DISTRIBUTION OF ANTIBODY-FORMING CELLS OF DIFFERENT SPECIFICITIES IN THE LYMPH NODES AND SPLEENS OF GUINEA PIGS*

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(Received for publication 17 May 1968)

Although the precise mechanisms responsible for antibody formation are unknown, it is now generally accepted that most if not all individual plasma cells produce antibodies of a single specificity (1–5) and immunoglobulin molecules of a single allotypic type (6–8). We can therefore ask how antibody-forming cells of different specificities are arranged and distributed in the lymph nodes and spleen. The answer to this question may provide some insight into the mechanism of antibody production, and it has some relevance to the clonal selection hypothesis of antibody formation (9–11). According to this hypothesis, a very small number of preexisting antibody-bearing cells are present in an animal prior to antigen administration. Contact of these cells with administered antigen would then lead to a rapid proliferation of these cells and thus to the production of a large amount of that antibody initially present on the cell. If such a hypothesis were correct, one might expect to see, especially soon after immunization, groups or clones of plasma cells, each producing antibody of the same specificity, in lymph node sections of animals immunized with two different antigens. Studies of the distribution of cells producing antibodies of different specificities were first performed by White in a brief study in 1958 (1). He found, by employing immunofluorescent techniques in animals immunized with protein antigens, that cells of different specificities were randomly intermixed. In contrast, in more recent studies by Braun and Nakano (12) and Young and Friedman (13) using the Jerne plaque technique, it was found that antibody-forming cells making antibodies to sheep and chicken red blood cells appeared to be clonally distributed in the spleens of mice.

In the present study, the distribution in lymph node and spleen sections of antibody-forming cells of different specificities was examined by means of double label immunofluorescent techniques. In some experiments, guinea pigs

* Supported by Grant No. CA 4788-06 from the National Cancer Institute, United States Public Health Service.

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were immunized with two separate antigens, ovalbumin (OVA) and bovine γ-globulin (BGG); here the antigenic determinants are on separate molecules. In other experiments, animals were immunized with dinitrophenylated bovine γ-globulin, or dinitrophenylated-poly-L-lysine bovine serum albumin complexes (DNP-PLL·BSA); here two antigenic determinants are on the same molecule or immunogenic complex. Antibody-forming cells with different specificities were found both randomly mixed and separately segregated. Which of these distribution patterns was obtained depended upon the method of immunization and the physical state of the antigens employed.

Materials and Methods

Guinea pigs (400–500 g) were obtained from Camm Research Laboratories, Wayne, N. J.; bovine serum albumin (BSA) and ovalbumin (OVA) from General Biochemicals, Chagrin Falls, Ohio; and bovine γ-globulin (BGG) from Armour Pharmaceutical Co., Kankakee, Ill. Guinea pig γ2-globulin was purified from a commercially obtained fraction II (Pentex, Inc., Kankakee, Ill.) by passing it through a DEAE-cellulose column equillibrated with a sodium phosphate buffer, .01 M pH 7.6. Poly-L-lysine (PLL), molecular weight 115,000 was purchased from Pilot Chemical Co., Watertown, Mass.; Freund's complete adjuvant (CFA) from Difco Laboratories, Inc., Detroit, Mich.; and pertussis vaccine from Eli Lilly & Co., Indianapolis, Ind. 1-Fluoro-2,4-dinitrobenzene (DNFB) was obtained from Eastman Organic Chemicals, Rochester, N. Y. Fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (RITC) were purchased from Baltimore Biological Laboratories, Baltimore, Md. DNP-PLL, DNP-BGG, and DNP guinea pig γ-globulin conjugates were prepared by reacting the PLL or the protein with DNFB under alkaline conditions (14) and the degree of DNP substitution calculated as previously described (15). The subscript refers to the number of DNP groups per molecule. The following conjugates were prepared: DNP_5-BGG, DNP_{16}BGG, DNP_{66}BGG, DNP_{25} guinea pig γ-globulin, and DNP_{23}PLL. DNP-PLL albumin electrostatic complexes were prepared as previously described (5). Lymph node and spleen sections were made using the cold alcohol fixation and wax-embedding technique of Guy Sainte-Marie (16).

Preparation of Fluorescein- and Rhodamine-Labeled Antibodies.—Rabbits were immunized with BSA, OVA, BGG, or DNP_{66}BGG in complete adjuvant. Anti-BSA, OVA, and BGG antibodies were obtained from the serum as the γ-globulin fraction after precipitation two times with 33% NH_4SO_4. Specific anti-DNP antibodies were isolated by the method of Farah et al. (17) from the serum of rabbits immunized with DNP_{66} BGG. The anti-BSA and anti-BGG antibodies were conjugated with FITC and purified on Sephadex and DEAE columns according to the technique of McDevitt et al. (18). The anti-OVA antibodies and the pure anti-DNP antibodies were conjugated with RITC according to the same method except that after Sephadex filtration the conjugate was absorbed with charcoal as described by Nairn (19).

Immunofluorescent Technique.—The sections to be examined were dewaxed in cold xylene, rehydrated in graded alcohols, and finally placed in pH 7.2 phosphate buffer. The anti-BSA, BGG, and OVA-producing cells were detected using the Coon's sandwich technique (20). BSA, BGG, and OVA at a concentration of 250 μg/ml were used as the middle layer antigens. The slides were then washed and treated with the appropriate fluoresceinated or rhodamine-labeled antibodies. Anti-DNP-producing cells were detected as previously described (4) using DNP_{16}BGG or DNP_{25} guinea pig γ-globulin as the middle layer detecting antigens. In animals immunized with DNP_5BGG, DNP_{25}, guinea pig γ-globulin was used as the middle

layer antigen. After washing, the slides were treated with rhodamine-labeled anti-DNP antibody.

Controls: Slides were incubated with saline instead of the middle layer antigens, the subsequent steps being identical with those described above. After this procedure, no stained cells were seen except when fluoresceinated anti-BGG antibody was used; here a very rare, and weakly stained green cell was seen in an occasional section.

With these techniques, in animals immunized so as to produce cells of different specificities, cells of one specificity were stained green and cells of the other specificity were stained orange; if a cell were producing antibodies of two different specificities it would be stained yellow. A detailed discussion of the validity of these methods as well as the problems associated with them is given in reference 4.

Microscopy: A Leitz Ortholux microscope equipped with an Hb 200 ultraviolet light source, a UG-1 exciter filter, and a GG-13 barrier filter was used. Photography was performed with either high speed Ektachrome daylight film or Anscochrome "500" daylight film. Exposure times were from 60 to 180 secs.

Immunization (general): Animals were immunized with the antigens mixed with either an equal volume of complete Freund's adjuvant or an equal volume of pertussis vaccine (20×10^9 or organisms/ml). In all the cases, the final administered volume of Freund's adjuvant–containing emulsion or pertussis vaccine antigen mixture was 0.1 ml/site. Animals were immunized with the two separate antigens, OVA and BGG, or with antigens containing two determinants are on the same molecule; either DNP_5BGG or DNP-PLL·BSA.

The amount of each antigen given, sites of immunization, and time intervals between immunization and sacrifice are shown in detail in the respective tables and are also discussed below in the section Experimental Design.

