of rabbits 11 weeks after immunization with the same antigen. Dinitrophenylated human serum albumin was then added so that approximately 20% of the serum anti-dinitrophenyl antibody, as well as 20% of the H^3-labeled antibody, was precipitated. The precipitate was removed, and additional antigen, again sufficient to precipitate approximately 20% of the remaining antibody, was added to the supernatant fluid. The procedure was repeated until (after the fourth addition of antigen) no precipitate appeared. Additional

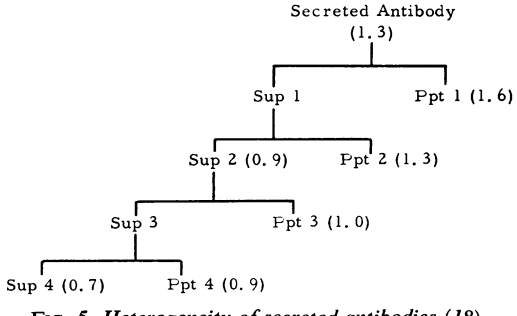

FIG. 5. *Heterogeneity of secreted antibodies (18).*

antiserum was added, and a final precipitate was formed containing some serum antibody, some labeled antibody, and most of the free antigen remaining in the sample.

The general scheme of the fractionation is shown in Fig. 5 (18). Portions of alternate supernatant solutions were saved and assayed for relative affinity with respect to the same pooled anti-dinitrophenyl serum obtained 11 weeks after immunization. H^3-anti-dinitrophenyl antibody in the precipitates was recovered by the following elution procedure. The precipitates were washed repeatedly in the cold, suspended in 12 ml of the antiserum, and incubated for 4 hr at 50 C. The precipitates remaining after incubation were removed and the supernatant solutions containing H^3-labeled antibody, which had been eluted out of the original precipitates, were assayed for affinity. The recovery of labeled antibody from the precipitates was approximately 40 to 50%. In another experiment, the elution was carried out at 37 C, with similar results. In Fig. 5, the numbers in parentheses are the relative affinities of the H^3-labeled antibodies with respect to the 11-week pooled antiserum. It is apparent that each successive precipitation resulted in the removal of antibodies of higher average affinity than those left behind. Therefore, the secreted antibodies in the extracellular medium must be heterogeneous with respect to affinity.

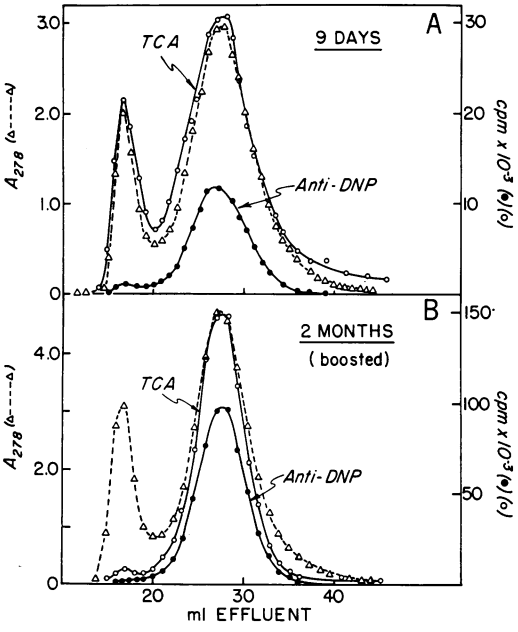

FIG. 6. *Gel filtration of secreted antibodies. Extracellular medium containing H^3-labeled secreted proteins was concentrated by precipitation with 50% saturated ammonium sulfate in the presence of normal serum carrier. Samples of approximately 0.6 ml, containing about 40 absorbance units (278 mμ), were added to a column (1.8 by 23 cm) of Sephadex G-200 in 0.1 M NaCl and 0.1 M potassium phosphate (pH 7.5). The recovery of absorbance units from the column was 100%. The recovery of H^3-labeled trichloroacetic acid-precipitable protein and H^3-anti-dinitrophenyl antibody was 85 to 100%.*

