INTRACELLULAR LOCALIZATION AND QUANTITATION OF TRITIATED ANTIGENS IN RETICULOENDOTHELIAL TISSUES OF MICE DURING SECONDARY AND HYPERIMMUNE RESPONSES*, ‡

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Plates 54 to 56

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A vast literature exists concerning the mechanism of antibody synthesis in which various cell types, including the plasma cell, the macrophage, and the lymphocyte have been described as antibody-synthesizing cells (1–4). While investigators have provided supportive data involving all 3 cell types in antibody synthesis, much less information has been accumulated on the role of antigen in the immune response. Early studies on the distribution and storage of colored azoprotein antigens in tissues of injected animals repeatedly demonstrated these antigens in the reticuloendothelial cells of the liver, spleen, lymph nodes, and bone marrow (5–12). Gitlin *et al.* (11) provided inconclusive, but suggestive, evidence that acriflavine azoprotein antigens, which were microscopically localized in the cytoplasm of liver Kupffer cells, were associated with the mitochondrial fraction of these cells.

More recently, isotopically labeled protein antigens have been used to study the disappearance rate of complete antigens or haptens from the circulation, following their injection into immunized and non-immunized animals (13–15). S^{35}-, C^{14}-, and I^{131}- labeled antigens have been employed in attempts to study localization of antigens, using radiochemical techniques to measure tissue-bound antigen (16–22) and autoradiography to study intracellular antigen localization (22, 23). The localization of I^{131}-labeled antigens in autoradiographs of tissue sections or tissue touch preparations is complicated by the high energy of the emitted β-particles which causes a wide scatter of the exposed silver grains. Better results are obtained with C^{14}- or S^{35}-labeled antigens with which a resolution of 2 μ can be attained. The radiation emitted by tritium is much softer than that originating from C^{14} or S^{35}. Since the tritium β-particle has an average range of only 1 μ (24), tritiated antigens have a resolution of less than 1 μ in autoradiographic studies.

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In the present investigation, H^3–aniline monohydrochloride and H^3–arsanilic acid were diazotized and coupled to protein, to form heavily substituted antigens. The intracellular localization of these H^3-antigens in cells of spleen, liver, lung, and lymph node was studied with autoradiography techniques. The autoradiographic findings were correlated to radiochemical measurements of tritium in the tissue sections using the windowless flow counter and 2 methods of scintillation counting. The *surface activities* of the intact tissue sections and the total activities of dissolved tissue sections were determined. An attempt was made to correlate sites of H^3-antigen deposition as determined with autoradiography, to sites of antibody synthesis, detected with fluorescence microscopy. An approximate proportional value was derived between the counts measured with the windowless flow counter and the grains exposed in an autoradiograph of each tissue section per unit time.

Materials and Methods

Preparation of Tritium-Labeled Aniline Azo Porcine Gamma Globulin (PGG).—In preparation of the heavily substituted antigen, 14.8 mg of aniline-H^3 monohydrochloride (New England Nuclear Corp., Boston) were diazotized and coupled to 0.1 gm of PGG at 2°C. The H^3-antigen was incubated at 2°C for 30 minutes to allow completion of the coupling reaction. The resulting H^3-aniline azo PGG was then dialyzed against cold distilled water (5°C) for 48 hours to remove residual dye, after which the isotonicity of the H^3-antigen was restored with NaCl. The final concentration of the H^3-antigen was 6.7 mg/ml.

A windowless gas flow counter was employed to determine the activities of infinitely thin preparations of H^3-antigen dilutions. Counting efficiency of the instrument was determined to be roughly 38 per cent, as described previously (25). 0.1 ml aliquots of a $1:2 \times 10^4$ dilution of H^3-aniline azo PGG averaged 380 counts per minute (CPM). Corrected for efficiency, this would yield 2.0×10^8 disintegrations per minute (DPM) per ml of undiluted H^3-antigen. The total 15.0 ml H^3-antigen preparation would give approximately 3.0×10^9 DPM, representing 1.36 mc of H^3/0.1 gm PGG.

Preparation of Tritium-Labeled Arsanilazo Porcine Gamma Globulin.—Arsanilic acid (111 mg) was tritiated by Tracerlab, Inc., Lyndhurst, New York, by exposure to tritium gas for 75.4 Curie-days (26). Approximately 28 mg of H^3-arsanilic acid were diazotized and coupled to 0.2 gm PGG, to yield a heavily substituted antigen. Residual dye was removed from the H^3-antigen preparation by dialysis against phosphate buffered saline (PBS), pH 7.2, for 24 hours. Final concentration of the 28 ml of H^3-arsanilazo PGG was 7 mg/ml.

The tritium content of the H^3-arsanilazo PGG was determined with the windowless gas flow counter in the manner described above for the H^3-aniline azo PGG. 0.1 ml of a $1:2 \times 10^3$ dilution of H^3-arsanilazo PGG yielded 756 CPM. Corrected for 38 per cent efficiency, 28.0 ml of undilute H^3-antigen would yield 1.1×10^9 DPM, equaling 0.5 mc/0.2 gm PGG.

Non-Radioactive Antigen Preparations.—Non-labeled aniline azo PGG and arsanilazo PGG were prepared for rabbit and control mouse immunizations, following the techniques and quantities described above for the tritiated antigens. These unlabeled antigens were used in their soluble forms and as alum-precipitated antigens (27).

Mouse Immunization.—Adult male white Swiss mice were employed throughout these investigations. Mice in group I received 4 intravenous injections of H^3-aniline azo PGG into the tail vein, 0.15 ml (13.6 μc) per injection, on days 1, 3, 6, and 8. These hyperimmunized mice thus received a total of 54.4 μc of H^3-antigen and were sacrificed 7 days following the final

injection. Mice in group II received the same quantities of H^3-aniline azo PGG in 4 subcutaneous injections on days 1, 3, 6, and 8, and were sacrificed on day 15. The mouse sera were collected and retained for titration of antibody against aniline azo PGG.

Mice in group III received intramuscular hip injections of 0.1 ml (0.7 mg) and subcutaneous foot-pad injections of 0.04 ml (0.28 mg) arsanilazo PGG on days 1, 3, and 6. Mice in group IV received intramuscular hip injections of 0.1 ml (0.7 mg) arsanilazo PGG and subcutaneous foot-pad injections of 0.04 ml (0.7 μc) H^3-arsanilazo PGG on days 1, 3, and 6. A secondary response was induced in each mouse of groups III and IV on day 35 by injecting 0.10 ml (1.8 μc) and 0.04 ml (0.7 μc) of H^3-arsanilazo PGG into the hip and foot-pad, respectively. All mice were sacrificed 72 hours after the final antigen injections, and their sera were retained for antibody determination.

