

CELLULAR LOCALIZATION OF ANTI-DNP-PLL AND ANTICON-
VEYOR ALBUMIN ANTIBODIES IN GENETIC NONRESPONDER
GUINEA PIGS IMMUNIZED WITH DNP-PLL
ALBUMIN COMPLEXES*

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About 30-40% of random-bred Hartley (1) and all strain 2 (2) guinea pigs recognize poly-L-lysine (PLL)¹ and hapten-PLL conjugates as antigens. These "responder" animals develop delayed hypersensitivity to the conjugated polymer and produce specific antihapten antibodies when immunized with hapten-PLL conjugates. The ability to respond to these antigens was shown to be controlled by a dominant autosomal gene (2, 3). Hapten-PLL conjugates which are complete antigens only for responder animals can behave as haptens in genetic nonresponder guinea pigs. Thus, insoluble electrostatic complexes formed by the reaction of positively charged 2,4-dinitrophenyl-PLL (DNP-PLL) with negatively charged foreign albumins, acting as conveyor or *Schlepper* molecules, induce the formation of high serum levels of anti-DNP antibodies in nonresponder guinea pigs (4). These animals, however, in contrast with responder guinea pigs, do not show delayed hypersensitivity reactions to DNP-PLL, which is not surprising, since it is well known that unconjugated haptens cannot elicit delayed hypersensitivity reaction (5). In these systems, the recognition of antigenicity and the capacity to show delayed hypersensitivity reactions therefore involve some identical immunological recognition mechanism in which the specificity of the carrier molecule plays an important part. This relationship is further illustrated by the observations of Schlossman on the α DNP-oligo-L-lysines that the same minimum number of 7-8 lysine residues is essential both for immunization of responder guinea pigs and for the elicitation of delayed hypersensitivity reactions in these animals (6, 7).

An understanding of the immune response to hapten-protein and hapten-polypeptide conjugates must depend upon a better knowledge of the hapten-carrier rela-

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¹ The following abbreviations are used throughout the paper: PLL, poly-L-lysine; DNP, 2,4-dinitrophenyl hapten; BSA, bovine serum albumin; BGG, bovine gamma globulin; OVA, hen ovalbumin (5 × recrystallized); GPA, guinea pig albumin; DNFB, 1-fluoro-2,4-dinitrobenzene; FITC, fluorescein isothiocyanate; RITC, tetramethylrhodamine isothiocyanate.

tionship. Since the definition of hapten and of complete antigens by Landsteiner (8) and his demonstration that the immune response could be directed towards well-defined chemical structures, provided they were covalently bound or complexed by ionic forces to protein antigens, great advances have been made with the help of these immunological systems. But very little work has been directed to the understanding of the hapten-carrier relationship with regard to the mechanism by which an immunogenic carrier molecule renders a hapten capable of inducing the formation of specific antibodies. The immune response to DNP-PLL complexed with foreign albumins in guinea pigs genetically unable to recognize DNP-PLL as antigen constitutes a particularly suitable system in which to study the processes by which the carrier confers antigenicity to a hapten (4). Two mechanisms can be proposed to explain this phenomenon:

(a) The carrier molecule allows antibody to the hapten to be synthesized by forming with the hapten a new and complete antigenic determinant. This interpretation postulates that the specificity of the antihapten antibodies involve both the hapten and adjacent areas of the carrier molecule. According to this view, the energy that will allow binding of a sufficient amount of antigen to induce the immune response is only provided by adding the contribution of the carrier to the immunological specificity of the hapten. In support of this interpretation, there has been ample evidence for carrier specificity in hapten-protein and hapten-polypeptide systems for the reactions of actively sensitized cells with antigen such as delayed hypersensitivity (9, 10, 11) and anamnestic reactions (12). In the hapten-PLL system, an unequivocal example of antibody with carrier specificity has been reported in the case of anti-DNP-PLL antibodies produced by genetic responder guinea pigs. These antibodies show partial specificity for the PLL carrier molecule (13). However, the specificity of the DNP-PLL antibodies produced by genetic nonresponder guinea pigs immunized with DNP-PLL complexed with ovalbumin is not consistent with the above hypothesis: these antibodies were shown by fluorescent-quenching technique to have identical specificity towards PLL as the antibodies produced by responder guinea pigs immunized with DNP-PLL alone. No contribution of the foreign albumin conveyor molecule to the specificity of these antibodies could be detected (4).

