SEQUENTIAL CHANGES IN THE RELATIVE AFFINITY OF ANTIBODIES SYNTHESIZED DURING THE IMMUNE RESPONSE*

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The antibodies that are isolated from a rabbit's serum after immunization with dinitrophenylated proteins are heterogeneous with respect to their affinity for dinitrophenyl ligands. In addition, the average affinity increases markedly with time after antigen administration (1). Thus, when anti-dinitrophenyl antibodies specifically purified from serum obtained 8 wk after immunization were compared with those from serum obtained 2 wk after immunization, at least a 100-fold increase in average association constant was observed. The increase in affinity was shown to be related to the quantity of antigen administrated: large amounts delayed the change (1).

However, serum antibodies of a particular specificity are a mixture of molecules synthesized at different times and subject, perhaps, to varying rates of elimination from the circulation. Moreover, the isolation of antibodies from serum may result in some degree of selection of certain antibody molecules at the expense of others. Thus the purified antibody may contain, preferentially, molecules that are more easily precipitated by antigen or that are eluted more efficiently from precipitates. In order to determine the properties of all the antibody molecules formed at any particular time it seemed desirable, accordingly, to obtain antibodies directly from the antibody-forming cells themselves.

Antibody synthesis generally takes place mainly in the lymph nodes draining sites of antigen injection (2–4). Studies were initiated therefore to determine the binding properties of the anti-DNP[1] molecules formed in vitro by suspensions of cells obtained from the axillary and popliteal lymph nodes of rabbits

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[1] Abbreviations used are: DNP, 2,4-dinitrophenyl; BγG, bovine γ-globulin; HSA, human serum albumin; TCA, trichloroacetic acid.

after the injection of dinitrophenylated antigens into the foot pads. This antibody, although produced in amounts insufficient for standard binding studies, can be highly labeled by providing the cells with radioactive amino acids as precursors (5). An assay for measuring the affinity of trace amounts of labeled antibodies was developed and is described in an accompanying paper (6). With this assay it has been possible to show that after immunization with dinitrophenylated bovine γ-globulin, the antibodies synthesized by rabbit lymph node cells gradually increase in their affinity for the dinitrophenyl determinant. This change accompanies, and is apparently responsible for, the similar change in antibodies isolated from the serum. These systematic variations in binding occur among anti-DNP molecules belonging to the γG-immunoglobulin class.

Materials and Methods

Reagents.—Antigens, antisera, and purified antibodies were prepared and characterized by methods referred to in an accompanying paper (6). Immunizations were carried out with antigens in complete Freund's adjuvants, and rabbits were injected in the footpads.

Rabbit γG-immunoglobulin was purified by chromatography on DEAE-cellulose (Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N.Y.) (7). Antiserum against rabbit γG-immunoglobulin was prepared by injecting chickens with rabbit γG-immunoglobulin. Goat antisera prepared against crude globulin fractions of pooled rabbit sera were contributed by E. S. Simms. Antisera specific for rabbit immunoglobulin μ- and α-chains, as well as antiserum specifically reactive with rabbit immunoglobulin light chains, were the generous gift of J. J. Cebra and J. B. Robbins. The preparation of these sera has been described (8, 9). Antiserum specific for the Fc fragment of rabbit γG-immunoglobulin was kindly provided by J. B. Fleischman.

Purity of antibody and γG-immunoglobulin preparations, and the specificity of the various antisera, were verified by immunoelectrophoresis.

^{3}H-L-leucine and ^{3}H-DL-leucine (specific activity about 5 c/mmole) and ^{14}C-L-leucine (specific activity about 250 mc/mmole) were obtained from the New England Nuclear Corp., Boston, Mass.

Preparation and Incubation of Lymph Node Cell Suspensions.—Rabbits were exsanguinated by heart puncture, the axillary and popliteal lymph nodes were removed, and suspensions of the lymph node cells were prepared essentially as described by Helmreich et al. (5). The cells were suspended in 15–20 ml medium in 500 ml siliconized flasks and incubated without shaking in a moist atmosphere of 95% O_2 and 5% CO_2 at 37°C. The cell concentration (determined after centrifugation at 1300 g for 5 min) was approximately 10 μl packed cells per milliliter of medium (about 2 to 3 $\times$ 10^7 cells/ml). The incubation medium, largely based on Eagle's medium (10), contained amino acids, salts, and glucose as described by Helmreich et al. (5). Unlabeled L-leucine was omitted, and the medium was not supplemented with serum protein. Vitamins were added as a 100-fold concentrate (MEM, Eagle Vitamin Concentrate, Baltimore Biological Laboratory, Baltimore, Md.). G-penicillin (K-salt) and phenol red were added in the amounts recommended by Eagle (10). Glass-distilled water was used. At the beginning of the incubation, ^{3}H-L-leucine or ^{3}H-DL-leucine was added to a final concentration of 30 μc/ml. In a few experiments, ^{14}C-L-leucine (1.5 μc/ml) was used instead. 5–8 hr later, the cells were sedimented by centrifugation at 140 g for 15 min, and after the addition of unlabeled L-leucine to a final concentration of 2 mg/ml the supernatant, hereafter called ***extracellular medium***, was stored at -10°C. Before assay, the extracellular medium was thawed and centrifuged at 30,000 g for 1–2 hr to remove cellular debris and other insoluble material.

After an initial lag period of about 30 min (5), the rate of secretion of labeled proteins into

the extracellular medium was uniform for approximately 4 hr, and then gradually decreased. The amount of radioactive protein found in the medium at the end of the incubation period varied greatly according to the conditions of immunization; there were also marked differences in cells from different rabbits. However, in a typical experiment, after 4 hr incubation with ^{3}H-L-leucine, each milliliter of extracellular medium contained the following: total TCA-precipitable protein, 150,000 cpm; rabbit immunoglobulin, 120,000 cpm; anti-2,4-dinitrophenyl antibody, 15,000 cpm.

Determination of Radioactive Proteins.—TCA-precipitable protein was measured essentially as described by Helmreich et al. (5). Extracellular medium containing ^{3}H-labeled proteins was added to 2 mg carrier BγG and the mixture was precipitated with 5% TCA. The precipitate was held for at least 1 hr at 4°C, washed three times with cold 5% TCA, extracted for 15 min at 85–90°C in 5% TCA, extracted for 15 min at 45°C in a 3:1 (v/v) mixture of ethanol and ether, washed with ether, dried briefly, and dissolved by warming for about 30 min at 50°C in 0.5 ml of hydroxide of Hyamine 10X (Rohm and Haas, Philadelphia, Pa.). Finally, 10 ml of toluene containing PPO (2,5-diphenyloxazole, 4 g/liter), and POPOP [1,4-bis-2-(5-phenyloxazolyl)-benzene, 0.1 g/liter] were added, and the sample was counted in a Packard Tri-Carb liquid scintillation counter. Counting efficiency for ^{3}H was 13-18% depending on the type of optical chamber in the counter. The background was 60 cpm in one counter and 30 cpm in another.