Mouse Serum Antibody Titrations.—Mouse serum antibody titers against aniline azo PGG and arsanilazo PGG were determined using the Stavitsky (28) modification of the tannic acid hemagglutination technique (29). The erythrocytes employed in the hemagglutination test and the normal rabbit serum which was added in a concentration of 1:100 to the diluent (PBS, pH 7.2) for red cell stabilization were obtained from an uninoculated rabbit. The heavily substituted, unlabeled aniline azo PGG and arsanilazo PGG were used in a concentration of 1.0 mg/ml for erythrocyte sensitization. Following overnight absorption with rabbit erythrocytes, duplicate titrations were made of each antiserum, and the titers were indicated by the highest serum dilution to show complete agglutination of the sensitized cells.

Histological Techniques.—The tissues examined using autoradiography, histological stains, and fluorescence microscopy included spleen, liver, lung, and various lymph nodes (mesenteric, popliteal). These tissues were cut into blocks of up to 3 mm in thickness and fixed by the freeze-substitution technique (30). The small lymph nodes were fixed intact. After quick-freezing in an isopentane–liquid nitrogen bath (−170°C), the tissues were transferred to acetone or absolute ethanol-$HgCl_2$ solution at −70°C, and were fixed for 3 to 7 days. The tissues were then brought to room temperature, cleared in a paraffin solvent (benzene, chloroform), and vacuum-embedded in paraffin (mp 56°C). 5 μ sections of each tissue were individually placed on acid-clean slides, floated out and dried at 45°C, decerated in xylene, and rehydrated. The sections were coated with a thin layer of Kodak nuclear track type NTB3 emulsion, after which the emulsion was dried, and the slides were exposed over desiccant for various time intervals at 5°C.

All tissue sections were stained after development of the photographic emulsion. The methyl green–pyronine technique (30) was used to observe cell–ribonucleic acid concentrations, and the May-Grünwald–Giemsa stain was employed for general histological studies.

Antibody-synthesizing cells were detected with fluorescence microscopy of tissue touch preparations. These touch preparations were made by application of a freshly cut tissue surface to the glass slide and rapid fixation in acetone.

Preparation of Fluorescein-Labeled Anti-Arsanilazo PGG.—A rabbit received intramuscular injections of alum-precipitated arsanilazo PGG at weekly intervals for 3 weeks, 2.0 ml per week, 1.0 ml in each hip. The antiserum was collected 10 days following the final antigen injection, and the antibody titer was found to be $1:2 \times 10^4$ with the ring precipitin test.

The globulin fraction of the immune serum was precipitated by 50 per cent $(NH_4)_2SO_4$ saturation. Following dialysis against PBS (pH 7.2), the protein content of the purified globulin was determined with the Folin-Ciocalteu technique (31). The globulin was then conjugated with fluorescein isothiocyanate (32) and dialyzed to remove the excess dye. Before use, the labeled globulin was absorbed overnight with mouse liver powder (33). After absorption, the labeled globulin was cleared by centrifugation at 10,000 *G*.

Detection of cellular antibody specific for arsanilazo PGG was accomplished by the layer technique of Coons *et al.* (33). Slides containing tissue touch preparations were flooded with a

1:10 dilution of unlabeled arsanilazo PGG in PBS (pH 7.2) and incubated in a moist chamber for 30 minutes at 37°C. The slides were drained and washed through four 5 minute changes of PBS. They were then flooded with the fluorescent-labeled anti-arsanilazo PGG, incubated, washed, and mounted as described by Riggs *et al.* (34).

Tissue Radioactivity Determinations.—The tritium content of tissues from mice injected with H^3-aniline azo PGG or H^3-arsanilazo PGG was demonstrated with autoradiography and histological techniques as well as with counting procedures using the windowless gas flow counter and liquid scintillation counting systems. For windowless gas flow counting, 5 μ tissue sections were prepared on glass coverslips fitted into stainless steel planchets. The number of sections per coverslip depended upon the size of the tissue being sectioned. The paraffin was removed with xylene, and the tissue sections were rehydrated as described above. The planchets containing coverslips were dried *in vacuo* over a desiccant and were weighed before and after the sections were placed upon them, in order to determine the exact tissue weight. Tissue sections from uninoculated mice and empty planchet coverslips were carried through the entire procedure as controls for the radioactivity and weight determinations, respectively.

Since the tritium β-particles of average energy have a range of only 1 μ (24), only a fraction of the tritium in or near the surface of 5 μ sections would be detectable with either autoradiography or the windowless gas flow counter. Hence, 2 methods of liquid scintillation counting were employed to obtain a measurement of the total radioactivity present in the various tissue sections. The first of these 2 methods again measured only surface radioactivity. The same sections on coverslips counted above were fitted vertically into vials of low potassium glass to minimize background, immersed in scintillation solution composed of 4.0 gm of 2,5-diphenyloxazole (PPO) + 0.1 gm of 1,4-bis-2-(5-phenyloxazolyl)benzene (POPOP) per liter of toluene and counted in a Packard tri-carb liquid scintillation spectrometer at −5°C. Normal tissue sections and empty coverslips were included as controls which demonstrated no increase in background counts. The counting efficiency of the instrument was determined by adding a prepared H^3-standard (stearic acid-9, 10-H^3) in toluene of known activity to the scintillation solution. The instrument detected 13.8 per cent of the calculated number of disintegrations for the H^3-standard.

In order to determine the total radioactivity content of the tissue sections counted above, the fixed sections were dissolved from the coverslips by shaking for 24 to 96 hours in 3.0 ml of a 1:1 solution of hydroxide of hyamine 10X-absolute methanol (MeOH) at 50°C. After this treatment, the fixed tissues appeared to be completely dissolved, leaving no residue on the coverslips. Each solution was then added dropwise into a vial containing 10.0 ml of scintillation solution, swirling vigorously following addition of each drop. If this procedure was reversed, an immiscible mixture was obtained, and the sample was lost. The quenching effect of the MeOH-hyamine-hydroxide solution was determined by adding H^3-standard to vials containing the 3.0 ml of digestion solution and 10.0 ml of scintillation solution. These controls showed that the presence of the MeOH-hyamine-hydroxide reduced the counting efficiency to 3.6 per cent. The presence of dissolved non-radioactive tissue sections had no additional quenching effects.