Preparation of Heat Denatured Antigens.—OVA or BGG solutions at a concentration of 10 mg/ml were added dropwise to boiling 0.15 N saline. Boiling was continued for 10 min. The precipitated proteins were then washed by centrifugation three times in 0.15 N saline and the precipitate was diluted to the appropriate concentration.

Antigens Carrying Two Determinants.—Two different antigens were used, each of which carried two determinants on the same molecule or immunogenic complex. The first was DNP_5BGG. It has been previously demonstrated that when animals are immunized with DNP-BGG conjugates bearing only a few DNP groups, anti-DNP antibodies are produced as well as antibodies to the unmodified BGG carrier, and that these two kinds of antibody are produced in separate cells (4).

The second type of antigen employed carrying two determinants was $DNP_{23}PLL$ complexed to BSA. The immune response to DNP-PLL is under genetic control. Only 30% of Hartley guinea pigs can make an immune response to DNP-PLL (21–23). However, animals genetically unable to respond to DNP-PLL can be made to produce anti-DNP antibodies if the positively charged DNP-PLL is electrostatically complexed to a negatively charged foreign albumin. Thus, in genetic nonresponder animals DNP-PLL behaves as a hapten and the BSA acts as the carrier (24). After immunization of animals with this complex, anti-DNP antibodies are produced as well as antibodies to the BSA. Again it had been shown that these antibodies are produced in separate cells (5). The distribution of these antibody-producing cells was determined both in genetic nonresponder animals immunized with DNP_{23}-PLL·BSA (here the presence of the BSA is absolutely essential for the production of anti-DNP antibodies) and also in genetic responder animals immunized with $DNP_{23}PLL$·BSA.

The number, density, location, and arrangement of antibody-forming cells of each specificity was determined by inspection; in selected cases, counts of cells of each specificity were performed. The density of antibody-forming cells in the lymph nodes and spleen is described in the tables as low density, moderate density, and high density. These are defined arbitrarily

as follows at a magnification of ×250: *low density*, only a few cells (2–4) seen per field, many fields contain no antibody-forming cells; *moderate density*, from 15–70 cells seen per field, occasional fields show no cells; *high density*, several hundred cells per field, almost all areas of section contain antibody-forming cells.

Animals were bled from the retroorbital plexus on the day of sacrifice and the presence of circulating antibody was determined by gel diffusion using Hyland Immunoplates, Pattern C, Hyland Laboratories, Los Angeles, Calif. Antigens were used at a concentration of 0.25 mg/ml.

Experimental Design

In the animals immunized with the two separate antigens, OVA and BGG, an attempt was made to see if varying the conditions of immunization would influence the degree of mixing of the cells of separate specificities.

1. The degree of in vitro mixing of the two antigens was varied prior to injection. This experimental design is shown in Table I.

2. The two separate antigens, OVA and BGG, were not mixed in vitro but were injected into separate sites, these sites being either closely or distantly spaced. This experimental design is also shown in Table I.

3. Since administration of Freund's complete adjuvant produces first a severe degree of hyperplasia and granuloma formation and then extensive fibrosis and distortion of the node architecture, immunization of animals with BGG and OVA in pertussis vaccine was performed. Here, although hyperplasia and secondary follicle formation are prominent, severe distortion of the node architecture does not take place. Again these two antigens were either mixed and injected into the same sites or were separated and injected into separate sites. This experimental design is shown in Table II.

4. Another variation in the method of immunization was to make the antigen insoluble. Here it was found that immunization with heat-denatured OVA and BGG did not lead to a significant degree of antibody production to soluble OVA and BGG, but did result in an immune state in these animals in which a rapid and vigorous booster response could be elicited by subsequently immunizing these animals with soluble OVA and BGG. The experimental design of these experiments is shown in Tables IV, V, VI, and VII.

In this fourth variation of the method of immunization, the animals were therefore immunized first with the denatured OVA and BGG and then boosted with the soluble OVA and BGG. In order to see if the sites of injection of the insoluble antigens would influence the number and distribution of each type of antibody-forming cell which resulted from boosting with soluble antigen, the animals were immunized with the insoluble antigens and boosted with the soluble antigens in a variety of combinations:

a. The insoluble antigens were injected separately on opposite sides of the body and the soluble boosting antigens subsequently injected either ipsilaterally, contralaterally, or mixed.

b. The two insoluble antigens, OVA and BGG, were mixed in vitro and injected symmetrically into the four footpads, and the soluble boosting antigens were subsequently injected unilaterally or were mixed and injected symmetrically into the four footpads.

As a control for the above experiment, in which the ability of immunization with insoluble OVA and BGG to prepare the animal for a booster response with soluble OVA and BGG was tested, the following experiment was also performed: Animals were immunized only with Freund's complete adjuvant and saline; the animals were subsequently boosted with soluble OVA and BGG.

In animals injected with antigens containing two determinants on the same molecule, there was no variation in the method of immunization. Antigens were always incorporated into CFA, and the fourfoot pads were injected.

RESULTS

General Observations.—All the animals responded with the production of antibodies of two specificities as determined by fluorescein (green) or rhodamine (orange) labeling of antibody-producing cells. In no instance were there yellow plasma cells, indicating antibodies of two specificities. However, plasma-containing interstitial areas of the lymph node, as well as the interior of blood vessels, were frequently stained yellow, indicating that sites containing antibodies of both specificities were indeed stained yellow. This is shown in Figs. 1 *a* and *b*.

There was a correlation between the sites of antigen injection and the density of antibody-forming cells in the draining lymph nodes. The antibody-forming cell density was highest in the immediate draining lymph nodes and was considerably less in lymph nodes distant from the site of antigen injection. For example, in those animals immunized in the rear footpads and posterior flank, high antibody-forming cell densities were present in the popliteal, flank, and inguinal nodes; low cell densities were seen in the axillary and scapular nodes. Examples of this observation are seen in Table II.

In the animals immunized with Freund's adjuvant, the density of antibody-forming cells in lymph node sections was far greater than that seen in animals immunized with the same antigen and pertussis vaccine.

In the lymph nodes, antibody-forming cells were seen within the diffuse cortex between lymphoid follicles and also in the medullary areas of the lymph node. An occasional antibody-forming cell was also seen in the area of closely packed small lymphocytes of the lymphoid nodule. Yellow staining in a dendritic pattern due to adsorbed γ-globulin over germinal center areas was only rarely seen. Why this type of staining was not more commonly seen is not known.

In the spleen, antibody-forming cells were seen in the lymphoid cuffs surrounding the central arteries, and also at the periphery of lymphoid follicles in the red pulp.

Distribution of Antibody-Forming Cells.—In all animals immunized with soluble OVA and BGG, either in CFA or pertussis vaccine, the anti-OVA–producing cells and the anti-BGG–producing cells were always randomly and intimately mixed. This is illustrated in Figs. 2 *a–c*. As can be seen in Tables I and II, this result was not influenced by the degree of in vitro mixing of the antigens prior to injection or by the sites of antigen injection.