^{3}H-anti-DNP-antibody was measured by adding to an aliquot of extracellular medium carrier anti-DNP serum (usually anti-DNP-BγG, sometimes anti-DNP-hemocyanin) and sufficient DNP-HSA to give maximum precipitation of the serum antibodies. After incubation at 37°C for 1 hr and at 4°C for 12–36 hr, the specific precipitates were collected and washed five times with cold buffer containing 0.15 M NaCl, 0.01 M potassium phosphate, pH 7.5, and unlabeled L-leucine, 1 mg/ml. The final precipitates were washed once with 3:1 (v/v) ethanol-ether, dried, and then dissolved and counted as described above. (Occasionally, the washed antigen-antibody precipitates were dissolved in sodium dodecyl sulfate, 5 mg/ml, 2 mg BγG were added as carrier, and the protein was precipitated with 10% TCA, washed with ethanol-ether, dissolved, and counted.) To ensure complete precipitation of labeled anti-DNP antibodies, the carrier antiserum was selected so that the relative affinity for antigen of the ^{3}H-antibodies with respect to the antibodies in the carrier serum was about 1.0 (6).

^{3}H-immunoglobulins were measured by adding carrier rabbit immunoglobulins to an aliquot of extracellular medium, followed by an excess of goat or chicken antiserum prepared against rabbit immunoglobulins. The precipitates were then treated as outlined above for immune precipitates in the DNP system.

Assay for Relative Affinity of ^{3}H-Anti-2,4-Dinitrophenyl Antibodies.—The assay, described in detail in an accompanying paper (6), measures the relative ability of labeled anti-DNP antibodies to combine and precipitate with antigen in the presence of a large amount of unlabeled "reference" antibody of the same specificity. Aliquots of extracellular medium containing ^{3}H-anti-DNP antibody were mixed with 1 ml samples of reference anti-DNP antiserum. Sufficient antigen (usually DNP-HSA) was then added to duplicate samples to precipitate approximately 50% of the serum antibody; enough antigen was also added to another pair of samples to precipitate approximately 100% of the serum antibody. A third pair of samples (blanks) received no antigen. After incubation at 37°C for 1 hr, and at 4°C for 12–36 hr, the precipitates were collected, washed, and analyzed for protein content and for radioactivity. The relative affinity of the labeled antibodies in the extracellular medium is defined as the ratio of the specific activity of the smaller precipitate to the specific activity of the larger precipitate:

$$\text{Relative affinity} = \frac{\text{Specific activity at 50\% precipitation of reference antiserum}}{\text{Specific activity at 100\% precipitation of reference antiserum}}$$

If the capacity of the ^{3}H-antibody to precipitate with antigen is the same as that of the reference antiserum, the two precipitates have the same specific activity and the relative affinity of the secreted antibody is 1.0. If the ^{3}H-antibody precipitates more readily than the reference antibody, its relative affinity is greater than 1.0; if it precipitates less readily, its relative affinity is less than 1.0. Although the relative affinity values measure strength of binding, they cannot be converted directly into association constants. However, it is possible to estimate the average binding constant of a preparation of labeled antibody by procedures described in an accompanying paper (6).

Gel Filtration.—A column (1.8 × 22 cm) containing approximately 1.2 g of Sephadex G-200 (Pharmacia Fine Chemicals, New Market, N. J.) was equilibrated with 0.1 M sodium chloride, 0.1 M potassium phosphate buffer, pH 7.5. The samples, consisting of a mixture of normal rabbit serum and extracellular medium (containing ^{3}H-proteins) were precipitated and washed with cold 50% saturated ammonium sulfate. (Between 62 and 98% of the TCA-precipitable ^{3}H-proteins and at least 97% of the ^{3}H-anti-DNP antibodies were precipitated by ammonium sulfate under these conditions.) The precipitates were then dissolved and dialyzed against 0.1 M NaCl, 0.1 M potassium phosphate, pH 7.5, before being applied on the column. Fractions of approximately 1.0 ml were collected and analyzed for absorbance at 278 mμ, TCA-precipitable ^{3}H-protein, ^{3}H-immunoglobulins, ^{3}H-anti-DNP antibody, and relative affinity.

DEAE-Cellulose Chromatography.—Columns of DEAE-cellulose (0.6 × 22 cm) were equilibrated with the starting buffer, 0.02 M potassium phosphate, pH 8.0. The samples, consisting of a mixture of normal rabbit serum and extracellular medium, were precipitated and washed with cold 50% saturated ammonium sulfate, dissolved, and then dialyzed against the starting buffer. Normal serum proteins, isolated in the same way, were added to bring the total absorbance (278 mμ) of the sample added to the column to 12.1–12.2. Before each change of buffer, the column was washed until the absorbance at 278 mμ and the amount of ^{3}H-protein that emerged was negligible. The effluent fractions were analyzed for absorbance at 278 mμ, TCA-precipitable ^{3}H-protein, ^{3}H-immunoglobulins, ^{3}H-anti-DNP antibody, and relative affinity.

RESULTS

Variation in Relative Affinity with Time.—The relative affinities of labeled antibodies produced by lymph node cells taken 13 days and 12 wk after the immunogen was injected were determined by comparing them to the same reference antiserum. In the experiment summarized in Fig. 1, the cells taken at 13 days from one rabbit were incubated with ^{14}C-L-leucine; those removed at 12 wk from another rabbit were incubated with ^{3}H-D,L-leucine. The two extracellular media were mixed with a reference antiserum, various amounts of DNP-HSA were added, and the resulting precipitates were analyzed for protein, ^{14}C, and ^{3}H. For each precipitate, the fraction of the total specifically precipitable protein or radioactivity (θ or θ': see reference 6) was determined and plotted against antigen added. It is evident in each panel of Fig. 1 that the ^{3}H-antibody obtained 12 wk after immunization precipitated much more readily with the antigen than did the ^{14}C-antibody obtained at 13 days. This result demonstrates an increase during the period after immunization in the ability of secreted antibodies to bind and precipitate with antigen.

With respect to the 12 wk serum, the relative affinities were 0.29 and 1.0 for the ^{14}C- and ^{3}H-antibodies respectively; compared to the 13 day serum, the cor-

responding relative affinities were 1.6 and 1.9 (Fig. 1 A and B). These results are consistent with the fact that two labeled antibody preparations are differentiated more distinctly by a reference antiserum of intermediate affinity (Fig. 1 A) than by one with affinity either higher or lower (Fig. 1 B) than that of both labeled antibodies (6).

More detailed data on the change in relative affinity with time were obtained

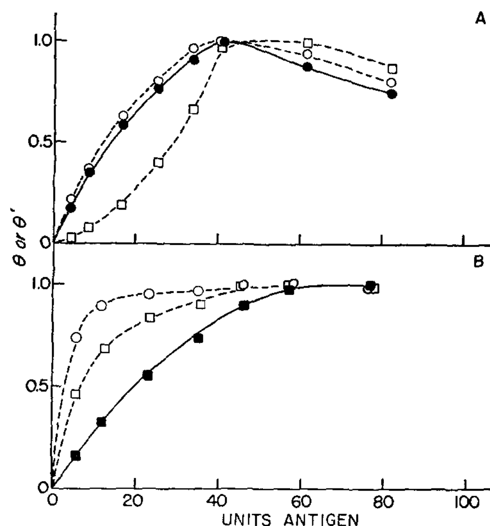

FIG. 1. Simultaneous precipitation of two secreted antibody preparations with a reference antiserum. □, ^{14}C-antibodies were obtained from the lymph node cell suspension of a rabbit 13 days after immunization with 2 mg DNP-BγG; ○, ^{3}H-antibodies were obtained from the lymph node cell suspension of a rabbit 12 wk after immunization with 2 mg DNP-BγG; A: —●—, reference antiserum was obtained from the rabbit that furnished the ^{3}H antibodies, at the same time that its lymph nodes were removed (12 wk after immunization). B: —■—, reference antiserum was obtained from the rabbit that furnished the ^{14}C antibodies, at the same time its lymph nodes were removed (13 days after immunization).