Gel Filtration of Secreted Antibodies

Some of the antibodies produced in the rabbit in the early period after immunization may belong to the γM immunoglobulin class (2, 3, 19, 22, 23). It seemed possible, therefore, that the gradual change in affinity of the antibodies formed by the lymph nodes might be related to a change in immunoglobulin class from γM to γG. To evaluate this possibility, secreted antibodies obtained early and later after immunization were subjected to gel filtration under conditions which separate the 19*S* γM immunoglobulins from the 7*S* γG immunoglobulins.

Extracellular medium containing antibody and other secreted proteins (mainly nonspecific immunoglobulins) was concentrated by precipitation with 50% saturated ammonium sulfate in the presence of normal serum carrier, and was passed through a column of Sephadex G200 (Fig. 6). Analysis of the fractions by analytical ultracentrifugation indicated that the 19*S* or γM immunoglobulins were found in the first peak which emerged at the column front, whereas the 7*S* or γG immunoglobulins were eluted in the second peak. Figure 6A shows the pattern obtained when the lymph nodes were removed 9 days after immunization; Fig. 6B shows the corresponding pattern when the cells were obtained after 2 months. In both experiments, virtually all of the H^3-anti-dinitrophenyl antibody appeared in the second peak eluted. Furthermore, in each case the relative affinity of this antibody was the same as that of the antibody in the unfractionated material applied to the column. These results indicate that the change in affinity with time is not the result of a change in immunoglobulin type from γM to γG.

There is, however, one striking difference between the two elution patterns. In the early sample (Fig. 6A), a considerable fraction of the total trichloroacetic acid-precipitable material emerged at the column front. In contrast, the late sample (Fig. 6B) contained hardly any radioactive material in the first peak. The H^3-labeled protein in the first peak of the early sample was completely precipitated by goat antiserum prepared against purified rabbit γG immunoglobulin, thus indicating that it is an immunoglobulin. Its presence in the early sample, but not