Similar mixing of cells of different specificities was always seen in animals immunized with antigens bearing two determinants, and DNP_5BGG or DNP-PLL·BSA gave the same results These are shown in Table III. Accordingly, an attempt was made to determine the effect of a more insoluble antigen. It was found that animals immunized with heat-denatured OVA and BGG produced no antibodies as detected by gel diffusion and only in an occasional

TABLE I

Distribution in Lymph Node Sections of Cells Producing Anti-Ovalbumin Antibodies and Cells Producing Anti-BGG Antibodies in Guinea Pigs Immunized with Ovalbumin and Bovine γ-Globulin in Complete Freund's Adjuvant

Animal No.	Method of immunization	Amount of each separate antigen per site	Days after immunization	Nodes studied	Result	Comment
		μg				
9-2	OVA and BGG in saline, mixed with CFA and injected into four footpads	50	20	Popliteal and flank	Cells of each specificity intermixed	High cell density
9-4	As above	50	29	Popliteal and flank	Cells of each specificity intermixed	More anti-BGG cells, high cell density
9-5	As above	50	29	Popliteal and flank	Cells of each specificity intermixed	More anti-BGG cells, high cell density
17-1	OVA in CFA, BGG in CFA, mix both Freund's adjuvant emulsions gently as to give a marble cake effect. Inject into four footpads	25	16	Popliteal, flank, inguinal, axillary, and scapular	Cells of each specificity intermixed	Low to moderate cell density
17-3	Same as above	25	16	Popliteal, flank, inguinal, scapular	Cells of each specificity intermixed	High cell density, more anti-OVA cells than anti-BGG cells
19-2	OVA in CFA, BGG in CFA, each separate emulsion injected into separate sites of same footpad. Four footpads injected	12.5	16	Popliteal, flank, and scapular	Cells of each specificity intermixed	More anti-BGG cells than anti-OVA cells
19-5	As above	12.5	16	Popliteal, flank, inguinal, and scapular	Cells of each specificity intermixed	Low cell density
19-1	As above	12.5	21	Flank, inguinal, axillary, scapular, and popliteal	Cells of each specificity intermixed	High cell density, equal number of anti-BGG cells and anti OVA cells
20-3	BGG in CFA injected into both footpads. OVA in CFA injected into both posterior flanks	25	14	Inguinal, flank, popliteal, axillary, and scapular	Cells of each specificity intermixed	Moderate cell density. In the popliteal, flank, and inguinal nodes there were more anti-BGG cells than anti-OVA cells. In the axillary and scapular nodes there were equal number of each kind of cell

TABLE I (*Concluded*)

Animal No.	Method of immunization	Amount of each separate antigen per site	Days after immunization	Nodes studied	Result	Comment
		μg				
20-5	As above	25	20	Inguinal, flank, popliteal, axillary, and scapular	Cells of each specificity intermixed	Low cell density. The anti-BGG cells were more numerous than anti-OVA cells in all nodes. In axillary and scapular nodes very few antibody forming cells were seen
20-1	OVA in CFA injected into both footpads. BGG in CFA injected into both posterior flanks	25	18	Inguinal, flank, popliteal, axillary, and scapular	Cells of each specificity intermixed	Moderate to high cell density. In the popliteal, flank, and inguinal nodes the anti-OVA cells were more numerous than anti-BGG cells. In the axillary node the anti-BGG cells were more numerous than the anti-OVA cells
20-4	As above	25	26	Inguinal, popliteal, upper and lower flank node	Cells of each specificity intermixed	Moderate cell density. Equal numbers of anti-OVA cells and anti-BGG cells were seen, except that in the lower flank node there were more anti-BGG cells than anti-OVA cells
20-2	As above	25	26	Inguinal, flank	Cells of each specificity intermixed	Moderate cell density. In the flank node many more anti-OVA cells than anti-BGG cells

animal were a few weak anti-BGG–producing cells seen in lymph node sections. In no case were anti-OVA–producing cells found. These results are shown in Table IV.

However, other animals immunized with insoluble OVA and BGG were then boosted with soluble OVA and BGG. This lead to the appearance of a large number of antibody-forming cells in the lymph nodes and spleen and to the presence of detectable anti-BGG and anti-OVA antibodies in the serum of these animals. These results are shown in Tables V and VI. In the first set of these experiments shown in Table V, the animals were immunized first with insoluble OVA in the right half of the body and with insoluble BGG in the left

TABLE II

Distribution in Lymph Node Sections of Cells Producing Anti-Ovalbumin Antibodies and Cells Producing Anti-BGG Antibodies in Animals Immunized with Ovalbumin and Bovine γ-Globulin with Pertussis Vaccine

Animal No.	Method of immunization	Amount of each separate antigen per site	Days after immunization	Nodes studied	Result	Comment
		μg				
31-1	OVA and BGG in saline mixed with equal volumes of pertussis vaccine,* mixture injected into four footpads	50	13	Flank, popliteal, axillary and scapular	Cells of each specificity intermixed	More anti-BGG cells than anti-OVA cells except in one node where there were equal numbers of both kinds of cells
31-2	As above	50	13	Flank, axillary, and scapular	Cells of each specificity intermixed	More anti-OVA cells than BGG cells
31-3	As above	50	16	Flank, scapular, and popliteal	Cells of each specificity intermixed	Moderate cell density, more anti-OVA cells than anti-BGG cells
31-4	As above	50	16	Flank, scapular, and popliteal	Cells of each specificity intermixed	Low cell density, more anti-OVA cells than anti-BGG cells
31-5	As above	50	19	Flank, scapular, and popliteal	Cells of each specificity intermixed	Moderate cell density. A large number of anti-OVA cells were present plus a few anti-BGG cells
30-1	OVA in saline mixed with equal volume of pertussis vaccine, injected into posterior flank. BGG in saline mixed with equal volume pertussis vaccine, injected into rear footpads	50	10	Flank, inguinal, popliteal, axillary, and scapular	Cells of each specificity intermixed	Low cell density. In flank node more antiOVA cells than anti-BGG cells. In popliteal node more anti-BGG cells than anti-OVA cells; axillary and scapular nodes did not show antibody-forming cells
30-5	As above	50	19	Popliteal and flank nodes	Cells of each specificity intermixed	Low cell densities in the flank and popliteal nodes; anti-OVA cells outnumbered the anti-BGG cells
30-2	OVA in saline mixed with equal volume of pertussis vaccine, injected into rear footpads. BGG in saline mixed with equal volume pertussis vaccine, injected into posterior flank	50	10	Popliteal, flank, and axillary	Cells of each specificity intermixed	Antibody-forming cells were seen only in flank node and axillary node. In flank node cell density was moderate, in this node some areas anti-BGG cells were more numerous in other areas anti-OVA cells were more numerous. Axillary node had a low density of anti-OVA and anti-BGG cells, weakly stained

* 20×10^9 organisms/ml.

TABLE II (*Concluded*)

Animal No.	Method of immunization	Amount of each separate antigen per site	Days after immunization	Nodes studied	Result	Comment
		μg				
30-3	As above	50	15	Flank, axillary, and popliteal	Cells of each specificity intermixed	Axillary and popliteal nodes had low cell densities. Two flank nodes were found, in one node anti-BGG cells outnumbered anti-OVA cells by 20:1, in the other node this ratio was reversed
30-4	As above	50	15	Flank, popliteal, and scapular	Only anti-OVA cells were seen	Low cell densities. Scapular node had no antibody-forming cells. Flank and popliteal nodes had only anti-OVA-producing cells

half of the body; these animals were then subsequently boosted with soluble OVA and BGG. The soluble antigens were given either ipsilaterally or contralaterally in respect to the first immunization, or were given mixed together. As can be seen, the ratios of the numbers of BGG to OVA cells in the nodes in the right and left sides of the body bore no constant relationship to the sites of either the first or second immunization. In general there were more anti-BGG cells than anti-OVA cells. Here, however, for the first time in four of these eight animals, lymph nodes were found in which some areas of the node showes cells of a single specificity grouped together. This is illustrated in Figs. 3 *a–b* and Figs. 4 *a–c*. In the spleens of these animals, cells of different specificitied were always randomly mixed.