θ and θ' are the fractions of reference and labeled antibodies precipitated. The amount of antigen added (abscissa) is normalized with respect to the maximum amount of antibody precipitated. For a sample calculation see accompanying paper (6).

by comparing several different secreted antibodies with the same reference serum. Table I shows the variation in affinity when ^{3}H-antibodies obtained at intervals from 6 to 88 days after immunization were compared to two reference sera. With respect to the antiserum obtained 5 wk after immunization, the relative affinities of the secreted ^{3}H-antibodies ranged from 0.09 to 1.7; with respect to a 13 day serum (from rabbit 5, Table I), the spread in relative affinity values was 0.69–2.0. Although there was some variation among rabbits unrelated to time changes, the results show a general tendency toward higher rela-

tive affinity with passage of time after immunization. The data are also consistent with the rule that the relative affinity value of a given labeled antibody preparation varies inversely with the average association constant for the reaction of antibodies in the reference sera with simple dinitrophenyl substances (6).

It is of interest that the antibodies formed by the lymph node cells taken 13

TABLE I

Change in Relative Affinity of Secreted Antibodies with Time after Immunization

Rabbit No.	Days after immunization*	Relative affinity‡	
		Assayed with a 5 wk serum§	Assayed with serum from rabbit 5‖
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	—
10	88	1.6	—

* Immunization was with 2 mg DNP-BγG.

‡ For reasons already discussed (6), the values shown cannot, without additional experiment, be converted directly to association constants for the binding of a univalent ligand. However, if the labeled antibodies were "matched" with suitable reference antisera (6), it would be found that these values correspond to a spread in association constant of about 5×10^5 to $>10^8$ M^{-1} for the binding of ε-DNP-L-lysine.

§ Pooled reference antiserum was obtained 5 wk after injecting rabbits with 1 mg DNP-hemocyanin. Antibodies from comparable sera have association constants for ε-DNP-L-lysine of approximately 1×10^7 M^{-1}.

‖ Reference antiserum was obtained from rabbit 5 at the same time that its lymph nodes were removed (13 days after immunization). Antibodies isolated from comparable antisera have association constants for ε-DNP-L-lysine of about 1×10^6 M^{-1}.

days after immunization from rabbit 5 were higher in relative affinity (1.6) than the antibodies circulating at the same time in this rabbit's serum (Table I). This finding is doubtless related to the rapid change in affinity of the secreted antibodies in the first few weeks after immunization compared to the relatively slow turnover of serum antibody. Because the half-life of γG-immunoglobulin in the rabbit is approximately 6 days (11, 12), the serum at any particular time contains antibodies that have accumulated for several days and are of lower average affinity than those currently secreted. Many weeks after immunization the affinity of the secreted antibody approaches a plateau, and the difference

between secreted and serum antibodies becomes negligible (e.g., compare Figs. 1 B and 1 A).

Variation in Relative Affinity with Dose of Immunogen.—The effect on relative affinity of variation in the amount of immunogen administered is shown in Table II. The affinity increased more rapidly in the group of rabbits that received a small dose (0.5 mg) than in the group that was injected with a 200-fold larger dose.

TABLE II

Variation in Relative Affinity of Secreted Antibodies with Time and Immunizing Dose

DNP-BγG injected at zero time	Rabbit No.	Days after immunization	Relative affinity*
mg			
0.5	11	12	0.4
	12	"	0.3
	13	13	0.6
	14	"	0.8
	15	25	1.4
	16	"	1.1
	17	26	1.2
	18	"	1.4
	19	56	1.7
	20	"	1.7
100	21	12	0.2
	22	"	0.4
	23	13	0.2
	24	"	0.4
	25	25	0.7
	26	"	0.5
	27	26	0.5
	28	56	1.1
	29	"	0.7

* Assayed with a pooled reference antiserum obtained 5 wk after immunization with 2 mg DNP-BγG. The average association constant for ε-DNP-L-lysine of antibodies isolated from this pool was 9.3×10^6 M^{-1}.

Heterogeneity of Secreted Antibodies with Respect to Relative Affinity.—Since the relative affinity of secreted anti-DNP molecules changes with time, it seemed possible that the antibody formed at any given moment is actually homogeneous with respect to affinity, and the heterogeneity of the serum antibodies (1) due merely to the accumulation of antibody molecules formed at different times. Fig. 2 shows the results of an experiment designed to determine whether or not the antibody synthesized during a relatively short interval is homogeneous with respect to affinity. The experiment was based on the sup-

position that if the secreted antibody were heterogeneous, it should be possible to divide it into several fractions that differ in affinity.

Extracellular medium containing ^{3}H-anti-DNP antibody was obtained after a 5 hr incubation of lymph node cells taken from a rabbit 19 days after immunization with 2 mg DNP-BγG. The medium was mixed with carrier antiserum and sufficient DNP-HSA was added to precipitate approximately 15% of the serum anti-DNP antibodies as well as 15% of the ^{3}H-antibodies. The precipitate was removed and additional antigen was added to the supernatant,

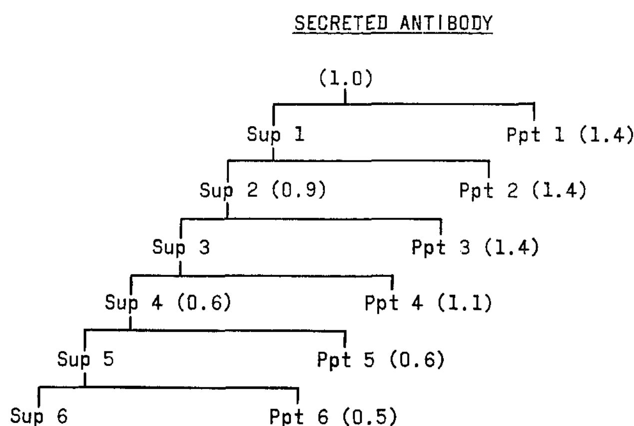

FIG. 2. Heterogeneity of secreted antibodies. The extracellular medium was mixed with carrier antiserum obtained 5 wk after injecting rabbits with 2 mg DNP-hemocyanin. Several precipitates (Ppt) were obtained by the successive addition of small amounts of antigen as described in the text. Alternate supernatant (Sup) fractions were assayed for relative affinity with respect to the carrier antiserum as reference. In addition, some of the ^{3}H-antibody was isolated from the precipitates by the following procedure. The precipitates were washed repeatedly with cold saline and then were suspended in 13 ml of the carrier antiserum containing 1 mg streptomycin sulfate. The suspensions were incubated with occasional mixing at 37°C for 24 hr. The precipitates remaining at the end of this period were removed and the eluted ^{3}H-antibodies were assayed for relative affinity with respect to the carrier antiserum. The amount of radioactivity recovered by elution ranged from 20–35% of that present in the original precipitates. The relative affinity values are indicated by the numbers in parentheses.

again precipitating approximately 15% of the antibodies. The second precipitate was removed, more antigen was added to the supernatant, and the process was repeated several times. After the sixth addition of antigen no precipitate formed. Another aliquot of the antiserum was then added to precipitate the antigen remaining in solution. ^{3}H-antibodies were isolated from the precipitates by the procedure outlined in the legend of Fig. 2, and they and the ^{3}H-antibodies in alternate supernatants were assayed for relative affinity with respect to the carrier antiserum as reference.