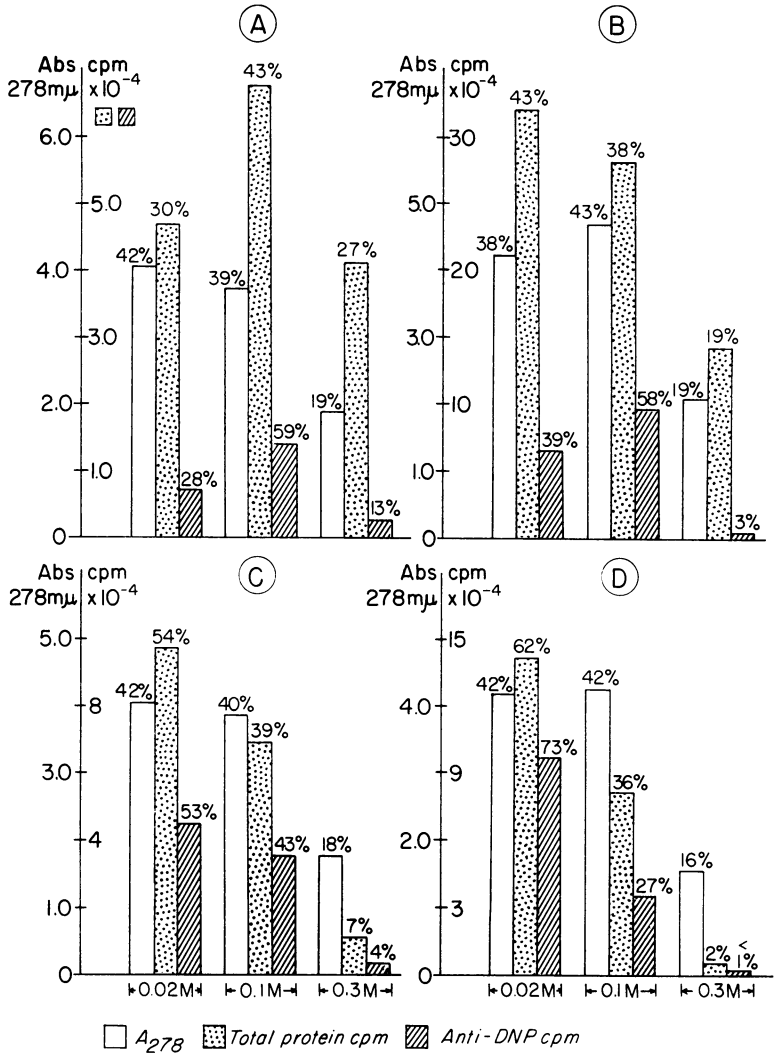

FIG. 7. *DEAE-cellulose chromatography of secreted antibodies. Extracellular medium was obtained from lymph node cell suspensions: (A) 12 days after immunization with 100 mg of dinitrophenyl-bovine γ globulin; (B) 13 days after immunization with 2 mg; (C) 9 weeks after initial immunization with 1 mg; (D) 10 weeks after initial immunization with 1 mg. In each case, the medium containing H^3-labeled secreted proteins was concentrated by precipitation with 50% saturated ammonium sulfate in the presence of normal serum carrier. Each sample containing 12.2 absorbance units (278 mμ) was equilibrated with 0.02 M potassium phosphate buffer (pH 8.0), and was added to a DEAE-cellulose column (0.6 by 20 cm) in the same buffer. After the unretarded fraction was collected, the column was eluted first with 0.1 and then with 0.3 M phosphate (pH 8.0). The percentages indicate material recovered in each step, expressed as the fraction of that recovered in the three steps. In terms of the material added to the column, the total recovery in the three elution steps was: absorbance units, 80 to 90%; H^3-labeled trichloroacetic acid-precipitable protein, 40 to 85%; H^3-anti-dinitrophenyl antibody, 45 to 80%. Almost no additional radioactivity was obtained by subsequent elution with 0.9 M KH_2PO_4. However, washing the column with 0.1 M NaOH–0.85 M NaCl released an additional 20 to 30% protein.*

in the late one, suggests that it may actually be high-molecular-weight antibody produced in response to the immunization. However, it does not coprecipitate with anti-dinitrophenyl serum and dinitrophenyl-human serum albumin, and does not contribute to the relative affinity as measured in the present assay.

Chromatography of Secreted Antibodies

Although the experiments summarized in Fig. 6 indicate that the variation in affinity is not the result of a shift from γM to γG immunoglobulin, it is nevertheless possible that, with time, other changes in immunoglobulin class might occur

and that these might be related to the affinity change. Accordingly, secreted proteins obtained at several intervals after immunization were fractionated by stepwise elution from diethylaminoethyl (DEAE)-cellulose columns under conditions which separate the immunoglobulins into groups primarily, although not entirely, on the basis of charge.

Extracellular medium was mixed with carrier serum, precipitated with 50% saturated ammonium sulfate, and applied to a DEAE-cellulose column equilibrated with 0.02 M phosphate buffer (*p*H 8.0). After collecting the first fraction, the molarity of the eluting buffer was increased to 0.1 M and then to 0.3 M at the same *p*H. The results of four such fractionations are shown in Fig. 7. The heights of the columns indicate the total protein, H^3-protein, and H^3-anti-dinitrophenyl antibody recovered in each step. The recoveries are also expressed as percentages of the total recovered in the three steps. Absolute recoveries are indicated in the legend to the figure.