In the next set of experiments, with insoluble antigens, shown in Table VI, the insoluble OVA and BGG were mixed together and injected symmetrically into the four footpads. The animals were then boosted with soluble OVA and BGG either symmetrically or asymmetrically. Again there was no constant relationship between the ratios of the anti-BGG cells to anti-OVA cells in terms of the sites of either the first or second antigen injections. However, in three of these six animals, lymph nodes were again found which in some areas showed cells of one specificity grouped together. As in the previous experiment, the spleens of these animals showed cells of different specificities to be always

TABLE III

Distribution in Lymph Node Sections of Cells Producing Antibodies to Two Separate Determinants on the same Antigenic Molecule

Animal No.	Antigen and method of immunization	Days after immunization	Nodes studied	Result	Comment
23-1, genetic nonresponder animal	DNP-PLL·BSA* in CFA 200 μg/animal, four footpads injected	18	Popliteal, axillary, flank	Cells of each specificity intermixed	High cell density
25-1, genetic nonresponder animal	As above	13	Inguinal, popliteal, scapular	Cells of each specificity intermixed	Low cell density
25-2, genetic nonresponder animal	As above	13	Inguinal, popliteal, scapular, axillary, flank	Cells of each specificity intermixed	Moderate cell density
25-A, genetic responder animal	As above	19	Axillary and inguinal	Cells of each specificity intermixed	Moderate cell density, anti-DNP cells more numerous than anti-BSA cells
25-B, genetic responder animal	As above	19	Flank, popliteal, and scapular	Cells of each specificity intermixed	Moderate cell density, number of DNP cells and anti-BSA cells were equal
25-C, genetic responder animal	As above	19	Flank, popliteal, and axillary	Cells of each specificity intermixed	Low cell density. The number of anti-DNP cells and anti-BSA cells are equal
26	DNP_5BGG‡ in CFA 100 μg/animal, injected in four footpads	15	Flank and inguinal	Cells of each specificity intermixed	High to moderate cell density, anti-BGG cells outnumber anti-DNP cells
34-3	As above	24	Flank, inguinal, popliteal, and scapular	Cells of each specificity intermixed	High cell density. Anti-BGG cells outnumber anti-DNP cells by ratio 10:1
34-5	As above	24	Flank, popliteal, and scapular	Cells of each specificity intermixed	Moderate cell density. Anti-BGG cells are more numerous than anti-DNP cells
34-1	As above	26	Popliteal, flank, scapular, and axillary	Cells of each specificity intermixed	Moderate to high cell density. Anti-BGG cells outnumber anti-DNP cells by ratio of 10:1
34-2	As above	36	Inguinal, flank, axillary, and popliteal	Cells of each specificity intermixed	Moderate cell density. Anti-BGG cells outnumber anti-DNP cells by ratio of 20:1
34-4	As above	36	Inguinal, axillary, flank, and popliteal	Cells of each specificity intermixed	Moderate density. Anti-BGG cells outnumber anti-DNP cells by ratio of 20:1

* Here the animals produce anti-DNP-PLL antibodies and anti-BSA antibodies (see reference 5).

‡ Here the animals produce anti-DNP antibodies and antibodies to the determinants on the BGG molecule (see reference 4).

randomly mixed. All these animals made anti-OVA antibody and anti-BGG antibody as detected by gel diffusion.

To determine the degree of response to soluble antigens alone in animals not previously immunized with insoluble antigens, a group of five animals was injected with only saline in CFA. They were then boosted with soluble OVA and BGG as shown in Table VII. Here, only a few antibody forming cells were seen and no anti-OVA or anti-BGG antibodies were detected in the sera of these animals by gel diffusion. Thus, immunization with these insoluble protein antigens produces an immune response characterized by little or no antibody

TABLE IV

Effect of Immunizing Guinea Pigs with Heat-Denatured Ovalbumin and Heat-Denatured Bovine γ-Globulin

No. of Animals	Immunizing Antigen	Days after immunization	Over-all result*
5	Denatured OVA and denatured BGG in CFA, 50 μg/site/antigen. Antigens given either together at same site or separately into footpads and posterior flanks	13-14	In three animals a very few weakly stained anti-BGG cells were seen in lymph node sections. No anti-BGG or anti-OVA antibodies were detected by gel diffusion
4	Denatured OVA and denatured BGG mixed with pertussis vaccine, 50 μg/site/antigen. Antigens given either together at same site or injected separately into footpads and posterior flanks	13-14	No antibody forming-cells seen. No anti-BGG or anti-OVA antibodies were detected by gel diffusion

* Popliteal, flank, inguinal, axillary, and scapular nodes examined.

production but one which prepares the animal for a vigorous booster response to soluble antigens.

DISCUSSION

The observations in the present study are in agreement with the concept that individual antibody-forming cells produce antibodies of a single specificity. In addition, it was found that cells responding to different soluble antigens were always randomly intermixed. These results were obtained whether the animals were immunized with the soluble OVA and BGG mixed together in varying degrees or whether the soluble OVA and BGG were injected separately into closely or distantly separated sites. Identical, random intermixing

TABLE V

Effect of Immunizing Guinea Pigs with Heat-Denatured Ovalbumin and Heat-Denatured Bovine γ-Globulin on Opposite Sides of the Body Followed by Ipsilateral, Contralateral, and Mixed Boosting with Soluble Ovalbumin and Soluble Bovine γ-Globulin

Animal No.	First immunizing antigen	Interval	Second immunizing antigen	Days after first immunization nodes examined	Distribution of cells of each specificity	Density of cells	Over-all ratio Anti-BGG cells: Anti-OVA cells
		days					
39-1	Right: Denat. OVA 50 μg/site in CFA right paw and right footpad Left: Denat. BGG 50 μg/site in CFA left paw and left footpad	10	Right: Soluble OVA 10 μg in right paw and right footpad Left: Soluble BGG 10 μg in left paw and left footpad	17	Right:* Mixed	High to moderate	2:1
					Left:* Mixed	High	5:1
					Spleen: Mixed	Moderate	4:1
39-5	As above	10	As above	20	Right: Mixed	High	1.5:1
					Left: Mixed	High	5:1
					Spleen: Mixed	Low	10:1
39-2	As above	10	Right: Soluble BGG 10 μg in right paw and right footpad Left: Soluble OVA 10 μg in left paw and left footpad	17	Right: Mixed except in axillary and scapular node there was same grouping of anti-OVA cells	High	2:1
					Left: Mixed in general, however, in axillary and scapular nodes there was grouping of anti-OVA cells	High	10:1
					Spleen: Mixed	Moderate	2:1
39-7	As above	10	As above	20	Right: Generally mixed, however, in inguinal and flank node there were separate areas of anti-OVA and separate areas of anti-BGG	Moderate	1.5:1
					Left: Generally mixed, however, in left popliteal node there were areas of all anti-BGG cells	Moderate	1:6
					Spleen: Mixed	Moderate	1:1
39-3	As above	10	Right: Soluble OVA and BGG mixed, 10 μg/each per foot pad Left: Soluble OVA and BGG mixed, 10 μg/each per footpad	20	Right: Mixed	High	6:1
					Left: In general mixed, but in left inguinal node there were areas of only anti-OVA cells	High	10:1
					Spleen: Mixed	Moderate	20:1

* In all cases right and left popliteal, flank, inguinal, axillary, and scapular nodes were examined.