The relative affinities of the various fractions are indicated by the numbers in parentheses in Fig. 2. Evidently, each precipitation resulted in the removal of antibodies of higher average affinity than those remaining behind in the

supernatant. The original extracellular medium, therefore, contained antibody molecules of different relative affinities.

In a parallel experiment, the first, second, and fifth precipitates were analyzed for total protein and for radioactivity. The specific activities of these three precipitates were the same. Thus the antibodies in the extracellular medium had not only the same average relative affinity (1.0) as those in the carrier antiserum, they also had a grossly similar distribution of affinities. Since there is independent evidence that serum antibodies are heterogeneous with respect to average intrinsic association constant (1), it follows that the secreted antibodies must be similarly heterogeneous.

Gel Filtration of ^{3}H-Proteins.—In the rabbit, as well as in other species, the immune response to a variety of immunogens is characterized by the production of antibodies of high molecular weight (γM-immunoglobulins) in the early period after immunization (13–22). Since the anti-DNP antibodies formed in the first 2 wk after antigen administration were lower in average affinity than the antibodies formed later, it seemed possible that the variation in affinity might be related to a shift in the proportions of γM- and γG-immunoglobulins. Accordingly, the ^{3}H-proteins synthesized early (9 days) and late (10 wk) after immunization were subjected to gel filtration on Sephadex G-200, as indicated in Fig. 3. Under these conditions, the γM-immunoglobulins are ordinarily found in the first peak, which emerges at the column front, whereas the γG-immunoglobulins are mainly eluted in the second peak. (This distribution was verified by analysis of the proteins in both peaks in the ultracentrifuge and by immunoelectrophoresis.) Both in the early and late samples, almost all the ^{3}H-anti-DNP antibodies appeared in the second peak eluted. The affinity of this antibody was the same, within experimental error, as that of the unfractionated antibody applied to the column (Table III). Thus, the change in affinity with time is not the result of a shift in immunoglobulin class from γM- to γG-immunoglobulin.

There was one notable difference between the elution patterns of the early and late ^{3}H-proteins in Fig. 3. A significant fraction (22%) of the TCA-precipitable counts of the early sample emerged in the first peak. This material was precipitated by chicken antiserum prepared against rabbit γG-immunoglobulin, but it coprecipitated poorly with anti-DNP serum and DNP-HSA. Whereas 40% of the TCA-precipitable protein in the second peak of the 9 day sample was anti-DNP antibody, only about 5% of the TCA-precipitable counts in the first peak had anti-DNP precipitating activity. Nevertheless, the synthesis of this immunoglobulin predominantly in the early period after immunization suggests that it may be antibody of the γM-immunoglobulin class. Possibly it is anti-DNP antibody that does not precipitate well with DNP-HSA either because of extremely low affinity for the DNP group or because of other structural features that interfere with precipitation. Alternatively, it could be antibody directed against some other component in the immunizing

inoculum, e.g., mycobacterial antigens. In any event, this radioactive immunoglobulin does not contribute significantly to the relative affinity as measured in the precipitation assay.

The gel filtration experiments were repeated and the immunoglobulin class of the eluted proteins was verified by quantitative immune precipitation with antisera specific for the

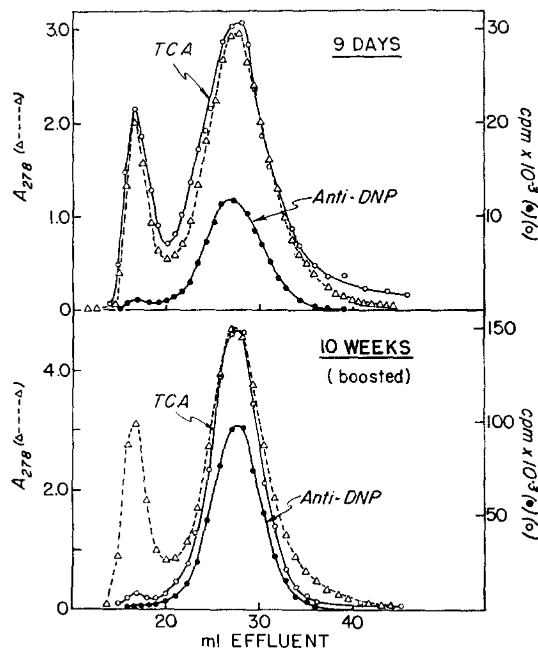

FIG. 3. Gel filtration of ^{3}H-proteins. *Upper*, rabbit 30: Extracellular medium was obtained from the incubation of pooled axillary and popliteal lymph node cells removed 9 days after immunization with 2 mg DNP-BγG. The sample (extracellular medium plus serum globulins as carrier) was 0.5 ml and contained: 32.6 absorbance units at 278 mμ; 4.4 × 10^{5} cpm as TCA-precipitable protein; 4.2 × 10^{5} cpm precipitable with chicken antiserum to rabbit γG-immunoglobulin; 8.6 × 10^{4} cpm as anti-DNP antibody. Recoveries from the column were: absorbance units, 99%; TCA-precipitable ^{3}H-protein, 82%; ^{3}H-anti-DNP antibody, 100%. *Lower*, rabbit 31: Extracellular medium was obtained from the incubation of axillary lymph node cells removed 10 wk after primary immunization with 1 mg DNP-BγG. The rabbit also received a booster injection of 1 mg DNP-BγG 8 days before removal of the lymph nodes. The sample added to the column was 0.8 ml and contained: 48.6 absorbance units at 278 mμ; 1.1 × 10^{6} cpm as TCA-precipitable protein; 7.2 × 10^{5} cpm as anti-DNP antibody. Recoveries from the column were: absorbance units, 100%; TCA-precipitable ^{3}H-protein, 95%; ^{3}H-anti-DNP antibody, 90%.

several classes of rabbit immunoglobulins. The extracellular ^{3}H-protein secreted by the lymph node cells of a rabbit 12 days after immunization with 100 mg DNP-BγG gave a pattern similar to that in Fig. 3 (upper), 18% of the total counts appearing in the first peak eluted from G-200 Sephadex. In contrast, 10 wk after immunization with 1 mg DNP-BγG and 4 days after a booster injection of the same antigen, the secreted ^{3}H-protein was eluted almost entirely

in the second peak, as in Fig. 3 (lower). In each case, the two or three maximum fractions in the peaks were pooled and, after addition of normal rabbit serum as carrier, specific precipitates were formed by the addition of the various antisera. To ensure complete precipitation of the labeled proteins, sufficient antiserum was added so that uncombined antibody was present in the supernatant remaining after removal of the precipitates. The counts in these precipitates (Table IV) indicated that the first peak consisted largely of γM-immunoglobulin, whereas the

TABLE III

Relative Affinities of Secreted Antibodies before and after Gel Filtration on Sephadex G-200

Rabbit No.	Time after immunization	Relative affinity*		
		Extracellular medium	Sample applied to column	Second peak‡
30	9 days	0.79	0.72	0.66
31	10 wk	1.1	1.1	1.0

* The following reference sera were used. For rabbit 30: the reference antiserum was obtained 10 days after immunization with 1 mg DNP-hemocyanin. The average association constant for ε-DNP-L-lysine of antibodies isolated from this serum was 1.0×10^6 M^{-1}. For rabbit 31: the reference antiserum was obtained 11 wk after immunization with 2 mg DNP-BγG. The average association constant for ε-DNP-L-lysine of antibodies isolated from this serum was $>1 \times 10^8$ M^{-1}.