The amount of total protein, measured by absorbance at 278 mμ, emerging in each of the three fractions was approximately the same in all the experiments, indicating that the conditions of elution were uniform. In the two early samples, A and B, approximately 30 to 40% of the H^3-anti-dinitrophenyl antibody which was eluted in the three steps emerged with the starting buffer, 0.02 M phosphate. Nearly 60% of the antibody was eluted with the second buffer, 0.1 M phosphate, and very little emerged with the 0.3 M buffer. The elution pattern was somewhat shifted in the case of the two later samples, C and D, in that more than half of the antibody emerged with the first buffer and most of the rest with the second. Again, only traces of H^3-anti-dinitrophenyl antibody emerged with the third buffer.

The relative affinities of the labeled antibodies in the extracellular medium, in the unfractionated ammonium sulfate concentrate applied to the column, and in the first two fractions eluted are listed in Table 3. Except for a somewhat lower relative affinity for the 0.1 M fraction in experiment D, the affinities of antibodies in both of the major fractions eluted from the column are the same as those in the corresponding samples before chromatography. Apparently, chromatography on DEAE-cellulose does not separate the secreted antibody into classes that vary significantly in relative affinity.

As with the gel filtration experiments, the effluent obtained from chromatography of the early samples contained certain radioactive proteins that were virtually absent in the effluent from the later samples. Thus, in experiments A and B (Fig. 7), approximately 20 to 25% of the total trichloroacetic acid-precipitable radioactive material emerged in the third step of the elution; in C and D, hardly any appeared in this step. Again, as in the gel filtration experiments, this radioactive protein, which appeared uniquely in the early samples, did not coprecipitate with anti-dinitrophenyl serum and dinitrophenyl antigen. The nature of this material was investigated by immune precipitation with antisera prepared against various immunoglobulin fractions. It was established that, although the radioactive material in the first two fractions was entirely γG immunoglobulin, that in the third fraction was mainly an immunoglobulin which shared some but not all antigenic determinants with γG globulin. It seems likely, although it was not established definitely, that it is γM immunoglobulin. It could not be demonstrated this material had precipitating antibody activity directed against the dinitrophenyl group or against other components of the immunizing mixture, such as the carrier protein, bovine γ-globulin, or the mycobacteria in Freund's adjuvant. However, the possibility remains that these immunoglobulins, as well as the correspond-

TABLE 3. *Relative affinities of secreted antibodies in various fractions from DEAE-cellulose*

Expt	Relative affinity*			
	Extracellular medium	Sample on column	Eluted in 0.02 M buffer	Eluted in 0.1 M buffer
A	0.67	—†	—†	—†
B	1.0	1.0	0.97	1.0
C	1.0	0.96	1.0	0.95
D	1.1	0.93	1.0	0.75

* In experiment A, the reference antiserum was pooled serum obtained 3 weeks after immunization with 2 mg of dinitrophenyl-bovine γ-globulin. The average association constant for ε-2,4-dinitrophenyl-L-lysine of antibodies isolated from this serum was 4.5×10^6 M^{-1}. In experiment B, the reference serum was obtained 3 weeks after immunization with 1 mg of dinitrophenyl-hemocyanin. The binding characteristics of antibodies in this serum were approximately the same as those in the reference serum of experiment A. In experiments C and D, the reference antiserum was obtained 11 weeks after immunization with 2 mg of dinitrophenyl-bovine γ globulin. The anti-dinitrophenyl antibodies isolated from this serum had an average association constant for ε-2,4-dinitrophenyl-L-lysine in excess of 10^8 M^{-1}.

† The relative affinities of the samples added to and eluted from the DEAE-cellulose column in experiment A were not determined because of a technical error. The relative affinity of the extracellular medium is included here for comparison with the other samples.