EXPERIMENTAL RESULTS

Localization and Quantitation of H^3-Aniline Azo PGG.—

Mice in group I, which had been hyperimmunized with 4 intravenous injections of H^3-aniline azo PGG, totaling 54.4 μc per mouse, yielded specific serum antibody titers of 1:320 to 1:640 with the hemagglutination technique. Autoradiographs were prepared with 5 μ sections of spleen, lung, liver, and mesenteric lymph nodes. A 3 week period yielded optimum time for exposure of the photographic emulsion, after which the autoradiographs were developed, histologically stained, and examined for sites of grain localization.

Few grains were observed over sections of mesenteric lymph node. These exposed grains were extracellularly scattered throughout the lymph node sinuses. A few grains were localized over the cytoplasm of the phagocytic reticular cells or fixed macrophages, which help to form the walls of the sinuses. Grains were also scattered over the cytoplasm of cells found in the medullary cords and germinal centers. However, these germinal centers did not show enhanced development when compared with control nodes from uninoculated mice. Both mature and immature plasma cells showing enhanced pyroninophilia were seen in the nodes of immunized mice. While there was no strict correlation between grain localization and pyroninophilia, the scattered grain distribution over these hyperactive cells suggested the association of H^3-labeled material with enhanced synthesis of ribonucleic acid and protein (gamma globulin?). Autoradiography results also showed that little radioactive material was present in the liver cells. No exposed grains were noted over the hepatic cells, and very few grains could be detected over the undifferentiated lining cells of the hepatic sinusoids. However, there was an occasional Kupffer cell (fixed macrophage) which possessed dense cytoplasmic localization of radioactivity (Fig. 1).

Examination of spleen section autoradiographs revealed very few grains over cells of the lymphatic tissue forming the white pulp. There was no distinct concentration of radioactivity in any particular cell type. However, heavy concentration of grains was found over the cytoplasm of some fixed macrophages lining the venous sinuses which penetrate the red pulp. No grains were observed over the nuclei of these cells, but in many cases the grains were in close proximity to the nuclear membranes (Fig. 2).

The highest concentration of exposed grains was found over the lung sections. The primarily involved cells demonstrated no enhanced pyroninophilia. They were almost exclusively "septal cells" or fixed macrophages. The lung section in Fig. 3 shows the exposed grains clustered over the cytoplasm of these macrophages, while no grains appear over the nuclei. Background grains were absent in the cavities of the alveoli, but radioactive material was evident in free macrophages (alveolar phagocytes) found within the alveoli.

Mice in group II, which received 54.4 μc of H^3-aniline azo PGG subcutaneously, demonstrated hemagglutinin titers of 1:320 to 1:640, when reacted with the tanned-sensitized red cells. Autoradiography of the various mouse tissue sections showed no distinct differentiation in radioactivity localization from the histological preparations of the above intravenously immunized animals. Although the subcutaneous injections were administered in the peritoneal region of each mouse, enhanced H^3-antigen localization in the mesenteric lymph node cells was not observed. The extracellular radioactivity content was very high in spotted areas throughout the lymph node sections examined. These extracellular grains rendered intracellular localization of activity difficult, and thus it could not be concluded with certainty whether grains overlying lymph node cells represented intracellular, extracellular or membrane-fixed H^3-antigen. There were several small germinal centers of pyroninophilic cells evident with loosely scattered overlying grains. These cells were identified as immature lymphocytes or cells of the plasmacytic series.

As with the intravenously injected mice, autoradiography revealed only small amounts of radioactivity in cells of the liver. Background radioactivity was extremely

TABLE I

*Radioactivity of Mouse Tissues Following Intravenous or Subcutaneous Injections of H^3-Aniline Azo Porcine Gamma Globulin**

Injection route‡	Tissue	No. of sections§	Wt./section ± SD	CPM/section‖			DPM/section
				WF¶	SCI**	SCD‡‡	SCD‡‡
			μg				
Intravenous	Spleen	82	14 ± 13	1.5 (36)	1.1 (36)	1.1 (27)	31
"	Lung	76	11 ± 2	51.0 (25)	24.0 (25)	35.7 (25)	990
"	Liver	70	29 ± 6	3.1 (23)	1.2 (23)	1.2 (25)	34
"	Lymph node	89	15 ± 9	0.7 (30)	0.4 (30)	1.1 (28)	31
Subcutaneous	Spleen	66	11 ± 2	0.8 (44)	0.6 (44)	0.7 (22)	19
"	Lung	60	18 ± 12	63.0 (18)	30.0 (18)	41.6 (20)	1155
"	Liver	54	18 ± 8	1.2 (36)	0.4 (36)	0.2 (18)	7
"	Lymph node	35	4 ± 1	0.8 (23)	0.3 (23)	0.1 (12)	2

* The background counts for each of the three counting techniques employed were determined with 62, 71, 59, and 73 control tissue sections of spleen, lung, liver, and lymph node, respectively, from uninoculated mice. Since the size and antigen content of the different tissue sections varied considerably, the standard deviation (SD) from each recorded mean value usually was larger than the counting error. In sections with 10 or more CPM, the SD varied from 10 to 25 per cent; in sections with less than 2 CPM, the SD was ±1 CPM. Accordingly, the recorded values of less than 2 CPM indicate only the approximate activity of the sections. Their significance is based on the large number of sections counted. The low activities found in lymph node after subcutaneous injection are due in part to the low weight of the tissue sections.

‡ Mice in group I received 4 intravenous injections totaling 54.4 μc of H^3-aniline azo PGG per mouse. Mice in group II received the same amounts of H^3-antigen by subcutaneous injections.

§ Findings represent total number of sections of each tissue studied.

‖ The number in parenthesis after each value tabulated represents the number of tissue sections counted to obtain the CPM/section or DPM/section.

¶ WF, windowless flow counting of 5 μ sections on coverslips to determine surface activities. Values have been corrected for average background of 20 CPM.

** SCI, scintillation counting of intact sections on coverslips immersed in scintillation solution (see Materials and Methods). Values have been corrected for average background of 82 CPM.