TABLE V (*Concluded*)

Animal No.	First immunizing antigen	Interval	Second immunizing antigen	Days after first immunization nodes examined	Distribution of cells of each specificity	Density of cells	Over-all ratio Anti-BGG cells: Anti-OVA cells
		days					
39-8	As above	10	As above	20	Right: Mixed, but in right axillary and scapular node there were areas of only anti-OVA and areas of only anti-BGG	Moderate	1:1
					Left: Mixed, but in inguinal node there were areas of only one or the other type of cell	Moderate	2:1
					Spleen: Mixed	Moderate	3:1
39-9	As above	10	As above	20	Right: Mixed	Moderate to low	1:2
					Left: Mixed	Moderate to low	1:1
					Spleen:	Low	1:1
39-10	As above	10	As above	20	Right: Mixed	Low	2:1
					Left: Mixed	Low	1:1
					Spleen: Mixed	Moderate	3:1

of cells of different specificities was obtained in animals also immunized with antigens in which the two antigenic determinants were on the same molecule or immunogenic complex.

However, when animals were immunized with heat-denatured particulate OVA and BGG and then boosted with soluble OVA and BGG a somewhat different result was obtained. Here, although cells of different specificities were also found to be intermixed in some lymph nodes, other lymph nodes in the same animal often showed areas in which cells of one specificity were grouped together. Of interest was the fact that animals immunized only with heat-denatured OVA and BGG produced very few anti-BGG cells and no anti-OVA cells, yet such animals were prepared for a rapid booster response to soluble OVA and BGG. Gell and Benacerraf (25) had previously observed that, although animals immunized with heat-denatured proteins produced no antibody to the soluble forms of these proteins, they did manifest delayed reactions to the soluble proteins. Steiner and Eisen (26) recently demonstrated that injection of hapten protein conjugates in rabbits, in amounts too small to elicit detectable antibody formation, nevertheless prepared the animal for partial secondary response.

Another observation which was made was that in animals immunized unilaterally with the priming insoluble antigens OVA and BGG, and then boosted with the soluble antigen either ipsilaterally or contralaterally or

TABLE VI

Effect of Immunizing Guinea Pigs with Heat-Denatured Ovalbumin and Heat-Denatured Bovine γ-Globulin in the Four Footpads Followed by Asymmetric or Symmetric Boosting with Soluble Ovalbumin and Soluble Bovine γ-Globulin

Animal No.	First immunizing antigen	Interval	Second immunizing antigen	Days after first immunization nodes examined	Distribution of cells of each specificity	Density of cells	Over-all ratio Anti-BGG cells: Anti-OVA cells
		days					
43-2	Insoluble OVA and insoluble BGG mixed, 50 μg each in CFA injected into the four footpads	9	Right: Soluble OVA 10 μg in right paw and footpad Left: Soluble BGG 10 μg in left paw and footpad	16	Right:* Mixed Left:* Mixed Spleen: Mixed	High High Moderate	2:1 1:1 3:1
43-6	As above	9	As above	20	Right: Generally mixed, but in popliteal and flank node there were areas of only anti-OVA cells Left: Generally mixed, but in flank node there were areas of only anti-OVA cells Spleen: Mixed	Moderate to high Moderate Moderate	1:2 2:1 1:1
43-7	As above	9	As above	20	Right: Generally mixed, but in inguinal and axillary nodes there were areas of each kind of cell Left: Generally mixed, but in axillary node there was an area of only anti-BGG cells Spleen: Mixed	Low to moderate High Moderate	1:3 1:4 1:3
43-8	As above	9	As above	20	Right: Mixed Left: Mixed Spleen: Mixed	Moderate Low to moderate Low	1:7 1:7 1:10
43-1	As above	9	A mixture of soluble OVA and soluble BGG was injected symmetrically into the four footpads. 10 μg each antigen/site	16	Right: Mixed Left: Mixed Spleen: Mixed	High High Moderate	2:1 2:1 3:1
43-5	As above	9	As above	20	Right: Mixed Left: Mixed, however, in flank node there were areas of only anti-OVA cells Spleen: Mixed	Moderate Moderate to low Low	1:1 1:1 2:1

* In all cases right and left popliteal, flank, inguinal, axillary, and scapular nodes were examined.

mixed, there was no constant relationship found between the number of antibody-forming cells of each specificity in the draining lymph nodes and the sites of injection of the first insoluble antigen or the second soluble antigen. Similar findings confirming the wide dissemination of "memory cells" during

TABLE VII

Effect of Immunizing Guinea Pigs with Saline in Freund's Adjuvant Followed by Boosting with Soluble OVA and BGG

Animal no.	First immunizing antigen	Interval	Second immunizing antigen	Days after first immunization nodes examined	Over-all result*
44-1	Saline in CFA	12	Soluble OVA right foot pad and right paw 25 μg/site Soluble BGG left footpad and left paw 25 μg/site	19	Only a very rare anti-BGG cell seen. No antibody-forming cells seen in spleen
44-3	As above	12	As above	20	Density of antibody forming cells very low. In right popliteal node OVA cells were more numerous than anti-BGG cells. In other areas of this node anti-BGG cells were more numerous. Nodes on left side of body contained only anti-BGG cells—spleen had no antibody forming cells
44-2	As Above	12	Soluble OVA and soluble BGG mixed and injected into the four footpads. 25 μg/site for each antigen	19	Density of antibody-forming cells was low to moderate. Anti-BGG cells were more numerous than anti-OVA cells—spleen contained a rare anti BGG cell. Cells of each specificity were mixed
44-4	As above	12	As above	20	Density of antibody forming cells was low. BGG cells were more numerous than anti-OVA cells, except in left popliteal and axillary nodes anti-OVA cells were more numerous. Cells of each specificity were mixed—spleen contained no antibody forming cells
44-5	As above	12	As above	20	Density of antibody-forming cells was low. Anti-BGG cells were more numerous than anti-OVA cells. Spleen had a very few anti-BGG cells. Cells of each specificity were mixed

* Right and left popliteal, flank, inguinal, axillary scapular nodes, and spleen examined. No anti-OVA antibody or anti-BGG antibody detected by gel diffusion in any animal.

later phases of the immune response have been reported by Hall et al. (27) as well as by Steiner and Eisen (26). Nevertheless, in agreement with the previous investigators, the present data indicate that, early in the antibody response, the nodes immediately draining the sites of antigen injection are the most active in antibody production, and the distant nodes are less active (28–30).

The earlier observations of Askonas and White (31), which indicated that antibody production was more pronounced in nodes distant from the site of antigen injection, may have been due to the relatively large amounts of antigen administered (5 mg/site) and thus local tolerance may have been produced (32).