‡ The pooled maximum two fractions of the second peak in Fig. 3 were used (see Fig. 3, upper, for rabbit 30, and Fig. 3, lower, for rabbit 31).

TABLE IV

Immunoglobulin Class of ^{3}H Proteins Eluted from Sephadex G-200

^{3}H-protein precipitated with antiserum* specific for	^{3}H-protein precipitated		
	12 days after immunization‡		10 wk after immunization§
	First peak	Second peak	Second peak
	cpm‖	*cpm‖*	*cpm‖*
Light chains	178	349	6170
Fc fragments	48	414	6490
μ-chains	165	43	Not tested
α-chains	15	12	70
Nonspecific precipitate¶	8	13	93

* Since light chains are a common structural feature of the immunoglobulin family the anti–light chain serum reacts with all immunoglobulins. The anti–Fc fragment, anti–μ-chain, and anti–α-chain are specific for γG-, γM-, and γA-immunoglobulins respectively.

‡ Immunization was with 100 mg DNP-BγG.

§ Immunization was with 1 mg DNP-BγG. 4 days before removal of the lymph nodes, a second dose of 1 mg DNP-BγG was injected.

‖ The cpm were corrected for background (32 cpm).

¶ Prepared with chicken ovalbumin and goat anti-ovalbumin.

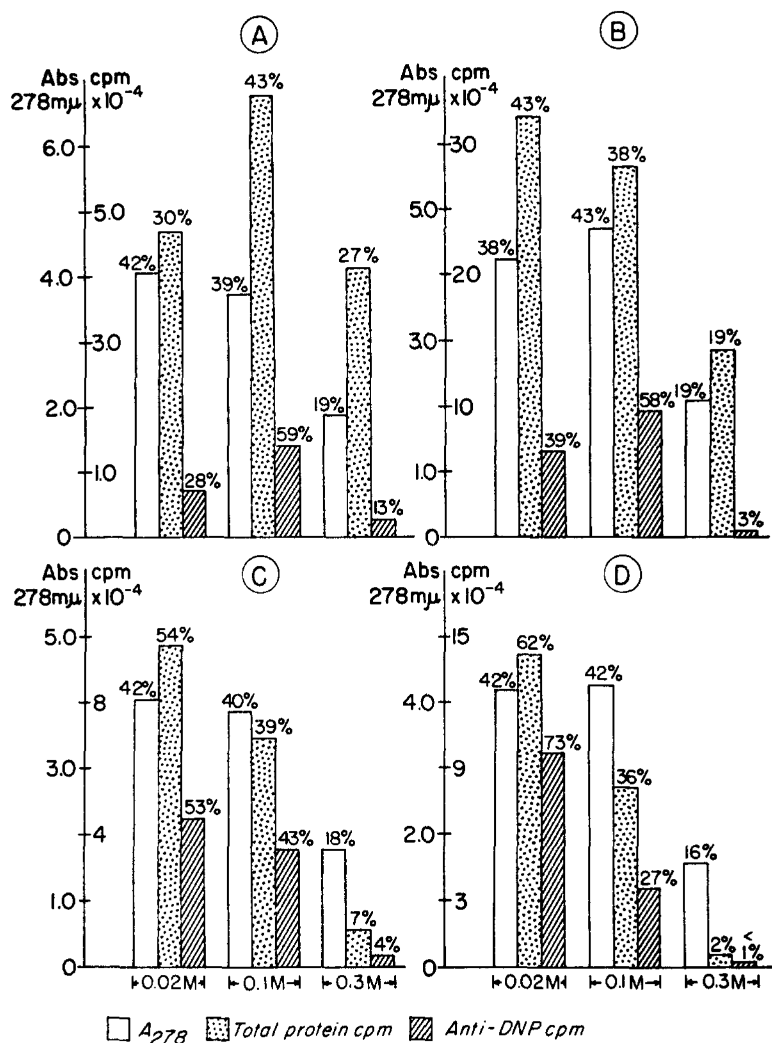

FIG. 4. DEAE-cellulose chromatography of ^{3}H-proteins. The samples added to all columns contained: 12.1–12.2 absorbance units at 278 mμ, 2.7–12.5 $\times$ 10^5 cpm TCA-precipitable protein, 0.6–2.6 $\times$ 10^5 cpm anti-DNP antibody. The heights of the bars indicate the absorption at 278 mμ and the cpm recovered in each fraction. Per cent figures above each bar represent the recovery in each step expressed as per cent of total recovery in all three steps. Absolute recoveries (relative to material applied to columns) are indicated below. A: Extracellular medium was obtained from the lymph node cells of a rabbit injected 12 days previously with 100 mg DNP-BγG. In the first three fractions the total recovery of absorbance units, TCA-precipitable cpm and anti-DNP antibody cpm was 79, 38, and 44%, respectively. After elution with 0.3 M buffer was completed, the column was washed with 0.85 M NaCl, 0.1 M NaOH. The remainder of the absorbance units as well as an additional 32% of the

second peak was almost exclusively γG-immunoglobulin. Virtually none of the radioactivity in either peak was precipitated by the antiserum specific for γA-immunoglobulin. Thus, a substantial amount of γM-immunoglobulin is formed in the early period after immunization.

DEAE-Cellulose Chromatography of ^{3}H-Proteins.—Although the variation in affinity did not appear to be the result of a shift from γM- to γG-immunoglobulin, it was nonetheless possible that other changes in immunoglobulin class might have occurred and that these might be related to the affinity changes. To detect such variations the ^{3}H-proteins synthesized at several intervals after immunization were fractionated by stepwise elution from DEAE-cellulose columns, thus separating the immunoglobulins into groups primarily, although not necessarily entirely, on the basis of charge.

The results of four such fractionations are shown in Fig. 4. In each experiment, the first three fractions were eluted with 0.02 M, 0.1 M, and 0.3 M potassium phosphate, pH 8.0. The amount of total protein (determined by absorbance at 278 mμ) emerging in each of the three fractions was approximately the same in all four experiments, indicating that the conditions of elution were uniform. In the two early samples (A and B) approximately 30–40% of the total ^{3}H-anti-DNP antibody eluted in all three fractions emerged with 0.02 M buffer; nearly 60% was then eluted with 0.1 M buffer; very little additional labeled antibody emerged with the 0.3 M buffer. The elution pattern was shifted in the two later samples: in C and D, 53 and 73% of the ^{3}H-antibodies found in all three fractions were eluted with the 0.02 M buffer; 43 and 27%, respectively, were eluted with the 0.1 M buffer. Again, very little ^{3}H antibody appeared with the third buffer.