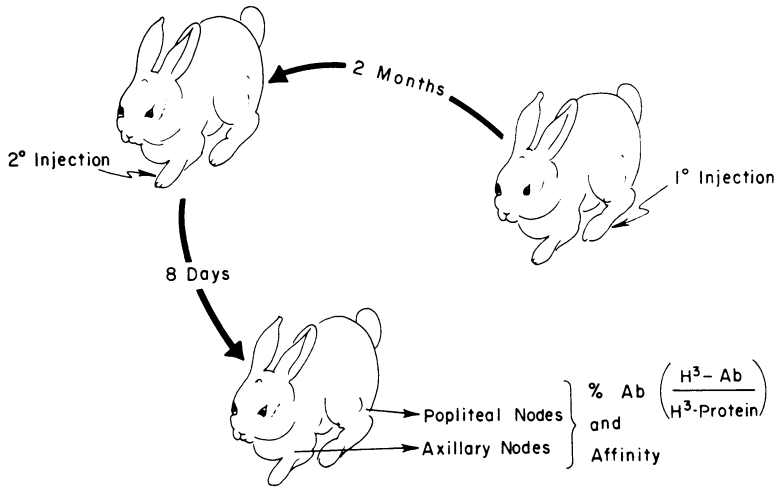

FIG. 8. *Design of experiment to evaluate the immune response to a second injection of antigen (18).*

TABLE 4. *Relative affinity of secreted antibodies in secondary response*

Group	First injection (rear feet)	Second injection (front feet)	Popliteal			Axillary		
			H^3-protein	H^3-anti-dinitrophenyl		H^3-protein	H^3-anti-dinitrophenyl	
			Activity*	Activity*	RA†	Activity	Activity	RA
1	Adjuvant alone	DNP-BγG‡	66,000	500	—	56,000	3,200	0.15
			78,000	800	—	80,000	14,000	0.19
2	DNP-BγG	Adjuvant alone	109,000	15,000	0.92	96,000	600	—
			139,000	15,000	1.1	141,000	1,500	—
			91,000	9,000	1.1	159,000	1,700	—
3	DNP-BγG	DNP-BγG	115,000	16,000	1.2	155,000	65,000	1.0
			61,000	3,000	—	85,000	31,000	1.1
			131,000	12,000	0.90	319,000	138,000	1.1

* Expressed as counts per minute per milliliter.

† The relative affinity (RA) was determined with respect to the same antiserum as in Table 3, experiments C and D. If the antibody activity was low compared to the relevant blanks, the relative affinity could not be determined, and a dash is inserted.

‡ Dinitrophenyl-bovine γ globulin.

ing proteins in the gel filtration experiments, are nonprecipitating γM immunoglobulin anti-dinitrophenyl molecules.

RELATIVE AFFINITY OF SECRETED ANTIBODIES IN THE SECONDARY RESPONSE

In a previous section it was shown that, after single injection of a dinitrophenylated antigen, the relative affinity of the antibody produced by lymph node cells is low at first and in time gradually becomes high. What will be the effect of a second injection of the same antigen given at a time when the animal is making antibody of high affinity? One possibility is that the animal would respond, as it had to the initial injection, with the production at first of antibody of relatively low efficiency in binding antigen. Alternatively, one might expect that the affinity of the antibody produced in response to the second injection would be modified by the animal's immunological experience, as is the case with other manifestations of the secondary response.

Experiments to investigate the effect on antibody affinity of a second injection of antigen were based on the finding, consistent with earlier observations (13, 17), that antibody synthesis is most active in the lymph nodes draining the site of antigen injection. Thus, if antigen is given in

the rear feet, the popliteal nodes are active in antibody production, whereas the axillary nodes are almost completely inactive. If a second injection of antigen is then given in the front feet, virtually all of the antibody now synthesized by the axillary nodes will be produced specifically in response to the second injection. Thus, by varying the injection site, the responses to two injections of the same antigen can be studied separately in the same animal.

The outline of the experimental design is shown in Fig. 8 (18). A primary injection is given in the rear feet. Approximately 2 months later, a secondary injection is given in the front feet. Eight days later, the axillary and popliteal nodes are removed and cell suspensions prepared. After the incubation period, the extracellular medium is assayed for total H^3-labeled trichloroacetic acid-precipitable protein, for total H^3-anti-dinitrophenyl antibody, and also for the relative affinity of the secreted antibody.