‡‡ SCD, scintillation counting of whole tissue activity following the dissolution of sections in MeOH-hyamine-hydroxide. Values have been corrected for average background of 76 CPM. Total DPM/section were calculated from the CPM on the basis of the 3.6 per cent counting efficiency for this technique.

low, with no grains over the hepatic cells. Some liver Kupffer cells possessed cytoplasmically localized radioactivity. Autoradiographs of spleen sections showed a concentration of grains over scattered groups of macrophages or histiocytes in the red pulp. These grains were found exclusively over the cytoplasm of the cells. Extracellular, or background, radioactivity was again very low.

The greatest amount of radioactivity was found in the lungs of subcutaneously injected mice. Large numbers of grains were clustered over the cytoplasm of the fixed macrophages, and few grains were observed over the cell nuclei and in the alveolar cavities.

Quantitative determination of the radioactivity of tissue sections with the windowless flow counter and the 2 methods of scintillation counting (see Materials and Methods) supported the above autoradiographic results and more accurately determined amounts of tritium present in the 4 tissues studied. The specific activities of the spleen, lung, liver, and lymph node sections from mice injected intravenously (group I) or subcutaneously (group II) with H^3-antigen are presented in Table I. Scintillation counting of dissolved tissue sections (SCD) was the most accurate method for determination of tissue radioactivity, yielding CPM from which the quantity of H^3-antigen per unit weight of tissue section could be calculated. Windowless flow counting (WF) and scintillation counting of undissolved tissue sections on coverslips (SCI) measured only a fraction of the radioactivity present within 1 μ of the tissue section surfaces.

As shown in Table I, highest radioactivity was found in lung sections of both group I and group II mice. Calculations from the results of the scintillation counting of dissolved sections and from the activity determinations of the H^3-aniline azo PGG (see Materials and Methods) revealed that lung tissue from intravenously injected mice and subcutaneously injected mice contained radioactivity equivalent to 3×10^{-3} μg and 2×10^{-3} μg H^3-antigen per μg of dried tissue, respectively. Quantities of radioactivity in spleen sections from mice of groups I and II were equivalent to 71×10^{-6} μg and 54×10^{-6} μg H^3-antigen per μg of delipidated tissue. Lowest activities were found in liver and mesenteric lymph node sections. Liver sections from group I mice contained radioactivity equivalent to 37×10^{-6} μg H^3-antigen/μg tissue, and the mesenteric node sections, 66×10^{-6} μg H^3-antigen/μg tissue. These values for liver and mesenteric node sections of group II mice were 12 and 16×10^{-6} μg H^3-antigen/μg tissue, respectively. Thus, more H^3-antigen had been deposited in the liver and mesenteric nodes of intravenously injected mice than in comparable tissues of mice inoculated subcutaneously.

Tritium Localization and Sites of Antibody Synthesis Following Foot-Pad Injections of H^3-Arsanilazo PGG.—

Mice in group III had received intramuscular and foot-pad injections of unlabeled arsanilazo PGG to induce the primary response, and 2.5 μc of H^3-antigen subcutaneously in the foot-pad to induce the secondary response. Mice in group IV had received intramuscular injections of unlabeled arsanilazo PGG and subcutaneous foot-pad injections of H^3-arsanilazo PGG, totaling 4.6 μc of H^3 per mouse. The mouse tissues were examined 72 hours after secondary response stimulation. Sections of spleen, lung, liver, regional popliteal node, and contralateral popliteal node were prepared from each mouse for autoradiography and windowless flow counting. Also, tissue touch preparations were made from each tissue before fixation and embedding. The touch preparations were examined by the layer technique of fluorescence microscopy to detect sites of specific antibody formation.

Serum antibody titers of mice in groups III and IV were determined by the tannic acid method of hemagglutination and varied from 1:640 to 1:10,240.

Histological examination of regional popliteal node sections from mice of groups III and IV, using the methyl green–pyronine stain, revealed well developed germinal centers and clusters of deeply pyroninophilic mature and immature plasma cells. Mitotic figures were seen in some of the immature plasma cells. These regional nodes were greatly enlarged, being 5 to 8 times larger than the contralateral popliteal nodes. Enhanced formation of plasmacytes and well developed germinal centers were not observed in the contralateral nodes. Autoradiography revealed large numbers of grains overlying regional node sections from group IV mice, which had received several foot-pad injections of H^3-antigen. Both intracellular and extracellular grain localization was evident, with many grains clustered over the cytoplasm of plasmacytes in various stages of development (Fig. 4). Grains were also noted over the cytoplasm of histiocytes, but few lymphocytes appeared to be involved. Fewer grains were observed over regional node sections from mice of group III, which had received only one injection of H^3-antigen to stimulate the anamnestic response. Contralateral node sections revealed almost complete absence of grains in both groups of mice studied.

Results of autoradiography also showed that the spleen, lung, and liver sections from groups III and IV contained much less radioactivity than the corresponding regional popliteal node sections. Spleen and lung sections from group IV appeared to contain more tritium than comparable tissues of group III. As in the study with H^3-aniline azo PGG, splenic localization of grains was almost exclusively over the cytoplasm of histiocytes in the red pulp. Many pyroninophilic plasmacytes were present, and were widely scattered through each spleen section. These plasmacytes were single or grouped in small clusters, with no apparent grain localization above them. Radioactivity was present in the lungs of both mouse groups, with greater amounts in sections from group IV mice. The exposed grains were found in clusters over the lymphoid tissue in the lungs. Few grains were associated with the lung histiocytes. Liver sections demonstrated negligible activity, the number of grains being too few to determine any type of localization.

A more quantitative measure of radioactivity in the various tissues of mouse groups III and IV was accomplished by counting tissue sections on coverslips in the windowless flow counter. The results of this study appear in Table II. Greatest radioactivity was found in the regional popliteal node sections after stimulation of the secondary response with a single foot-pad injection of H^3-antigen (group III). Very low amounts of tritium were detected in spleen and lung sections. Regional lymph node sections from mice of group IV demonstrated approximately 3 times as much activity as regional nodes from group III. Also, greater quantities of tritium were found in spleen and lung sections from group IV mice. All contralateral node sections were non-radioactive.