The relative affinities of the labeled antibodies in the extracellular medium, in the unfractionated ammonium sulfate concentrate applied to the DEAE-cellulose columns, and in the first two fractions eluted from the column, are

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TCA-precipitable cpm were then recovered. B: Extracellular medium was obtained from the lymph node cells of a rabbit injected 13 days previously with 2 mg DNP-BγG. The total recovery in the first three fractions of absorbance units, TCA-precipitable cpm and anti-DNP antibody cpm was 91, 60, and 65%, respectively. After elution with 0.3 M buffer was completed, the column was washed with 0.9 M KH_2PO_4 (pH 4.1), and less than 2% additional absorbance units, TCA-precipitable or antibody cpm were eluted. C: Extracellular medium was obtained from the axillary lymph node cells of a rabbit injected 9 wk previously with 1 mg DNP-BγG and 8 days previously with 1 mg DNP-BγG. The total recovery in the first three fractions of absorbance units, TCA-precipitable cpm and anti-DNP antibody cpm was 79, 57, and 67%, respectively. The remainder of the absorbance units as well as an additional 18% of the TCA-precipitable cpm were recovered by elution with 0.85 M NaCl, 0.1 M NaOH. D: Extracellular medium was the same as that described in Fig. 3, lower (rabbit 31). The total recovery in the first three fractions of absorbance units, TCA-precipitable cpm, and anti-DNP cpm was 82, 83, and 81%, respectively. Less than 2% additional absorbance units, TCA-precipitable or antibody cpm were then eluted by 0.9 M KH_2PO_4 .

given in Table V. Except for a somewhat lower relative affinity of the 0.1 M fraction in experiment D, the affinities of antibodies in both fractions eluted from the column were very similar to those in the corresponding samples before chromatography. Thus, chromatography on DEAE-cellulose did not separate the secreted antibody into fractions that varied significantly in relative affinity.

As in the gel filtration experiments, the early samples contained ^{3}H-proteins that were almost absent in the later samples. In A and B, 27 and 19% of the total ^{3}H-proteins were eluted with the 0.3 M phosphate buffer, whereas in C and D, only 7 and 2% of the total labeled proteins were eluted in this fraction. It seemed likely that the protein that was relatively retarded by DEAE-cellulose was the same as the protein that had emerged in the front on G-200 Sephadex filtration. The low level of anti-DNP–precipitating activity in both of these fractions also suggested that they might be similar. Accordingly, the immunoglobulin nature of the ^{3}H-proteins emerging in the various fraction eluted from DEAE-cellulose was investigated by precipitation with (*a*) a goat antiserum prepared against a crude euglobulin fraction of rabbit serum, and (*b*) the same goat antiserum after absorption with rabbit γG-immunoglobulin. (Fig. 5 shows the behavior in immunoelectrophoresis of the goat antiserum before and after absorption. The only apparent difference is the disappearance of the precipitin line corresponding to γG-immunoglobulin.)

TABLE V

*Relative Affinities of Secreted Antibodies Eluted in Various Chromatographic Fractions from DEAE-Cellulose**

Experiment	Time after immunization	Relative affinity‡			
		Extracellular medium	Sample applied on column	Eluted with 0.02 M buffer	Eluted with 0.1 M buffer
A	12 days	0.67§	—§	—§	—§
B	13 days	1.0	1.0	0.97	1.0
C	9 wk	1.0	0.96	1.0	0.95
D	10 wk	1.1	0.93	1.0	0.75

* See Fig. 4 for chromatographic fractions and details of experiments A–D.

‡ The following reference antisera were used: A, pooled serum obtained 3 wk after immunization with 2 mg DNP-BγG. The average association constant for ε-DNP-L-lysine of antibodies isolated from this serum was 4.5×10^6 M^{-1}. B, pooled serum obtained 3 wk after immunization with 1 mg DNP-hemocyanin. The affinity of antibodies in this serum is approximately the same as the reference serum antibodies in experiment A; C and D, pooled serum obtained 11 wk after immunization with 2 mg DNP-BγG. The average association constant for ε-DNP-L-lysine of antibodies isolated from this serum was $>1 \times 10^8$ M^{-1}.

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

The results of the precipitation by the two goat antisera are summarized in Table VI. All of the TCA-precipitable radioactivity in the three fractions was precipitated by the unabsorbed serum. After removing the antibodies to γG-immunoglobulin, none of the ^{3}H-protein in the first two fractions, but a substantial proportion of that in the third, was precipitated. Thus, the ^{3}H-proteins in the first two fractions were γG-immunoglobulins, whereas the third fraction contained labeled proteins that were not γG-immunoglobulins.

In experiment A (Fig. 4), the nature of the radioactive protein in the three fractions was also investigated by checking the reactivity of the fractions

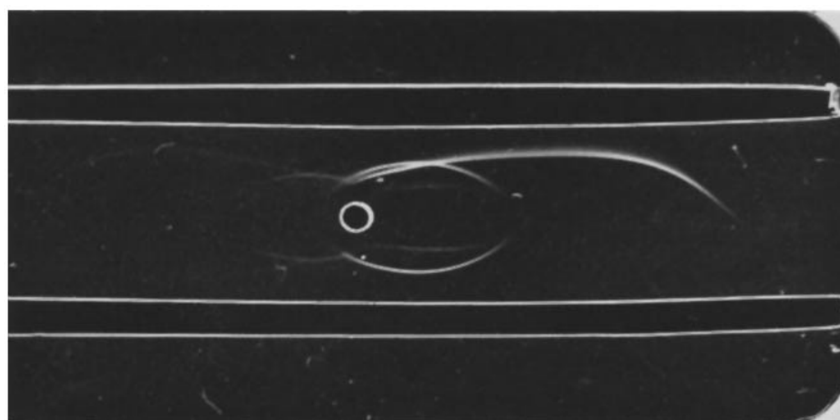

FIG. 5. Immunoelectrophoresis with goat antiserum prepared against a crude euglobulin fraction of rabbit serum, before and after absorption with rabbit γG-immunoglobulins. The upper trough contained the goat antiserum before absorption, the lower trough contained the same antiserum after absorption with rabbit γG-immunoglobulin. The well contained rabbit serum proteins prepared from normal rabbit serum by precipitating with cold 50% saturated ammonium sulfate and dissolving the precipitate in one-seventh the original volume. The agar was made up in sodium barbital acetate buffer, pH 8.6, $\Gamma/2 = 0.05$. The anode is on the left.

with a goat antiserum directed against the Fc fragment of rabbit γG-immunoglobulin. All the ^{3}H-protein in the first two fractions was precipitated by this antiserum, but only about half of the labeled protein in the third fraction was precipitated. This finding confirmed the observation that the third fraction, eluted with 0.3 M buffer, contained proteins that do not belong to the γG-immunoglobulin class.