Attempts to understand the mechanisms of antibody formation can be made at various levels. The present data apply best, perhaps, to the question whether the cell destined to produce a specific antibody in large amounts is a descendant of a cell precommitted to making this antibody, by virtue of having a small amount of the antibody on its surface prior to antigen administration. This restates the clonal selection hypothesis, which presumes proliferation of such committed cells in response to corresponding antigen. In addition, proponents of this view explain the kinetics of antibody formation as well as the changes in affinity of the antibody with time and amount of antigens administered, by the competition for antigen by cells bearing antibodies of different preexisting affinities (33–35).

Another view based upon a variety of physiological (36–40), morphological (41, 42), and theoretical considerations (43, 44) would suggest the possibility that a cell destined to make an antibody could have made any antibody. According to this view, the mechanism of antibody formation is more complicated and may entail a series of steps, each being performed by cells of a different lineage and function. The initiation of the immune response being performed by cells derived from the thymus, the final production of an antibody occurs in cells derived from another cell lineage, the bone marrow (38, 45). The products of this first interaction (be they an antigen-RNA complex, a viral particle, or episome) then transform uncommitted cells into cells which can produce specific antibodies. Whether this comes about as the result of a selection process at a subcellular level or by entirely different mechanisms must remain a matter for conjecture. Other evidence, recently obtained, suggests that the initiation of the immune response depends on a processing step which appeared to be genetically controlled, and the specificity of which may be different from that of the antibody which is later to be produced in the immune response (46).

The basic observations of the present paper and those of previous authors (12, 13) indicate that, in animals given two different particulate antigens such as sheep and chicken RBC or insoluble particulate OVA and BGG, antibody-forming cells of each separate specificity appear to occur in separate groups. In animals immunized with two different soluble protein antigens, antibody-forming cells of different specificities are randomly intermixed as shown by White (1) and confirmed here. These results suggest that the precursor cell is multipotential and that the microenvironmental site of deposition of the antigen (and therefore the site of the early processing step) determines where

antibody-forming cells of each specificity subsequently appear. It is reasonable that the soluble antigens become widely distributed and therefore the sites of processing are also widely distributed; insoluble antigens are more irregularly and less finely deposited. In the context of the present experiment, the injection of these particulate antigens would thus lead to focal and irregularly placed collections of cells capable of efficiently processing the soluble antigen which is given later. It must also be assumed that the products of the antigen-processing interaction must act at only very short distances and/or require actual cell to cell contact, as depicted for example by Thiery (41) and by McFarland et al. (42).

Also, although most investigators agree that antigen is not detectable in antibody-forming cells, (47, 48), and that antigen-containing macrophages may have no fixed relationship to any individual antibody-forming cells, the fact remains that large numbers of macrophages (but not the dendritic macrophages of the germinal center) and antibody-forming cells are found in the same general area of lymph nodes and spleen, namely in the areas immediately peripheral to the dense lymphoid nodule and in the medullary cords of lymph nodes and red pulp of the spleen (48–50).

The presence of areas of cell intermixing, as well as clusters of cells of single specificity in the lymph nodes of animals immunized with the two insoluble antigens followed by boosting with the two soluble antigens, can be interpreted to mean that some cells capable of processing antigen have become disseminated throughout the lymphoid system late in the immune response. The failure to find cell clusters of single specificity in the spleen of such animals—a site distant from the site of initial immunization—supports this idea.

If antibody production were based on the selection of a small number of precommitted cells followed by rapid proliferation of these cells, animals immunized with two separate antigens should have produced clones or group of cells of a single specificity, both in animals immunized with either soluble or insoluble antigens. Thus the observation made in this as well as in previous studies would favor the idea that which antibody is produced by a cell is determined by the location of that cell in relationship to the site of antigen processing. The production of a single antibody by each individual cell may therefore not be based on a precommitment, but rather preemption of the uncommitted cell by the postulated product of this antigen processing (51). The data do not clarify how the tremendous diversity of antibody structure is established at the level of the DNA, or whether after the initial interactions the persistence of some antigen is necessary for long continued antibody production.

Another observation which does not fit simple cell selection by antigen theories is that animals immunized with very small amounts of antigen (26), or insoluble antigens, produce little or no antibody; yet such animals, after a

booster injection of antigen, can rapidly form large amounts of antibody. This antibody also has a higher affinity than would have been expected to result from the boosting injection alone. The proposed mechanism for this phenomenon is the development of cell populations capable of binding antigen as the result of the initial immunization. If such postulated cell populations are developed, following immunization with small amounts of antigen or insoluble antigens, and are the basis for later cell selection by the boosting antigens, these cells are not detectable by current techniques. The alternative explanation of this phenomenon would be that the priming effect of immunization with very small amounts of antigen or heat-aggregated protein antigens might act by an amplification and increase in efficiency of the antigen-processing mechanism, rather than the production of antibody-forming cells.

Finally, this study has again shown that when animals are immunized with antigenic molecules bearing two separate determinants, the antibodies to these different determinants are made in separate cells. This fact alone suggests that the interaction of antigen with the antibody-forming mechanism is probably more complicated than simple cell selection by antigen.

SUMMARY

The distribution of antibody-forming cells of different specificities in the lymph nodes and spleens of guinea pigs immunized with two separate antigens or with antigens bearing two determinants was studied. When animals were immunized with two soluble protein antigens or antigens in which the two antigenic determinants were on the same molecule, antibody-forming cells of different specificities were always randomly intermixed. However, when animals were immunized with two heat-aggregated particulate protein antigens and then boosted with soluble protein antigens, cells of different specificities were often seen to occur in groups.

These results suggest that antibody-forming cells may not arise by the antigen-stimulated proliferation of precommitted antibody-forming cells, but rather antibody-forming cells arise by a transformation of uncommitted precursor cells as the result of their interactions with a locally produced material derived from the processing of antigen.

I would like to thank Patricia Kaplerchuk for her expert technical assistance.

BIBLIOGRAPHY

1. White, R. G. 1958. Antibody production of single cells. *Nature.* **182**:1383.
2. Mäkelä, O., and G. J. V. Nossal. 1961. Studies of antibody-producing capacity of single cells by bacterial adherence and immobilization. *J. Immunol.* **87**:457.
3. Nossal, G. J. V. 1962. Cellular genetics of immune responses. *In* Advances in Immunology. Academic Press Inc. New York 2.
4. Green, I., P. Vassalli, V. Nussenzweig, and B. Benacerraf. 1967. Specificity of the

antibodies produced by single cells following immunization with antigens bearing two types of antigenic determinants. *J. Exptl. Med.* **125**:511.