The presence of antibody-synthesizing cells in the various tissues examined was revealed with the layer technique of fluorescence microscopy. Tissue touch preparations of the regional popliteal nodes from mice of groups III and IV contained large

clusters or clones of antibody-synthesizing cells, comparable to the groups of pyroninophilic cells shown in autoradiographs to be associated with large quantities of tritium. These cells had brightly fluorescing cytoplasm, but no intranuclear fluorescence. Cells synthesizing antibody were: (*a*) cells with large nuclei and small amounts of cytoplasm identified as immature lymphoid cells or plasmacytes (Fig. 5), and (*b*) cells with small, non-fluorescent, eccentrically located nuclei and large amounts of cytoplasm, identified as mature plasmacytes. Groups of antibody-synthesizing cells contained non-fluorescent cell types, identified as histiocytes, with large,

TABLE II

*Radioactivity of Mouse Tissues Following Single or Multiple Foot-Pad Injections of H^3-Arsanilazo Porcine Gamma Globulin**

H^3 injected μc‡	Tissue	No. of sections§	CPM/section‖
2.5	Spleen	48	0.5
2.5	Lung	89	0.5
2.5	Regional node	16	2.0
2.5	Contralateral node	43	0
4.6	Spleen	75	3.0
4.6	Lung	90	2.8
4.6	Regional node	35	5.2
4.6	Contralateral node	31	0

* The 5 μ tissue sections in coverslip planchets were counted in the windowless flow counter. Each section which produced less than 2 CPM above background was counted for 4 to 6 hours. Thus, the total counting time of spleen sections from mice which received 2.5 μc H^3 was approximately 288 hours. The counting error and the standard deviation were given previously (Table I, footnote*).

‡ Total activity of the H^3-arsanilazo PGG received per mouse.

§ Findings indicate total number of sections counted.

‖ The values tabulated have been corrected for the average background counts obtained by counting 53 spleen sections and 72 lung sections from an uninoculated control mouse.

folded nuclei and large amounts of cytoplasm (Fig. 5). Touch preparations (imprints) from these lymph nodes treated only with the fluorescent-labeled anti-arsanilazo PGG, without previous incubation with the specific antigen, showed no positive fluorescence. Imprints from the contralateral popliteal nodes and from control nodes of uninoculated mice also showed no positive results with the fluorescence layer technique. These control results indicated the above findings to be specific.

Fluorescence observations of splenic touch preparations from group III and group IV mice revealed an occasional group of 4 to 5 antibody-synthesizing cells, possessing intracytoplasmic fluorescence. No intranuclear involvement was observed. As in the regional popliteal nodes, these cells resembled both mature and immature lymphoid cells, or plasmacytes. An interesting observation of these splenic preparations was the presence of an occasional Russell body plasma cell involved in antibody synthesis. These cells were enlarged and had the typical appearance of mature plasmacytes. The

nucleus was in an eccentric position, and the large amount of cytoplasm was filled with brightly fluorescing globules or Russell bodies (Fig. 6). No Russell body plasma cells were observed in the popliteal nodes or in the lungs.

No antibody-synthesizing cells were shown to be present in lung preparations. However, very few cell types, other than erythrocytes, were obtained in these imprints. Since clusters of lymphoid cells were found in lung sections, but very few in the lung

TABLE III

A Quantitative Comparison of Autoradiography and Windowless Flow Counting in the Detection of Tritium in Mouse Tissue Sections

Mouse group*	Tissue	Section No.‡	Grains/section/36 days§	Grains/CS/min.‖	CPM/CS¶	Counts/grain**
III	Spleen	1	9242	0.2	4	19
		2	1800			
		3	0			
III	Regional popliteal node	1	18738	0.6	11	18
		2	8377			
		3	4342			
IV	Spleen	1	25854	1.2	24	20
		2	7466			
		3	29560			
IV	Lung	1	31971	1.0	23	23
		2	4164			
		3	15892			
IV	Regional popliteal node	1	60613	2.7	52	19
		2	50968			
		3	26790			

* Mouse group III received 2.5 μc H^3-arsanilazo PGG and group IV, 4.6 μc of the same antigen.

‡ 3 sections of each tissue were placed on one coverslip, counted, and studied with autoradiography.

§ The number of grains over each section were microscopically counted at ×1000. The wide variation in numbers of grains per section indicates the irregular distribution of H^3-antigen throughout the tissue sections taken from different areas of each organ. This does not cause any error in the resulting ratio of counts to grains since the same sections were employed for autoradiography and windowless flow counting.

‖ Findings represent the total number of grains per coverslip (CS) after subtracting the per cent of background grains, which were averaged from autoradiographs of control tissue sections from uninoculated mice.

¶ Findings represent the CPM/coverslip in a windowless gas flow counter after correction for background counts.

** Since the half-life of H^3 is approximately 12 years, the radiation emitted by the sections would remain almost constant throughout this investigation.

imprints, the possible involvement of these cells in antibody synthesis should not be disregarded. No antibody-synthesizing cells were found in liver imprints, which were also predominantly composed of red cells.

Quantitative Comparisons of Grains on Autoradiographs to Counts Measured by Windowless Flow Counting per Unit Time.—

Spleen, lung, and right popliteal node sections from mice in groups III and IV were employed for this study. Three 5 μ sections of each tissue were placed on coverslips, dried, decerated, and rehydrated. The sections were then dried in a vacuum desiccator overnight and counted in a windowless flow counter. Tissue sections from uninoculated mice were used as normal controls.

After determination of the CPM/coverslip, the sections were coated with NTB3 photographic emulsion and exposed at 5°C for 36 days. After development of each autoradiograph, the tissue sections were stained with methyl green–pyronine, and the number of grains over each tissue section was microscopically counted at ×1000 (100 × oil immersion and 10 × eyepiece). Autoradiographs of control sections from uninoculated mice were used to determine the background grain formation. The grains over an average of 10 areas of each control section were counted, each area being 6400 μ^2. These counts were compared to the counts obtained over randomly selected equal areas of the above positive autoradiographs, and the per cent of background grain formation was thus determined.

The average per cent of background grains from 10 separate measurements with different tissue sections was calculated and subtracted from the total number of grains for each positive autoradiograph used in this study. The total number of grains formed over each tissue section after 36 days' exposure, and the number of grains exposed per minute for each coverslip of 3 sections are compared with the CPM per coverslip in Table III. The resulting ratio of CPM/grains was determined for the coverslips, and the average of these 5 calculated ratios was 19.76, or approximately 20.