The possibility was also considered that the fractions eluted with 0.3 M phosphate buffer might contain antibodies directed against components in the immunizing inoculum other than the DNP group. The results of preliminary experiments indicated that this was probably not the case. Thus, in one experiment, ^{3}H-proteins were mixed with either bovine γ-globulin or a saline extract of *Mycobacterium butyricum* and with antisera from rabbits that had been in-

jected with complete Freund's adjuvant, with or without BγG. Little if any of the ^{3}H-protein could be attributed to antibodies against the mycobacteria or against unsubstituted bovine γ-globulin. In addition, when DNP-BγG was added to a mixture of the ^{3}H-protein and antiserum to DNP-BγG, no more counts per minute were precipitated that when DNP-HSA was added to the same mixture of ^{3}H-protein and anti-DNP-BγG. Thus, the ^{3}H proteins did not contain a significant amount of ^{3}H-antibodies to nonhaptenic portions of DNP-BγG (the immunogen).

While the nature of the ^{3}H-proteins in the fraction eluted with 0.3 M buffer is thus not fully defined, it is very likely that these proteins correspond to the ^{3}H-γM-immunoglobulins that were eluted in the first peak in gel filtration on Sephadex G-200.

TABLE VI

Precipitation of DEAE-Cellulose Fractions by Absorbed and Unabsorbed Goat Antisera Prepared against Rabbit Euglobulins

Experiment*	Fraction eluted with potassium phosphate buffer pH 8.0	^{3}H-protein precipitated‡	
		Unabsorbed antiserum	Absorbed antiserum
	molarity	*cpm*	*cpm*
A	0.02	553	9
	0.1	637	7
	0.3	350	189
B	0.02	1024	30
	0.1	1510	7
	0.3	2226	1939
C	0.02	1292	16
	0.1	537	1
	0.3	499	346

* See Fig. 4 for experiments A, B, and C.

‡ The absolute number of cpm precipitated is irrelevant because different amounts of each fraction were tested. Only the difference between the last two vertical columns (absorbed vs. unabsorbed serum) is significant. See text and Fig. 5 for details concerning the absorption.

Heterogeneity in Charge of Anti-DNP Antibodies Isolated from Serum: Comparison with Secreted Antibodies.—Anti-dinitrophenyl antibodies isolated from serum are heterogeneous in charge (23) and in their affinity for haptens (1). However, these two manifestations of heterogeneity do not appear to be directly related since the average mobility in immunoelectrophoresis does not vary systematically with the binding constant. Fig. 6 shows the immunoelectrophoretic patterns of three antibody preparations isolated from sera obtained at different intervals after immunization of a group of rabbits. The average association constants for ε-DNP-lysine ranged from 1.2×10^6 to greater than 1×10^8 M^{-1}, but there was little difference in mobility.

In order to compare the chromatographic heterogeneity of secreted (^{3}H) and serum anti-DNP molecules, the latter had to be isolated from serum by a variation of the usual method for preparing anti-DNP antibodies. Antigen-antibody precipitates were prepared with DNP-HSA and anti-DNP antisera obtained 10 days and 8 wk after immunization with 2 mg DNP-hemo

cyanin. The precipitates were suspended in 0.3 M potassium phosphate, pH 8.0, saturated with 2,4-dinitrophenol, and the soluble material was passed through DEAE-cellulose and Dowex-1 equilibrated with 0.3 M phosphate, pH 8.0, thus removing free hapten and antigen. (In the usual purification method, the buffer used in these steps is 0.15 M NaCl, 0.01 M phosphate. Some antibody may be adsorbed to DEAE-cellulose in this buffer.) The two purified serum antibody preparations thus obtained were equilibrated with 0.02 M phosphate buffer and eluted from DEAE-cellulose columns by stepwise increase in salt concentration as in Fig. 4. The column size and sample load were the same as in Fig. 4. The elution patterns of the two preparations were virtually identical: 70% of the total protein emerged with 0.02 M buffer, whereas only 20% and 10% were eluted with 0.1 M and 0.3 M phosphate, repectively. The average association constants for ϵ-DNP-L-lysine of the antibodies eluted in the 0.02 M step were: 10 days,

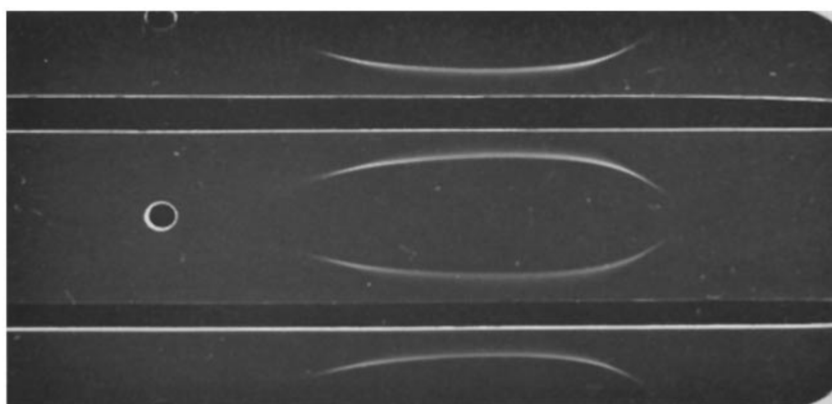

FIG. 6. Immunoelectrophoresis of preparations of anti-DNP antibody that differ in average affinity. The antibodies were isolated from the pooled sera from one group of rabbits bled at intervals after immunization with 2 mg DNP-BγG. Average association constants are given for the reaction with ϵ-DNP-L-lysine. Upper well: 10 days, $K_o = 1.2 \times 10^6$ M^{-1}; middle well: 5 wk, $K_o = 9.3 \times 10^6$ M^{-1}; lower well: 12 wk, $K_o > 1 \times 10^8$ M^{-1}. The troughs contained goat antiserum prepared against a crude rabbit globulin fraction. The agar was made up in sodium barbital acetate buffer, pH 8.6, $\Gamma/2 = 0.05$. The anode is on the left.

1.0×10^6 M^{-1}; 8 wk, 1×10^8 M^{-1}. The antibodies eluted by the 0.1 M buffer were present in insufficient amount for accurate determination of affinity, but the average affinity of the antibodies in the unfractionated samples added to the column was similar to that of the antibodies eluted with 0.02 M buffer.

Thus, antibodies of low and of high affinity, isolated respectively from an early and a late antiserum, were indistinguishable in their mobility on DEAE-cellulose and differed from the secreted ^{3}H-anti-DNP molecules in this respect. In particular, the antibodies isolated from serum obtained 10 days after immunization adhered less firmly (on the average) to DEAE-cellulose than did the antibodies secreted by the lymph node cells at about the same time after immunization. Evidently, the anti-DNP molecules isolated from early antisera differ from the early secreted anti-DNP molecules in charge distribution, or possibly in some other property that affects mobility on DEAE-cellulose.

DISCUSSION

These studies demonstrate that the anti-2,4-dinitrophenyl antibodies, synthesized and secreted by lymph node cell suspensions obtained from rabbits at various intervals after immunization with dinitrophenylated bovine γ-globulin, show a striking increase with time in their capacity to bind DNP ligands. These results confirm and extend the earlier observations made with antibodies isolated from serum (1).