The results of one such experiment are summarized in Table 4. The rabbits in group 1 received adjuvant alone in the first injection given in the rear feet. Eight weeks later, 2 mg of dinitrophenyl-bovine γ globulin was given in the front feet. Eight days after the second injection, cells taken from the popliteal nodes produced very little antibody, whereas the axillary nodes were moderately active in antibody production. The relative affinity of the antibody secreted by the axillary nodes was quite low, comparable to that usually found in the early period after immunization. The rabbits in group 2 were given 2 mg of dinitrophenyl-bovine γ globulin in the rear feet, followed after 9 weeks by adjuvant alone in the front feet. Eight days after the second injection, the popliteal nodes were moderately active in the synthesis of antibody of high relative affinity. The axillary nodes, however, were quite inactive.

The rabbits in group 3 received 2 mg of dinitrophenyl-bovine γ globulin in the rear feet, and 9 weeks later the same antigen in the front feet. The response of the popliteal nodes resembled that in group 2. The axillary nodes, however, were extremely active in antibody production. Furthermore, the affinity of this antibody was high. For example, over 40% of the total H^3-protein secreted by the axillary lymph nodes of one rabbit was anti-dinitrophenyl antibody of high affinity. Evidently, the primary injection prepared the animal for a secondary response even in a distant node. This secondary response is characterized not only by a burst of antibody synthesis, but by the formation of antibody that has much higher affinity than that formed in a comparable period after the first injection. Other experiments indicated that the capacity to respond in an accelerated fashion to a second injection of antigen with the production of large amounts of high-affinity antibody may be retained by the rabbit for more than 2 years after the first injection.

TABLE 5. *Relative affinity of antibodies secreted early in secondary response*

Days after second injection*	Popliteal			Axillary		
	H^3-protein	H^3-anti-dinitrophenyl		H^3-protein	H^3-anti-dinitrophenyl	
	Activity†	Activity†	RA‡	Activity	Activity	RA
2	186,000	7,000	1.2	19,600	700	—
	24,000	2,400	1.0	14,000	400	—
3	54,000	5,000	1.0	108,000	18,000	0.80
	65,000	2,800	0.96	38,000	1,200	0.82
4	155,000	17,000	1.0	459,000	261,000	0.93
	55,000	4,700	1.0	56,000	27,000	0.85
6	95,000	7,800	1.2	53,000	33,000	1.0
	163,000	8,500	0.94	515,000	237,000	0.59
8	103,000	12,000	1.1	500,000	340,000	1.1
	160,000	11,000	1.3	122,000	42,000	0.94

* The second injection was 2 mg of dinitrophenyl-bovine γ globulin given in the front feet 8 weeks after initial injection of the same antigen in the rear feet.

† Expressed as counts per minute per milliliter.

‡ The relative affinity (RA) was determined with respect to the same antiserum as in Table 4.

In the experiments summarized in Table 4, the interval between the second injection and removal of the lymph nodes was 8 days. Since it had been shown that in the response to a single injection there is a gradual increase in affinity, it seemed possible that a similar change occurs in the secondary response, but so rapidly that within 8 days the maximal affinity is already attained. Accordingly, the experiments were repeated, except that the interval between the second injection and removal of the lymph nodes was varied from 2 to 8 days. The results of these experiments are summarized in Table 5. Two days after the second injection, there was no change in the response of the axillary lymph nodes; the amount of total protein and specific antibody produced resembled that after a second injection of adjuvant only. (Compare the first two rabbits in Table 5 with the second group in Table 4.) However, already on the third day after the second injection the axillary lymph node cells removed from one of the rabbits synthesized a large amount of antibody of high relative affinity. (It is not usually possible to detect the antibody formed by isolated cells if the nodes are removed 3 days after a primary injection. By 6 or 8 days one can usually detect antibody, but its affinity relative to the antiserum used in this experiment would be in the range of 0.1 to 0.3, as