DISCUSSION

Studies on the radiochemical quantitation and autoradiographic localization of H^3-antigens in tissues of immunized mice have been described in this investigation. Following intravenous and subcutaneous injections of heavily substituted H^3-aniline azo PGG into mice, we studied the intracellular localization and quantitative tissue distribution of tritium during the hyperimmune response. Use of the tritium label provided excellent intracellular localization of exposed grains in the emulsion covering the labeled cells. 7 days following the final H^3-antigen injections, autoradiographs detected intracytoplasmic localization of tritium in the phagocytic reticular cells of mesenteric lymph nodes, liver Kupffer cells, fixed macrophages of the splenic red pulp, and "septal cells" or fixed macrophages of the lung. Comparable results were obtained by Kruse and McMaster (9) who diazotized and coupled Evans blue with various serum proteins and egg albumin. After injecting the colored azo protein antigen intravenously into mice, Kruse and McMaster found intracytoplasmic antigen

localization in cells of the reticuloendothelial system, including liver Kupffer cells and phagocytic cells of mesenteric nodes. Similar observations were made by others who also studied the localization of colored azo protein antigens during an immune response (10–12). These investigators reported no intranuclear localization of the colored azo proteins. Intranuclear localization of antigen in reticuloendothelial cells and hepatic parenchymal cells, detectable with fluorescence microscopy techniques was reported by Coons *et al.* (35). Our present autoradiographic studies revealed the presence of tritiated antigen in the cytoplasm, but did not indicate intranuclear antigen localization, although grains were observed in very close proximity to the nuclear membranes of phagocytic cells, particularly histiocytes of the spleen and lung.

The intracytoplasmic localization of H^3-antigen observed here correlates with previous studies on antigen localization in fractions of tissue homogenates of spleen, liver, bone marrow, and lung (18, 19). These studies revealed that isotopically labeled proteins were concentrated in the mitochondrial fraction of liver and spleen. Both Garvey and Campbell (36) and Hawkins and Haurowitz (37) submitted evidence that intracellular S^{35}-labeled antigens were retained in a bound or degraded form, perhaps in association with ribonucleic acid. However, these studies on tissue homogenates did not reveal in which cell types the antigens were localized.

In our present investigations, the autoradiographic observations of H^3-aniline azo PGG-injected mice were substantiated using 3 radioactivity counting techniques. Surface radioactivities of the tissue sections were measured on the windowless gas flow counter. Also, 2 new techniques of scintillation counting of tissue sections have been described. The first scintillation counting method was again a measure of tissue section surface activities, in which the fixed sections on coverslips were placed vertically in vials, immersed in scintillation solution, and counted. From the results shown in Table I, it was concluded that this scintillation method of measuring surface activities is less sensitive than windowless flow counting. The second scintillation counting method measured total tissue activities, following dissolution of the tissue sections in MeOH-hyamine-hydroxide. These quantitative studies revealed highest activities to be present in lungs of both intravenously and subcutaneously injected mice, with less amounts in liver, spleen, and mesenteric lymph nodes. The present results contrast with the findings of Cheng *et al.* (22), who showed that mice injected intravenously with single larger doses of S^{35}-antigens had greatest activities in liver and spleen, with less amounts in lung, heart, blood, and kidney 5 and 10 days following injection. This difference could be due to variation in the amounts of injected antigen, and to differences between singly injected and multiply injected animals. Autoradiographs of mouse liver sections following intravenous injection of S^{35}-antigen revealed high concentrations of grains over the cytoplasm of Kupffer cells (22). Rabbits which had received subcutaneous foot-pad injections of S^{35}-sulfanilazo BGG (22) showed intra-

cytoplasmic localization of activity in macrophages and lymphoid cells of the regional popliteal node. These observations correlate well with our present findings.

Mice which received subcutaneous foot-pad injections totaling 4.6 μc of H^3-arsanilazo PGG (group IV) showed hyperactivity of the regional popliteal node. Clusters of pyroninophilic plasmacytes were observed in tissue sections, and autoradiography displayed intracellular and extracellular grain localizations, with many grains over the cytoplasm of plasma cells (especially immature cell forms) and macrophages. The autoradiographic results were compared to fluorescence microscopy observations of tissue impressions from these same popliteal nodes, in which the clumps of plasmacytes were shown to be synthesizing antibody specific for arsanilazo PGG. Although not conclusive, this evidence suggests that in this study, antigen was associated with the antibody-synthesizing cells. Tissue sections from group III and group IV mice were counted in the windowless flow counter, and greatest activities were found in the regional popliteal nodes, with less in spleen and lung, and little or no activity in the contralateral lymph nodes.

Our autoradiographic observations gave further support to the role of the macrophage in antigen uptake following either intravenous or subcutaneous H^3-antigen injections. This correlates well with the studies on *in vitro* antibody synthesis by Fishman (38, 39), who suggested that macrophages function in the transfer of antigen to antibody-synthesizing cells. He has also suggested that macrophages biochemically prepare the antigen in such a manner as to render it capable of stimulating the lymphoid cells to produce homologous antibody. Fishman (38, 39) derived this conclusion from his inability to induce antibody formation by adding antigen directly to lymphoid cells. However, after addition of antigen to macrophage cultures, macrophage cell-free homogenates stimulated specific antibody formation in recipient lymphoid cells. Some investigators have suggested that macrophages can be converted into antibody-synthesizing cells (40, 41). At present, our findings are compatible with either of these views.

In the present studies, an attempt was made to correlate the grains produced in autoradiographs to the counts determined with the windowless gas flow counter per 5 μ section in a unit time. The resulting ratio of counts/grain using spleen, lung, and popliteal node sections was found to be approximately 20. Since 3.5×10^{-1} μg of H^3-arsanilazo PGG yielded 756 CPM (see Materials and Methods), 20 CPM equaled approximately 9.3×10^{-3} μg of the radioactive antigen. Therefore, each exposed grain represented the minimum presence of 9.3×10^{-3} μg of H^3-antigen in the upper 1 μ of a tissue section surface.

SUMMARY

Autoradiography and quantitative radiochemical techniques have been used to determine intracellular localization of tritium and the quantity of

tissue-bound tritium, respectively, following injections of H^3-aniline azo PGG or H^3-arsanilazo PGG to yield hyperimmune or secondary response stimulation in mice. Autoradiography revealed intracytoplasmic localization of grains in macrophages of spleen and lung sections, and in Kupffer cells of liver sections following intravenous and subcutaneous injections of H^3-aniline azo PGG. Quantitation of tissue section surface radioactivities in the windowless flow counter and scintillation counter, and of dissolved tissue section activities in the scintillation counter, showed that greatest radioactivity was present in lung tissue, with less in spleen, liver, and mesenteric lymph nodes from these hyperimmunized mice.