5. Green, I., P. Vassalli, and B. Benacerraf. 1967. Cellular localization of anti-DNP-PLL and anticonveyor albumin antibodies in genetic nonresponder guinea pigs immunized with DNP-PLL albumin complexes. *J. Exptl. Med.* **125**:527.
6. Pernis, B., G. Chiappino, A. S. Kelus, and P. G. H. Gell. 1965. Cellular localization of immunoglobulins with different allotype specifications in rabbit lymphoid tissues. *J. Exptl. Med.* **122**:853.
7. Bernier, G., and J. J. Cebra. 1965. Frequency distribution of α, γ, κ, and λ polypeptide chains in human lymphoid tissues. *J. Immunol.* **95**:246.
8. Cebra, J. J., J. E. Colberg, and S. Dray. 1968. Rabbit lymphoid cells differentiated with respect to α-, γ-, and μ-heavy polypeptide chains and to allotypic markers Aa1 and Aa2. *J. Exptl. Med.* **123**:547.
9. Ehrlich, P. 1900. Croonian Lecture: On immunity with special reference to cell life. *Proc. Royal Soc. (London).* **66**:424.
10. Jerne, N. K. 1955. The natural selection theory of antibody formation. *Proc. Natl. Acad. Sci. U.S.* **41**:849.
11. Burnet, F. M. 1957. A modification of Jerne's theory of antibody production using the concept of clonal selection. *Australian J. Sci.* **20**:67.
12. Braun, W., and M. Nakano. 1966. Fluctuation tests with antibody-forming spleen cell populations. *Science.* **151**:338.
13. Young, I., and H. Friedman. 1967. The morphological demonstration of antibody formation in follicles of lymphoid tissue. *In* Germinal Centers in Immune Responses. Springer-Verlag. New York. 102.
14. Benacerraf, B., and B. B. Levine. 1962. Immunological specificity of delayed and immediate hypersensitivity reactions. *J. Exptl. Med.* **115**:1023.
15. Carsten, M. E., and H. N. Eisen. 1953. The interaction of dinitrobenzene derivatives with bovine serum albumin. *J. Am. Chem. Soc.* **75**:4551.
16. Sainte-Marie, G. 1962. A paraffin embedding technique for studies employing immunofluorescence. *J. Histochem. Cytochem.* **10**:250.
17. Farah, F. S., M. Kern, and H. N. Eisen. 1960. The preparation and some properties of purified antibody specific for the 2,4-dinitrophenyl group. *J. Exptl. Med.* **112**: 1195.
18. McDevitt, H. O., J. H. Peters, L. W. Pollard, J. G. Harter, and A. H. Coons. 1963. Purification and analysis of fluorescein labeled antisera by column chromatography. *J. Immunol.* **90**:634.
19. Fluorescent Protein Tracing. 1962. R. C. Nairn, editor. E. & S. Livingstone, Ltd., Edinburgh. 27.
20. Coons, A. H., E. H. Leduc, and J. M. Connolly. 1955. Studies on antibody production: 1. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *J. Exptl. Med.* **102**:49.
21. Kantor, F. S., A. Ojeda, and B. Benacerraf. 1963. Studies on artificial antigens. I. Antigenicity of DNP-polylysine and DNP copolymers of lysine and glutamic acid in guinea pigs. *J. Exptl. Med.* **117**:55.
22. Levine, B. B., A. Ojeda, and B. Benacerraf. 1963. Studies on artificial antigens. III. The genetic control of the immune response to hapten poly-L-lysine conjugates in guinea pigs. *J. Exptl. Med.* **118**:953.

23. Levine, B. B., and B. Benacerraf. 1964. Genetic control in guinea pigs of the immune respone to conjugates of haptens and poly-L-lysine. *Science.* **147**:517.
24. Green, I., W. E. Paul, and B. Benacerraf. 1966. The behavior of hapten-poly-L-lysine conjugates as complete antigens in genetic responders and as haptens in nonresponder guinea pigs. *J. Exptl. Med.* **123**:859.
25. Gell, P. G. H., and B. Benacerraf. 1959. Studies on hypersensitivity. II. Delayed hypersensitivity to denatured proteins in guinea pigs. *Immunol.* **2**:64.
26. Steiner, L., and H. N. Eisen. 1967. The relative affinity of antibodies synthesized in the secondary response. *J. Exptl. Med.* **126**:1185.
27. Hall, J. G., B. Morris, G. D. Moreno, and M. C. Bessis. 1967. The ultrastructure and function of the cells in the lymph following antigenic stimulation. *J. Exptl. Med.* **125**:91.
28. Ehrich, W. E., and T. N. Harris. 1942. The formation of antibodies in the popliteal lymph nodes in rabbits. *J. Exptl. Med.* **76**:335.
29. McMaster, P. D., and S. S. Hudack. 1935. The formation of agglutinins within lymph nodes. *J. Exptl. Med.* **61**:783.
30. Harris, T. N., and S. Harris. 1950. Biological and technical factors in the demonstration of antibody production by lymphatic tissue. *J. Immunol.* **64**:45.
31. Askonas, B. A., and R. G. White. 1956. Sites of antibody production in the guinea pig. The relationship between in vitro synthesis of anti ovalbumin and anti-γ-globulin and the distribution of antibody containing plasma cells. *Brit. J. Exptl. Pathol.* **37**:61.
32. Cohen, M. W., and G. J. Thorbecke. 1964. Specificity of reaction to antigenic stimulation in lymph nodes of immature rabbits. *J. Immunol.* **93**:629.
33. Eisen, H. N. 1966. The immune response to a simple antigenic determinant. Harvey Lectures, Ser. 60 (1964–1965). 1.
34. Paul, W. E., G. W. Siskind, B. Benacerraf, and Z. Ovary. 1967. Secondary antibody responses in haptenic systems: Cell population selection by antigen. *J. Immunol.* **99**:760.
35. Siskind, G. W., P. Dunn, and J. G. Walker. 1968. Studies on the control of antibody synthesis. II. Effect of antigen dose and suppression by passive antibody on the affinity of the antibody synthesized. *J. Exptl. Med.* **127**:55.
36. Trentin, J., N. Wolf, V. Cheng, W. Fahlberg, D. Weiss, and R. Bonhag. 1967. Antibody production by mice repopulated with limited members of clones of lymphoid cell precursors. *J. Immunol.* **98**:1326.
37. Gallily, R., and M. Feldman. 1967. The role of macrophages in the induction of antibody in X-irradiated animals. *Immunology.* **12**:197.
38. Mitchell, G. F., and J. F. A. P. Miller. 1968. Immunological activity of thymus and thoracic duct lymphocytes. *Proc. Natl. Acad. Sci. U.S.* **59**:296.
39. Schechter, I. 1968. Antigenic competition between polypeptidyl determinants in normal and tolerant rabbits. *J. Exptl. Med.* **127**:237.
40. Fishman, M., and F. L. Adler. 1963. Antibody formation initiated in vitro. *J. Exptl. Med.* **117**:595.
41. Thiéry, J. P. 1962. Étude au microscope électronique de l'Ilot plasmocytaire. *J. Microscop.* **1**:275.
42. McFarland, W., D. H. Heilman, and J. F. Moorhead. 1966. Functional anatomy of the lymphocyte in immunological reactions in vitro. *J. Exptl. Med.* **124**:851.