(b) According to an alternative mechanism, the conveyor foreign molecule is first recognized as antigenic by an unknown process, and then it allows an immune response to be induced to all specific determinants to which it is bound. One of the questions that may be asked in exploring this possibility is whether the antibody produced to DNP-PLL and to the antigenic determinants of the conveyor albumin by non-responder guinea pigs are synthesized in the same or in different plasma cells. This question is very appropriate since in nonresponder guinea pigs the presence of the conveyor molecule is an absolute requirement for the production of anti-DNP-PLL antibodies. For two reasons, this immunological system is also ideally suited to answer the general problem posed in the companion paper (14), whether individual plasma cells synthesize antibodies of different specificities; because in this case the animals are presented with an antigenic complex in which the response to the two types of determinants is intimately linked (4) and because after immunization of genetic non-responder guinea pigs with DNP-PLL albumin complexes only anti-DNP-PLL antibodies and anticarrier protein antibodies with distinct immunological specificities

are detected (13). Thus, in contrast with the antibodies elicited by hapten-protein conjugates, link antibody and anti-hapten antibody with specificity for the conveyer molecule have not been demonstrated in this system. As in the companion paper (14), the classical Coon's sandwich technique (15) using sequential double immunofluorescent labeling, and a combination of this technique with radioautography (16), were used to identify the immunological specificity of the antibodies produced by individual cells from nonresponder guinea pigs immunized with complexes of DNP-PLL with ovalbumin or with bovine serum albumin.

Material and Methods

Polypeptides and Protein.—Poly-L-lysine hydrobromide (PLL) with average molecular weight of 90,000 was obtained from Pilot Chemical Co., Watertown, Mass. Bovine serum albumin (BSA) and bovine gamma globulin (BGG) were obtained from Armour Pharmaceutical Co., Chicago, Ill. Five times recrystallized hen ovalbumin (OVA) was obtained from Pentex, Inc., Kankakee, Ill. Guinea pig albumin (GPA) was prepared by starch block electrophoresis separation of guinea pig serum (17).

Other Reagents.—Freund's complete adjuvant was obtained from Difco Laboratories, Inc., Detroit, Mich. 1-fluoro-2,4-dinitrobenzene (DNFB) was obtained from Eastman Organic Chemicals, Rochester, N. Y. ^{125}I , carrier-free, was obtained from the New England Nuclear Corp., Boston, Mass.

Fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (RITC) were purchased from the Baltimore Biological Laboratories, Baltimore, Md.

Preparation of Conjugates.—DNP-PLL was prepared by reacting PLL with DNFB in dioxane under alkaline conditions as previously described (9).

Polypeptide and protein concentrations were determined by micro-Kjeldahl nitrogen measurements. The degree of DNP substitution was calculated from the absorbancy at $360\text{ m}\mu$ on the basis of the molar extinction coefficient of free ϵ -DNP-L-lysine ($\epsilon\ 360\text{ m}\mu = 17,400$) (18).

A single DNP₂₀-PLL₄₃₀ conjugate was used for this study. The subscripts refer respectively to the average number of groups of haptens and the average number of lysine residues in the molecule.

Preparation of DNP-PLL Albumin Complexes.—Equal amounts by weight of OVA or BSA (at concentration of 5 mg/ml in 0.075 M NaCl) were added to a solution of 5 mg/ml of DNP-PLL in 0.075 M NaCl. 0.1 N NaOH was added dropwise until a precipitate formed.

Immunization.—Male and female Hartley guinea pigs weighing 300 to 400 g were purchased from Camm Research Laboratories, Wayne, N. J. The animals were immunized in the four footpads with 0.2 mg of DNP-PLL-albumin complex in 0.4 ml of emulsion containing equal volumes of the antigen and of complete Freund's adjuvant. Booster injections of 50 μg of DNP-PLL albumin complex and 10 μg of DNP-PLL were given intradermally at 7-8 days.