Autoradiographic studies on tissue sections from mice in secondary response stimulation after subcutaneous foot-pad injections of H^3-arsanilazo PGG, showed intracellular and extracellular grains over regional popliteal node sections, with intracytoplasmic grain localization over macrophages and pyroninophilic plasmacytes. Scattered macrophages in spleen and lung sections also contained intracytoplasmic radioactivity. Clusters of antibody-synthesizing cells in the regional lymph nodes were demonstrated with fluorescence microscopy, and these cells were compared to similar cells possessing radioactivity as observed in the section autoradiographs. An occasional Russell body plasma cell containing specific antibody was observed in splenic impressions. Windowless flow counting showed that greatest radioactivity was in regional node sections, with less in spleen and lung, and none in contralateral lymph nodes.

A quantitative comparison between windowless flow counting and autoradiography revealed that 20 counts were required to yield one silver grain.

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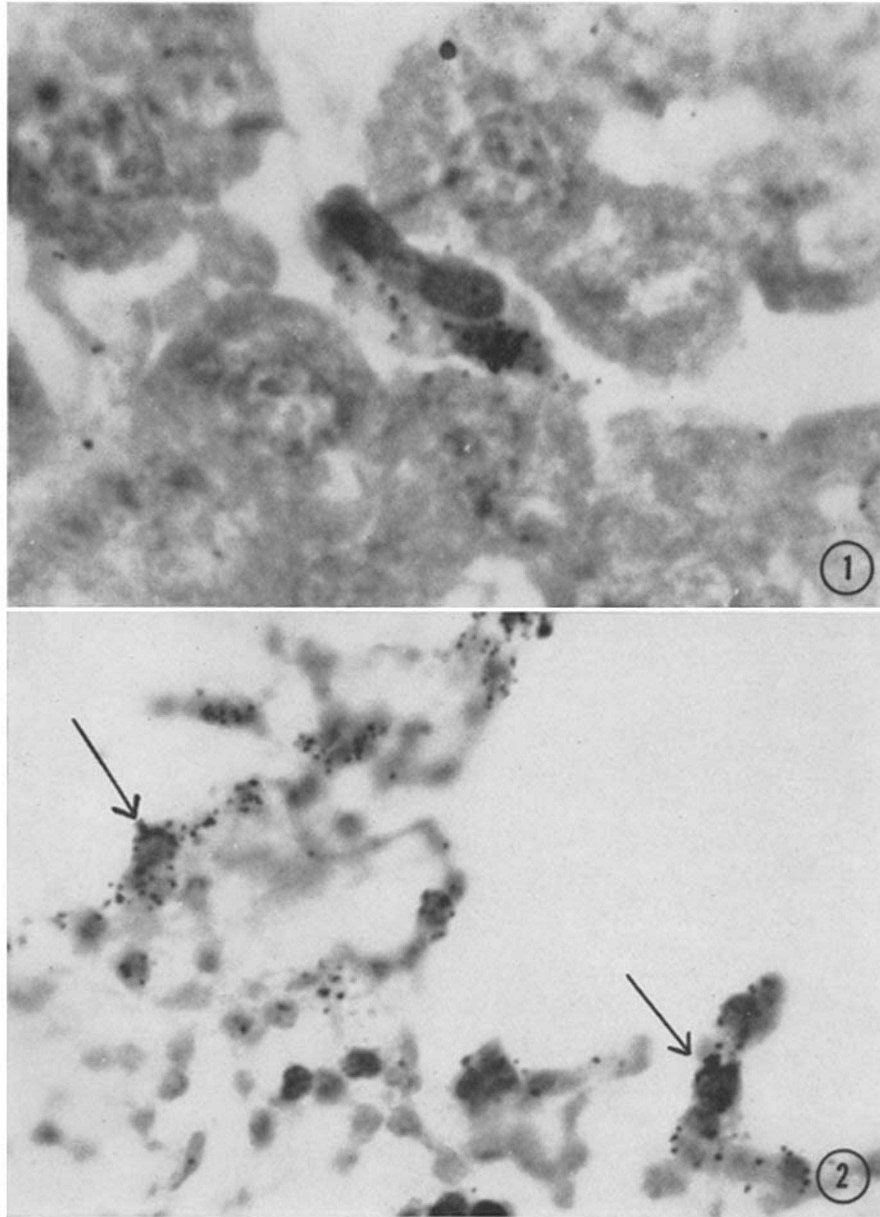
EXPLANATION OF PLATES

Plate 54

Fig. 1. Autoradiograph of liver Kupffer cell from mouse of group I, showing heavy intracytoplasmic localization of grains and stained with May-Grünwald–Giemsa. × 1250.

Fig. 2. Autoradiograph of spleen section from a group I mouse demonstrating the intracytoplasmic activity in macrophages with many grains in close proximity to the nuclear membranes (arrows). × 600.



(Roberts and Haurowitz: Localization and quantitation of H^3-antigens)

PLATE 55

FIG. 3. Autoradiograph of lung section which shows the intracytoplasmic accumulation of tritium in fixed macrophages. May-Grünwald–Giemsa stain. × 600.

FIG. 4. Autoradiograph of regional popliteal node section from a group IV mouse, showing intracellular and extracellular radioactivity. Arrows indicate the heavy accumulation of grains over strongly pyroninophilic cells of the plasma cell series. Methyl green–pyronine stain. × 1000.