TABLE 6. *Effect on secondary response of variation in carrier protein*

Group	First injection (rear feet)	Second injection (front feet)	Popliteal			Axillary		
			H^3-protein	H^3-anti-dinitrophenyl		H^3-protein	H^3-anti-dinitrophenyl	
			Activity[b]	Activity[b]	RA[c]	Activity	Activity	RA
1	Adj[d]	DNP-Hcy[e]	46,000	1,000	—	126,000	14,600	0.20
			61,000	600	—	99,000	10,000	0.16
			88,000	1,000	—	145,000	17,000	0.19
			22,000	300	—	67,000	3,400	0.30
2	DNP-BγG[f]	BγG[g]	17,000	600	—	31,000	240	—
			37,000	1,500	—	53,000	400	—
			11,000	4,000	1.2	85,000	1,300	—
			168,000	5,000	1.2	176,000	1,800	—
3	DNP-BγG	DNP-Hcy	29,000	4,100	1.1	207,000	103,000	1.2
			12,000	8,500	0.91	75,000	15,000	0.83
			286,000	41,000	0.70	168,000	32,000	0.78
			58,000	4,300	1.1	197,000	83,000	1.1

[a] The second injection was given 8 weeks after the first injection, and the lymph nodes were removed 8 days later.
[b] Expressed as counts per minute per milliliter.
[c] The relative affinity (RA) was determined with respect to the same antiserum as in Table 4.
[d] Adjuvant, given alone.
[e] Dinitrophenyl-hemocyanin, 1 mg.
[f] Dinitrophenyl-bovine γ globulin, 1 mg.
[g] Bovine γ globulin, 1 mg.

indicated in the first control group in Table 4.) At 4, 6, and 8 days after the second injection, all of the rabbits produced large quantities of high-affinity antibody. Evidently, the time-dependent affinity change characteristic of the primary response is not simply recapitaulated in the secondary response, since the earliest antibody detectable after secondary antigenic stimulation is already high in affinity.

In the experiments discussed so far, the antigen given in the second injection was identical to that given in the first injection. It is known that immunization with bovine γ globulin that has been extensively substituted with dinitrophenyl groups on lysyl residues induces an antibody response directed almost entirely against the dinitrophenyl group, with hardly any antibodies formed against the carrier protein (10). It seemed reasonable to inquire, therefore, whether a secondary response would be obtained if the booster antigen were another dinitrophenylated protein. Experiments were carried out with dinitrophenyl-bovine γ globulin as the first injection, given in the rear feet, and dinitrophenylated *Limulus polyphemus* hemocyanin as the second injection, given in the front feet. The results of these experiments are summarized in Table 6.

Group 1, a control group, demonstrates that an injection of dinitrophenyl-hemocyanin, following an initial injection of Freund's adjuvant alone, results 8 days later in the production of antibody of low affinity. The second control group indicates that the unsubstituted protein carrier bovine γ globulin is not effective in bringing about a secondary response after an initial injection of dinitrophenyl-bovine γ globulin. In contrast, the axillary nodes of the rabbits in group 3 responded to the second injection with the production of large amounts of antibody high in relative affinity. Moreover, the response to dinitrophenyl-hemocyanin resembled that of a second injection of dinitrophenyl-bovine γ globulin. (Compare group 3 in Table 6 with group 3 in Table 4.) These results indicate that dinitrophenyl-hemocyanin is as effective as the homologous antigen in eliciting a secondary response of high-affinity anti-dinitrophenyl antibody.

DISCUSSION AND CONCLUDING REMARKS

The capacity of lymph node cells removed from immunized animals to continue antibody formation in cell suspension has made it possible to investigate variations in the antibody response without many of the complexities inherent in studies with antibodies isolated from immune sera. Thus, the entire spectrum of antibodies synthesized by the cells can be examined, since the possible elimination of certain antibody

molecules by combination with extracellular antigen is largely avoided by the procedure of isolating and washing the cells. Moreover, the antibodies secreted during a short interval can be collected before they mix with the serum antibody pool. Although the amount of antibody produced by a cell suspension is minute, its activity has been studied by means of a sensitive assay which measures the precipitation by antigen of radioactively labeled secreted antibodies in the presence of excess serum antibody of the same specificity.