Immune Response to DNP-PLL Albumin Complexes.—The intensity of delayed hypersensitivity reactions to DNP-PLL was evaluated 24 hr after intradermal injection of 10 μg of DNP-PLL in 0.1 ml of 0.15 M NaCl. The presence of serum antibodies against DNP-PLL, BSA, or OVA was revealed by double diffusion analysis in agar gel using immunoplates from Hyland Laboratories, Los Angeles, Calif., and DNP₄₁-GPA, BSA, or OVA at the concentration of 0.25 mg/ml.

Detection of the Antibodies Produced by Individual Cells.—After immunization of random-bred guinea pigs with DNP-PLL albumin complex, the genetic nonresponder animals were identified by their lack of delayed hypersensitivity to DNP-PLL (4). These animals were killed from 13 to 23 days after immunization and cell smears were prepared from the peripheral lymph nodes draining the sites of injection, as described in the preceding paper (14). The anti-

body-producing cells were identified by a combination of immunofluorescent techniques and radioautography. Since the procedures and reagents used in these experiments have been described in detail in the companion paper (14), they will only be briefly discussed. The only new reagents used in the present study were ovalbumin 0.2 mg/ml as a middle layer antigen and rabbit anti-OVA antibodies labeled with fluorescein to identify anti-OVA antibody-producing cells. The rabbit anti-OVA antibodies were prepared by immunizing rabbits in the footpads with 2 mg of ovalbumin in complete adjuvant. The animals were bled 2-3 wk after immunization, the gamma globulin fraction was conjugated with fluorescein. In the case of guinea pigs immunized with DNP-PLL·BSA, the basic approach was first to expose fixed cell smears to a mixture of BSA and DNP₁₆-BGG. These middle layer antigens fixed to cells making antibody directed against BSA and the DNP determinant respectively. The smears were then washed and treated with a mixture of rabbit anti-BSA antibodies labeled with fluorescein and of anti-DNP purified rabbit antibodies labeled with rhodamine. Cells producing anti-BSA antibodies would be expected to stain green and cells producing anti-DNP antibodies would be expected to stain orange. Cells making both types of antibody would be stained yellow.

Two additional techniques were also employed as described in the companion paper (14): (a) Sequential double fluorescent staining. The slide was treated with one set of reagents, BSA middle layer followed by fluorescein-labeled anti-BSA antibody. Specific fields containing green cells were identified by Vernier readings and photographed. The same slides were then treated with the second set of reagents, DNP₁₆-BGG as the middle layer antigen followed by rhodamine-labeled anti-DNP antibody. The appearance of the newly stained cells or of a change of color, if any, of the previously green-stained cells was recorded. This type of experiment was also carried out using OVA and fluorescein-labeled rabbit anti-OVA antibodies in those animals immunized with DNP-PLL·OVA. (b) Combined double fluorescent and radioautographic technique. Here, a smear was treated with the double antigen middle layer mixture, DNP₁₆-BGG and BSA labeled with ¹²⁵I (BSA-¹²⁵I). The smear was then treated with a mixture of purified rabbit anti-DNP antibody labeled with rhodamine and rabbit anti-BSA antibody labeled with fluorescein. Fields were identified and photographed which contained green and orange cells. The slides were prepared for radioautographic examination by dipping in photographic emulsion. After a suitable time interval, the radioautographs were developed and the smears were stained with Giemsa. The same fields were again identified. It was noted which of the previously green and orange cells had grains localized over them. Only the anti-BSA-producing cells would be expected to have attracted BSA-¹²⁵I and thus to have grains over them. If any orange cells showed grains over them, this would indicate that they were making small amounts of BSA antibodies in addition to anti-DNP antibodies.

RESULTS

When the lymph node cells were reacted with the two sets of reagents to detect anti-DNP-producing cells and anti-BSA- or anti-OVA-producing cells, the stained plasma cells were stained either green (anti-BSA or anti-OVA) or orange (anti-DNP). No yellow plasma cells were seen. The only cellular material which stained yellow was some occasional eosinophile granules.

The results of sequential staining experiments are presented in Table I.