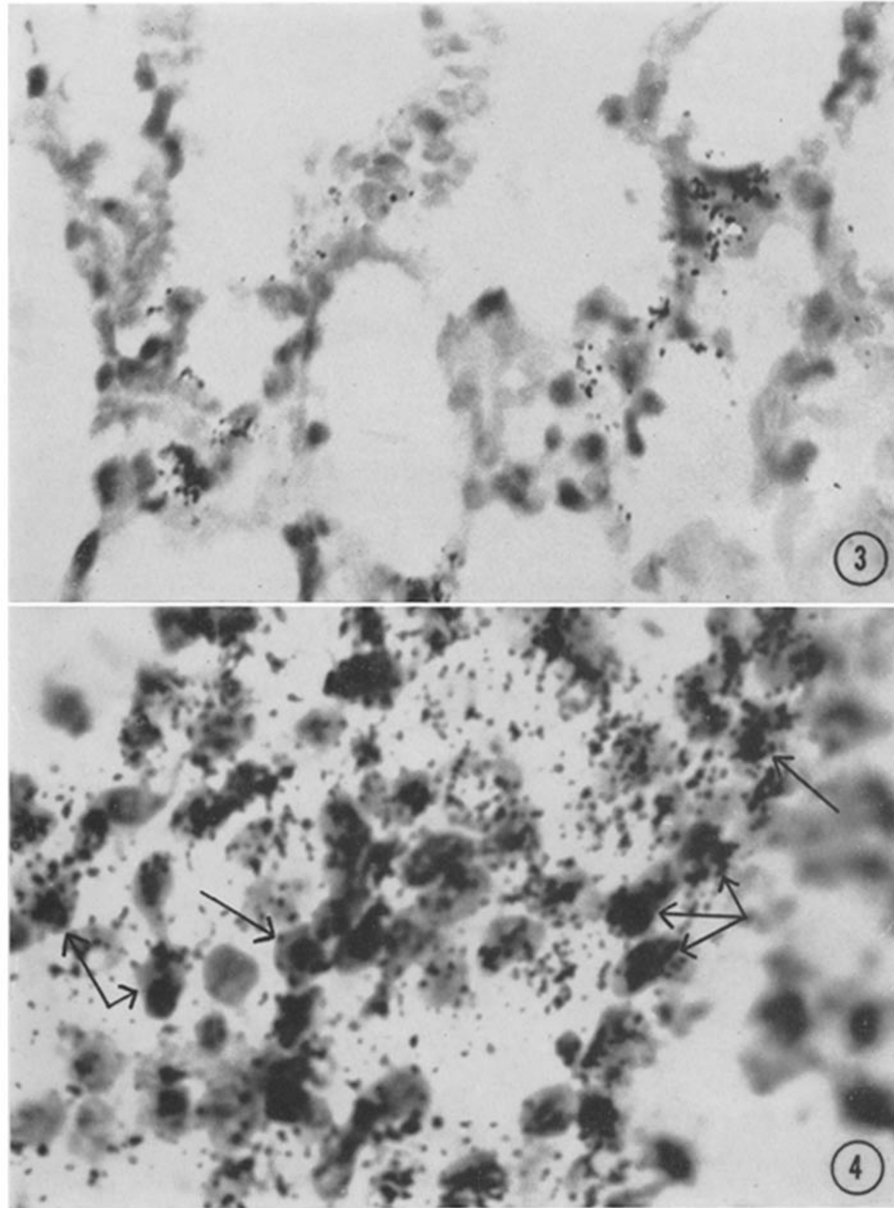


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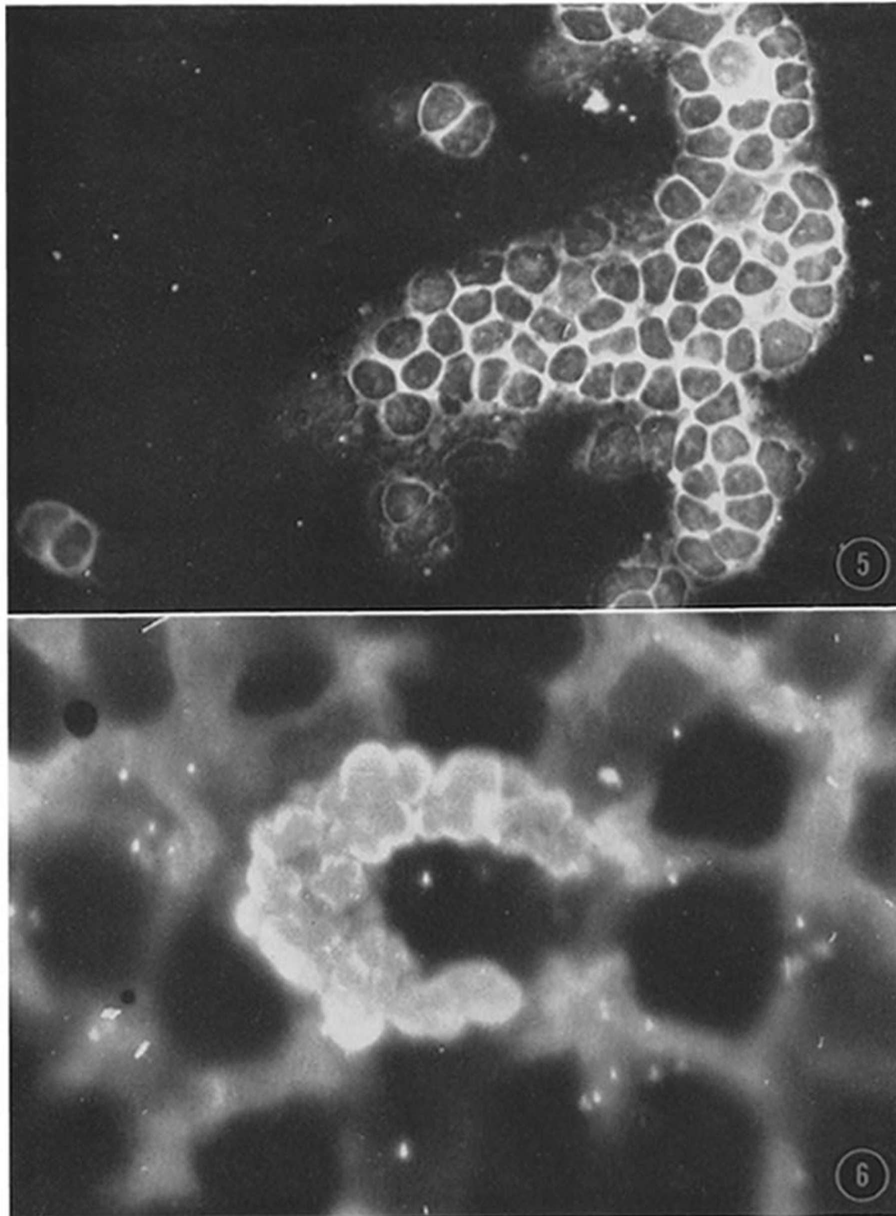
PLATE 56

FIG. 5. Photomicrograph of tissue touch preparation made from a regional popliteal node of a group IV mouse and treated with the layer technique of fluorescence microscopy to reveal specific antibody-producing cells. Note positive fluorescence in cytoplasm of immature plasmacytes and the absence of fluorescence in several visible macrophages. × 1000.

FIG. 6. Photomicrograph of Russell body plasma cell containing specific antibody and observed with the layer technique of fluorescence microscopy. The splenic impression was made from a group IV mouse. × 2700.



(Roberts and Haurowitz: Localization and quantitation of H^3-antigens)