Are these sequential variations in binding related to other forms of antibody heterogeneity? It has been known for some time that changes in the structural class of antibody molecules also occur during the course of the immune response. For example, in the early period after immunization with many different antigens, antibodies belonging to the γM-immunoglobulin class are often detected in the serum (13–22). Other shifts in immunoglobulin class after immunization have also been observed in the guinea pig (24) and horse (25, 26). Particularly pertinent to the studies reported here is the observation that antibodies formed by horses against the *p*-azophenyl-β-lactoside group may belong to six antigenically distinct classes and that the average affinity for the homologous hapten may vary from one class to another (26). Temporal changes in the predominant type of immunoglobulin could then result in changes in average affinity. However, it is noteworthy that a recent investigation of anti-DNP antibodies in the guinea pig demonstrated that antibodies belonging to the γ_1 and γ_2 electrophoretic classes had similar average affinities for ϵ-DNP-L-lysine (27).

Certain changes in the immunoglobulin class of the proteins secreted by the lymph node cell suspensions were also observed in the present study. In the early period after immunization, but not later, the extracellular medium contained a substantial proportion of γM-immunoglobulins. Their regular appearance in relation to antigenic stimulation suggested that they were anti-DNP antibodies. However, they hardly precipitated with DNP-HSA and thus did not contribute significantly to the measurement of relative affinity. Indeed, it was demonstrated that the precipitating antibodies were almost entirely γG-immunoglobulins. Furthermore, with one possible exception (see below), fractionation by gel filtration or by DEAE-cellulose chromatography did not divide the secreted antibodies into populations that differed significantly in relative affinity.

Our results also indicated that there was no relation between the average electrophoretic mobility of anti-DNP antibodies isolated from serum and their affinity for ϵ-DNP-L-lysine. However, in the early period after immunization, the antibodies isolated from the sera differed to some extent from the antibodies synthesized by the lymph node cells in their mobility on DEAE-cellulose. It is possible that the process of antibody purification—the formation of

antigen-antibody precipitates and the dissolution of these precipitates by excess hapten—favors the selection of certain antibody molecules at the expense of others. The secreted antibodies, in contrast, were not subjected to any purification procedures other than concentration by ammonium sulfate precipitation; therefore, they represent virtually the complete spectrum of antibodies actually produced by the lymph node cells. However, it is pertinent to note that the ^{3}H-antibodies that were relatively retarded on DEAE-cellulose (eluted with 0.1 M phosphate buffer, Fig. 4) did not, with the possible exception of experiment D, differ from the unretarded antibodies (eluted with 0.02 M buffer) in relative affinity (Table V). Evidently, although the antibodies isolated from the serum contained fewer of the relatively retarded molecules, the basis for this selection does not appear to be related to a lesser affinity for the DNP group on the part of these molecules.

It may well be that subclasses of rabbit γG-immunoglobulins, comparable to those found in other species, exist and will be observed as more discriminating antisera become available. Possible variations in affinity among the various subclasses would then have to be reconsidered. However, since immunoglobulin class and affinity are probably determined by different regions of the molecule it would be surprising if variations in binding were entirely the result of heterogeneity in immunoglobulin class.

It is possible to compare the amount of γM-immunoglobulins formed by our lymph node cell suspensions with the number of lymphoid cells found by Cebra et al. (9) to stain specifically for the heavy (μ) chain of the γM-immunoglobulins. In our studies, when the lymph nodes were removed in the early period after immunization, about 20% of the total immunoglobulins synthesized were γM-immunoglobulins. This agrees very well with the proportion (14–21%) of cells producing μ-chain in rabbits 2–3 wk after immunization with DNP-ferritin (9). However, in the study of Cebra et al., the time between immunization and bleeding was not varied. It cannot be determined, therefore, if, consistent with our observations, the proportion of μ-chain producers declines with time after antigen administration.

Studies on the molecular size of the antibodies formed in the secondary response by suspensions of lymphoid cells have also been reported by Nezlin (28). Specifically purified radioactively labeled antibodies formed on the 3rd and 5th days after restimulation of rabbits with human serum albumin were compared by gel filtration on Sephadex G-200. Much of the antibody obtained on the 3rd day was eluted in the peak at the column front in the position of the γM-immunoglobulins, but there was considerable variation in the proportion of material at this position in different rabbits. However, by the 5th day after restimulation nearly all the radioactivity appeared in the second peak, in the position of the γG-immunoglobulins. In our study, the immunoglobulins produced both on the 4th and 8th days after secondary injection of DNP-BγG in adjuvant were almost exclusively γG-immunoglobulin. Possibly, some of the anti-DNP antibodies synthesized even earlier than the 4th day after restimulation may be γM-immunoglobulins. In an accompanying paper, the binding characteristics of the earliest antibodies produced in the secondary response will be considered (29).

The systematic increase with time in the average affinity of secreted antibodies poses an intriguing puzzle in biosynthesis. Although an increase in binding

capacity is noted irrespective of the dose of immunogen, the rate of change is diminished when large doses are given. Indeed, the inverse relation between the amount of antigen present in the animal and the affinity of the antibodies produced suggests a possible critical role for antigen in selecting cells for antibody production. It has been postulated, for example, that the antibody response is initiated by combination of antigen with cell receptors (30–32). In particular, it has been suggested that antigen reacts with preexisting cellular receptors with affinity for ligand similar to that of the antibody ultimately formed (33–35). In the early period after immunization, the relatively large quantities of antigen present in the animal would then be sufficient to stimulate even those cells that have receptors of low affinity for the antigenic determinants. As the antigen is gradually eliminated, its concentration falls below that needed to stimulate the low affinity receptors, and only those cells with receptors of relatively high affinity would continue to respond to antigenic stimulation.

One might predict then that immunization with large amounts of antigen should induce the production of correspondingly large quantities of antibody, since all receptors, low as well as high affinity, would be stimulated. However, within rather broad limits, no direct relation between antigen dose and the total amount of antibody formed has been observed. Indeed, less high affinity antibody is apparently produced after immunization with a large amount of immunogen than after immunization with a smaller amount. Accordingly, a means for limiting the production of high affinity antibody in the presence of large quantities of immunogen is required. The basis for a restrictive mechanism has been proposed (36, 37), and if it operates also by means of cellular receptors, one might anticipate that the cells with high affinity receptors would be more easily inhibited (34). The transition from low to high affinity antibody would then result from both the selective stimulation and the selective inhibition of cells that bear receptors of varying affinity. In this way, the antigen would dictate not only the specificity, but also the affinity of the antibodies that are produced. The existence of such cellular receptors and their properties with respect to stimulation and inhibition by antigens obviously warrant further investigation.

SUMMARY

The anti-2,4-dinitrophenyl (DNP) antibodies synthesized by suspensions of lymph node cells obtained at various intervals from rabbits that had been immunized with DNP-bovine γ-globulin increased progressively in their affinity for the dinitrophenyl determinant. This change accompanied and was apparently responsible for a similar change in the binding properties of anti-DNP antibodies isolated from the serum. The rate of change in affinity was related to the dose of immunogen: increasing the dose delayed the change.

The antibodies formed during a brief (5 hr) incubation in vitro were heterogeneous in their binding properties. Therefore, the mixing in the circulation of molecules synthesized at different times may contribute to, but is not alone responsible for, the heterogeneity in the serum antibodies.

Variability in binding did not appear to be related to heterogeneity in immunoglobulin class. Indeed, the variations in relative affinity occurred entirely within the γG-immunoglobulins.

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