The cells of five individual animals were studied. The sequential staining was performed in both directions; in some experiments the antialbumin-producing cells were detected first, followed by the identification of the cells producing anti-DNP antibodies; in other experiments the order was reversed. A total of

TABLE I

Identification by Sequential Fluorescent Staining of Anti-BSA- or Anti-OVA-Producing Cells and of Anti-DNP-Producing Cells in Genetic Nonresponder Guinea Pigs Immunized with DNP-PLL·BSA or DNP-PLL·OVA

Experiment No.	Immunizing antigen	No. of days after immunization	No. of anti-OVA or anti-BSA-producing cells	No. of anti-DNP-producing cells seen	No. double producers
18-82*	DNP-PLL·OVA	16	80	19	0
19-45*	DNP-PLL·OVA	15	108	65	0
19-44‡	DNP-PLL·OVA	16	16	20	0
19-58§	DNP-PLL·BSA	23	7	15	0
20-14	DNP-PLL·BSA	17	11	56	0
Total			222	175	0

* The anti-OVA cells were identified first by fluorescein-labeled antibody.

‡ The anti-DNP cells were identified first by rhodamine-labeled antibody, then the same fields were reexamined after staining for OVA-producing cells with fluorescein-labeled antibody.

§ The anti-BSA cells were identified first by fluorescein-labeled antibody. The same fields were reexamined after staining for DNP-producing cells with rhodamine-labeled antibody.

|| The anti-DNP-producing cells were identified first with rhodamine-labeled antibody. The same fields were then reexamined after staining for BSA-producing cells using fluorescein-labeled antibody.

TABLE II

Identification by Combined Fluorescent and Radioautographic Technique of Anti-BSA-Producing Cells and Anti-DNP-Producing Cells in Genetic Nonresponder Guinea Pigs Immunized with DNP-PLL·BSA

Experiment No.	Immunizing antigen	No. of days after immunization	No. anti-BSA cells seen by fluorescent staining and radioautography*	No. of anti-DNP cells seen by rhodamine fluorescence	No. of anti-DNP cells exhibiting BSA specificity by radioautography‡
19-58	DNP-PLL·BSA	23	21	17	0/17
19-53	DNP-PLL·BSA	23	16	13	0/13
21-03	DNP-PLL·BSA	13	22	40	0/40
Total			59	70	0

* The same cells were always identified by both techniques.

‡ These are the same previously identified anti-DNP cells.

397 antibody-producing cells were identified. These cells produced either anti-DNP antibody (orange cells) or antibodies against the conveyor albumin (green cells). No cell (yellow cells) could be found which produced antibodies of both specificities. Figures 1 *a* and 1 *b* illustrate the sequential staining experiments.

The results of the second type of experiments are presented in Table II. Here, the anti-BSA-producing cells were identified by immunofluorescence and later by the radioautographic localization of ^{125}I -labeled BSA over the cell. In these very same fields, anti-DNP-producing cells were identified by their orange color. If the anti-DNP-producing cells were also producing small amounts of anti-BSA antibody, then some grains should have appeared over these cells as well. As indicated in Table II, a total of 59 anti-BSA-producing cells were localized by fluorescein fluorescence and radioautography. (It should be noted that there were always grains over the cells previously stained green.) In these same fields, a total of 70 orange anti-DNP-producing cells were seen; in no case did any grains appear over these anti-DNP-producing cells at a concentration higher than the background. Figures 2 *a* and 2 *b* are examples of the experiments combining immunofluorescent and radioautographic technique. Therefore, from the results of these two objective experiments no cells were seen which produced antibodies against DNP-PLL and the conveyor albumin molecule ovalbumin or BSA.

Several additional observations were made in the course of these experiments which deserve mention. First, the same two separate color classes of antibody-producing cells were seen in a few genetic responder animals that were studied. It was noted that, in the lymph nodes of genetic responder guinea pigs immunized with DNP-PLL·BSA, anti-DNP antibody-producing cells were seen very early, at 7–8 days, at which time few if any anti-BSA-producing cells could be seen. These latter cells appeared in large numbers at about 10–12 days. In genetic nonresponder animals immunized with DNP-PLL·BSA, neither anti-DNP-producing cells or anti-BSA-producing cells were seen at 7–8 days, but both types of cells were seen in large numbers at 10–12 days. The time sequence of the appearance of serum antibody to the dinitrophenyl hapten and to the conveyor molecules paralleled the observations made on the appearance of the lymph node cells producing these two respective antibodies.