The principal results of these studies are that the anti-dinitrophenyl antibodies secreted at any moment by lymph node cells are heterogeneous in their binding, and that their average affinity for the dinitrophenyl determinant increases the longer the interval between immunization and removal of the nodes. If very large doses of antigen are administered, the increase in relative affinity takes place more slowly. Thus, the properties of secreted antibodies resemble those previously described for serum anti-dinitrophenyl antibodies (9). It is possible to conclude that the variations in serum antibody result from changes in the antibodies formed by the cells themselves. The nature of these changes in structural terms is not understood at present, but it has been shown here that variations in binding are apparently not related to a change from one major class of immunoglobulins to another.

Since the capacity of antibody to react with antigenic determinants is a function of both its concentration and its affinity, these changes in binding amplify total antibody activity late in the immune response when antibody levels may be declining. Moreover, the copious synthesis in the secondary response of antibody of high affinity maximizes the effect of repeated antigenic stimulation. Although the possible benefits to the organism of the increased efficiency of combination with antigen are obvious, the nature of the mechanism regulating the change in affinity is less apparent. Nevertheless, the inverse relation between the amount of antigen present in the animal and the affinity of the antibody produced suggests a possible critical role for antigen in selecting cells for replication and for antibody production.

It has been proposed, for example, that antibody responses are stimulated by an equilibrium reaction between antigen and pre-existing cellular receptors with affinity for ligand similar to that of the antibody ultimately formed (20). However, more compatible with the properties of anti-dinitrophenyl antibodies is the recent suggestion that antibody synthesis takes place only when the concentration of antigen is within fixed limits, and that for each cell these limits are related to the affinity of the antibody produced (8). Thus, in the early period after immunization when antigen levels are high, only cells producing low-affinity antibody are stimulated. The high-affinity cells are not active because their upper threshold for stimulation by antigen has been exceeded. In the extreme, if antigen is in great excess, even the low-affinity cells become unresponsive, and all antibody production ceases; the result is immunological paralysis. As the level of antigen falls, the cells producing antibody of higher affinity escape from inhibition. If at this time the low-affinity producers continue to be active, the resulting antibody, although higher in average affinity than the earlier antibody, will also be more heterogeneous. Accordingly, one of the predictions of this model is that the early antibody of low average affinity will be less heterogeneous than the antibody produced later, and this has been observed (9).

These studies have been concerned with the antibodies formed against a simple synthetic determinant, the dinitrophenyl group. What is the relevance of these findings to the response to naturally occurring antigens? It has been mentioned already that antisera generally become more avid with time after immunization (1, 11, 12, 15, 21). The avidity or tendency to form stable antigen-antibody aggregates depends on complex and often poorly understood reactions involving antibodies directed against a variety of determinants on the antigen molecule. In addition to the specific reactions, antigen-antibody interaction may be influenced by intermolecular forces unrelated to the binding sites. The avidity of antisera for antigens, therefore, cannot be equated with the affinity of antibodies for a single antigenic determinant. Nevertheless, the availability of multivalent dinitrophenylated antigens makes feasible a comparison of their binding properties with those of univalent ligands, such as dinitrophenyl-lysine. It has been shown, for example, that there is a close correlation between the simple binding theory and the results obtained by the precipitin assay for relative affinity. Although the nature of the functional groups of natural antigens is generally not known, it is reasonable to suppose that their reactions with antibodies resemble those of the dinitrophenylated antigens. Accordingly, the formation and properties of most antigen-antibody complexes is no doubt in large measure determined by the affinity of the various antibody molecules for each of the antigenic determinant groups.

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