43. Smithies, O. 1965. Antibody induction and tolerance. *Science.* **149:**151.
44. Kabat, E. A. 1968. Unique features of the variable regions of Bence Jones proteins and their possible relation to antibody complementarity. *Proc. Natl. Acad. Sci. U.S.* **59:**613.
45. Davies, A. J. S., E. Leuchars, V. Wallis, R. Marchant, and E. V. Elliot. 1967. The failure of thymus-derived cells to produce antibody. *Transplantation.* **5:**222.
46. Green, I., W. E. Paul, and B. Benacerraf. 1968. Hapten carrier relationshps in the DNP-PLL. Foreign albumin complex system: Induction of tolerance and stimulation of cells in vitro. *J. Exptl. Med.* **127:**43.
47. Nossal, G. J. V., G. L. Ada, and C. M. Austin. 1965. Antigens in immunity. IX. The antigen content of a single antibody-forming cell. *J. Exptl. Med.* **121:**945.
48. McDevitt, H. O., B. A. Askonas, and J. H. Humphrey, I. Schechter, and M. Sela. 1966. The localization of antigen in relation to specific antibody producing cells. *Immunology.* **11:**337.
49. Leduc, E., A. H. Coons, and J. M. Connolly. 1955. Antibody production. II. The primary and secondary responses in the popliteal lymph node of the rabbit. *J. Exptl. Med.* **102:**61.
50. Langevoort, H. L. 1963. The histopathology of the antibody response. *Lab. Invest.* **12:**106.
51. Szilard, L. 1960. The molecular basis of antibody formation. *Proc. Natl. Acad. Sci. U.S.* **46:**293.

FIG. 1 *a* and 1 *b*. Sections from scapular lymph node of animals 19-5 (Table I) immunized with soluble OVA and BGG in CFA. Orange cells are producing anti-OVA antibody and green cells are producing anti-BGG antibody. Blood vessels and interstitial plasma-containing areas are stained yellow due to the presence of antibodies of both specificities. Moderate cell density. Anscochrome "500." × 338.

FIG. 2 *a*. Section from flank node of animals 9-2 (Table I) immunized with soluble OVA and BGG in CFA. Orange cells are producing anti-OVA antibody and green cells are producing anti-BGG antibody, cells of each specificity are randomly mixed. High cell density. High speed Ektachrome. × 63.

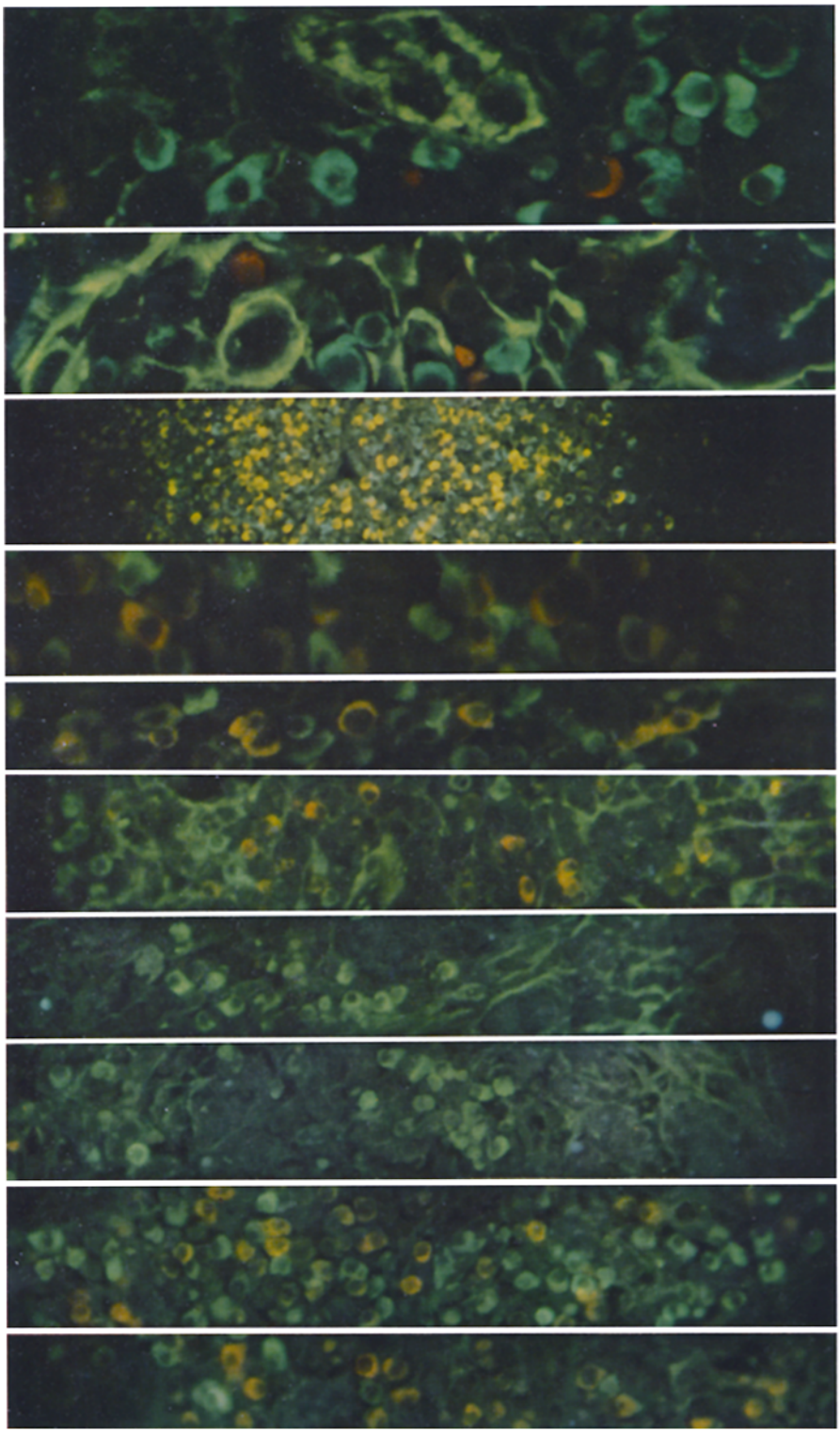
FIG. 2 *b*. Section from flank node of animals 9-5 (Table I) immunized with soluble OVA and BGG in CFA. Cells of each specificity are randomly mixed. High cell density. High speed Ektachrome. × 156.

FIG. 2 *c*. Section from popliteal node of animal 17-3 (Table I) immunized with soluble OVA and BGG in CFA. Cells of each specificity are randomly mixed. High speed Ektachrome. × 104.

FIGS. 3 *a* and 3 *b* show two different areas from the same section of the left inguinal node from animal number 39-3 (Table V) immunized first with heat-aggregated particulate OVA and BGG followed by boosting with soluble OVA and soluble BGG. Fig. 3 *a* shows mainly orange anti-OVA-producing cells. Fig. 3 *b* shows only green anti-BGG-producing cells. Figs. 3 *a* and 3 *b* show a moderate cell density. Taken with high speed Ektachrome. × 104.

FIGS. 4 *a*, 4 *b*, and 4 *c* show three different areas from the same section of the right inguinal lymph node of animal 39-7 (Table V) immunized first with heat-aggregated particulate OVA and BGG followed by boosting with soluble OVA and BGG. Fig. 4 *a* shows many green anti-BGG-producing cells and a rare orange anti-OVA-producing cell. Fig. 4 *b* shows an area where the anti-OVA cells and anti-BGG cells are randomly mixed. Fig. 4 *c* shows an area containing mainly orange anti-OVA-producing cells. There is a moderate cell density in Figs. 4 *a* and 4 *c* and a high cell density in Fig. 4 *b*. High speed Ektachrome. × 104.

It should be noted that the color values seen in the prints do not adequately represent the colors actually seen. This is especially true when high speed Ektachome is used; here orange cells appear yellow.



1*a*

1*b*

2*a*

2*b*

2*c*

3*a*

3*b*

4*a*

4*b*

4*c*