DISCUSSION

Genetic nonresponder guinea pigs unable to show an immune response to DNP-PLL can nevertheless recognize this molecule as a hapten when immunized with DNP-PLL complexed with a foreign albumin (4). These animals then produce two types of antibodies, antibodies against the DNP-PLL hapten and antibodies against antigenic determinants of the conveyor molecule. These two types of antibodies have completely different specificities; the anti-DNP-PLL antibodies could not be shown to have carrier specificity for the conveyor albu-

min (4). In spite of their different specificities, the synthesis of these two antibodies is clearly linked by some initial event involving the recognition of the immunogenicity of the conveyor molecule, since these genetic nonresponder animals are incapable of forming antibodies to DNP-PLL when the conjugate is injected alone and do not give a delayed response to DNP-PLL (4). This is further illustrated by the observation that after immunization of nonresponder guinea pigs with DNP-PLL·BSA, large numbers of plasma cells containing anti-DNP-PLL antibodies or anti-BSA antibodies appeared at the same time, at about 12 days, in the lymph nodes draining the injection site, while in responder guinea pigs, plasma cells containing anti-DNP-PLL antibodies were seen 4 days before the appearance of plasma cells containing anti-BSA antibodies. In spite of the fact that some common initial event controls the synthesis of DNP-PLL and of anti-BSA or anti-OVA antibodies in genetic nonresponder animals immunized with DNP-PLL·BSA or DNP-PLL·OVA, the results of these experiments demonstrate that the antibodies against DNP-PLL and the antibodies against the specific determinants of the conveyor albumins are synthesized in different plasma cells.

If some initial recognition of immunogenicity of a carrier molecule, as earlier hypothesized, is required and precedes the formation of antibodies against the specific determinants it bears, these findings indicate that this first step appears to be unrelated to the specificities of the antibodies later produced. These experiments also illustrate that when a molecule with several antigenic determinants is presented to the immunological mechanism, the different determinants are at some time identified by different immunocompetent cells.

Although the hapten-PLL albumin complexes can be considered as a special and simpler model of hapten-protein conjugates, the same relationships between hapten and conveyor molecule for the induction of immunogenicity is undoubtedly common to both types of antigen.

These experiments confirm and extend the findings presented in the companion paper that each antibody-producing cell synthesizes an antibody of only a single specificity, and probably only a single homogeneous immunoglobulin (14). This occurs even when the antibody-producing mechanism is presented with antigens with several determinants and when the response to these different determinants is linked, as is the case of DNP-PLL·BSA or DNP-PLL·OVA. All these results contrast sharply with those of Attardi et al. who observed that as many as 20% of antibody-producing cells in rabbits immunized with two different phages produced antibodies against antigenic determinants of both phages (19). If technical error is excluded in their experiments (20, 21, 22), the differences observed must depend upon the experimental conditions of both studies. The only notable difference between the design of our experiments and of those of Attardi et al. consists in the time after immunization when the antibody-producing cells were examined. In our studies, the experi-

ments were carried out up to 1 month after immunization, while Attardi et al. studied the cells of rabbits 6 months to 2 yr after repeated immunization. The appearance of cells producing two antibodies with different specificities on the basis of prolonged immunization can only be explained by the fact that very few cells were initially capable of producing antibodies against the two specificities studied, and that the cells producing these two immunoglobulins possessed a very distinct selective advantage of being able to proliferate over the cells producing only antibodies of a single specificity. This selective advantage would in time cause them to be present in large numbers. However, in Nossal's studies the duration and number of immunizations did not increase the number of cells producing antibodies against two separate antigens (23). Although there is no known mechanism in the dynamics of antibody synthesis to explain such a selective advantage, experiments with hapten-protein conjugates, of the type which have been done in these studies, should be repeated to resolve this problem very late in the course of immunization, after 6 months or 1 yr.

One may safely state on the basis of the evidence presented in this and the companion paper (14) that the production of two antibodies by a single plasma cell up to 1 month after immunization is at best an extremely rare event since it was not observed in a single cell out of 1569 cells examined in animals immunized with hapten-protein antigens or hapten-PLL albumin complex.

SUMMARY

Genetic nonresponder guinea pigs incapable of an immune response to DNP-PLL alone were immunized with DNP-PLL complexed to ovalbumin or bovine serum albumin. Under these circumstances the animals produce both anti-DNP-PLL antibodies and antibodies directed against the conveyor albumin. Thus immune response to DNP-PLL complexed to conveyor albumin molecules can serve as a simple model of hapten-carrier relationships. To explore these relationships the question whether these two types of antibody are synthesized by the same or by different plasma cells was investigated by a combination of a double immunofluorescent technique and radioautographic localization of radioactive antigen.

It was shown that the anti-DNP-PLL antibodies and the antibodies against the carrier albumin molecule were produced in separate cells. No cell-producing antibodies with both specificities were detected out of 526 cells studied.

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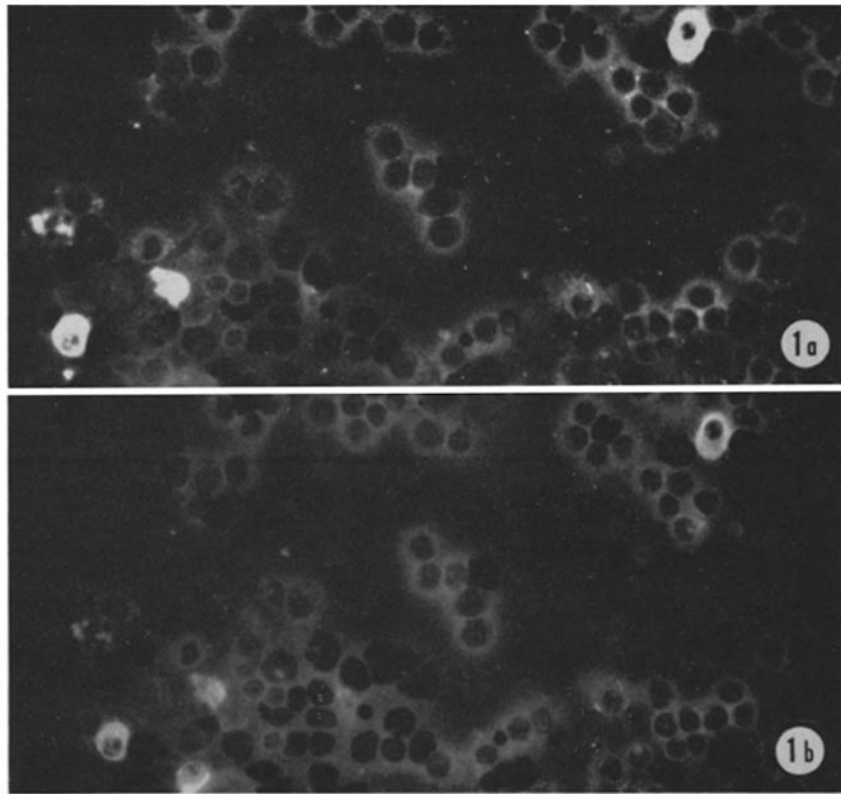
EXPLANATION OF PLATES

PLATE 68

FIG. 1. Lymph node smears of guinea pig 20-14 immunized with DNP-PLL·BSA.

In FIG. 1 *a* the smear has been stained only with rhodamine-labeled anti-DNP antibody. The two brightly stained cells in the upper right and lower left corner are anti-DNP-producing cells. The brightly stained object near the cell in the lower left corner is an artifact.

In FIG. 1 *b* the same smear has been subsequently stained with fluorescein-labeled anti-BSA antibody. The newly stained cell appears at the lower edge of the photograph, below and to the right of the cell previously seen in the lower left corner of FIG. 1 *a*. This new cell is a green anti-BSA-producing cell. The orange cells did not change in color.



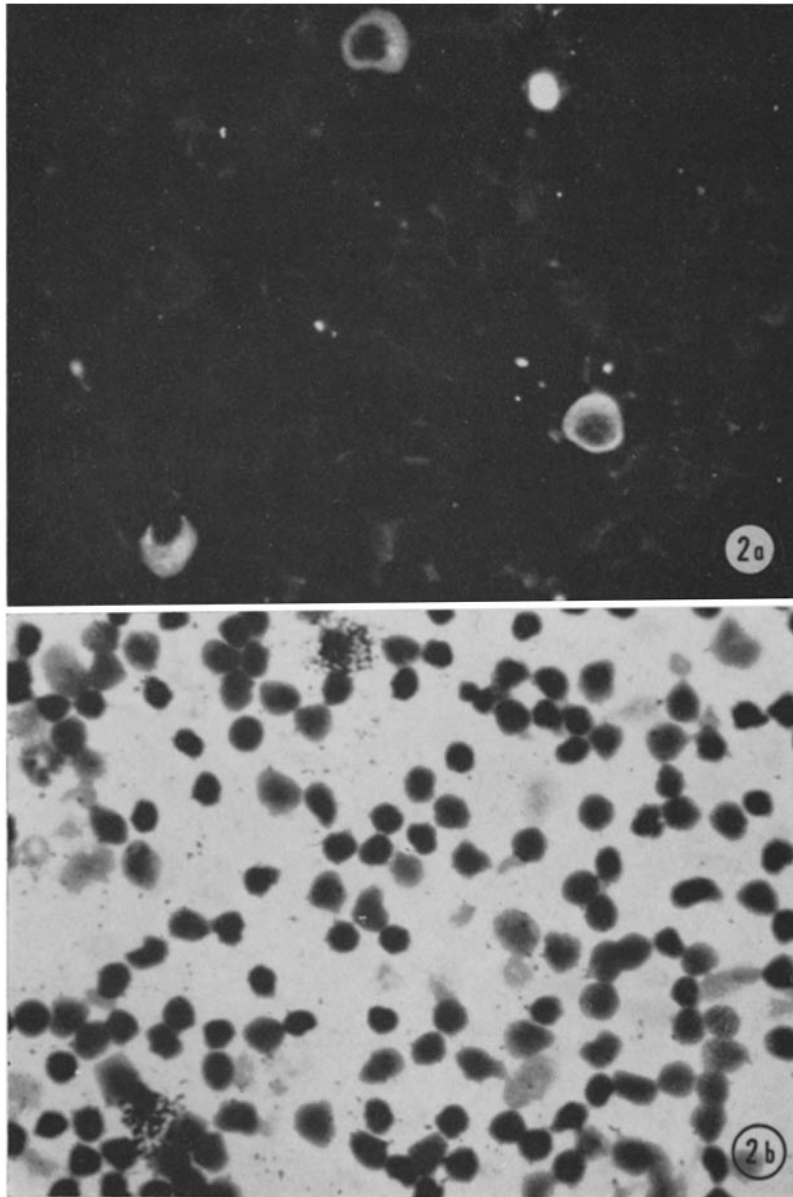
(Green et al.: Cellular localization of antibodies)

PLATE 69

FIG. 2. Lymph node smear from guinea pig 19-53 immunized with DNP-PLL·BSA· The smear has been stained for both anti-DNP- and anti-BSA-producing cells; however, in this instance the detecting antigen BSA used in the middle layer was tagged with ^{125}I .

In Fig. 2 *a* three stained cells are seen, the two in the left portion of the field were stained green indicating that they were producing anti-BSA antibodies. The single cell in the right portion of the field was stained orange indicating the presence of anti-DNP antibody.

In Fig. 2 *b* the same smear was processed for radioautography and stained with Giemsa. In the same field as Fig. 2 *a* it is seen that the two cells identified as having BSA specificity have many grains localized over them indicating the presence of bound BSA- ^{125}I . In contrast the cell on the right side of the field, containing anti-DNP antibodies, has no grains over it above background.



(Green et al.: Cellular localization of antibodies)