CELLULAR SITES OF SYNTHESIS OF RABBIT IMMUNOGLOBULINS DURING PRIMARY RESPONSE TO DIPHTHERIA TOXOID-FREUND'S ADJUVANT*

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PLATE 49

(Received for publication, January 4, 1965)

The initial immunization of rabbits with many protein, bacterial, and bacteriophage antigens results in the synthesis of γM- and γG-globulin hemagglutinating, agglutinating, and phage-neutralizing antibodies (1, 2).[1] On the basis of a number of observations it was suggested (1, 2) that different cells synthesize γM- and γG-globulin antibodies.[2] Further immunofluorescent studies (4, 5) of the spleens of rabbits which had been injected intravenously with diphtheria toxoid and Freund's adjuvant provided evidence for the participation of two types of cells in the synthesis of these two molecular species of antibody. Non-phagocytic mononuclear cells in the walls of the sinusoids of the red pulp contained antidiphtheria toxoid during the time when only γM-hemagglutinating antidiphtheria toxoid was found in the serum of these animals. Later plasma cells in the non-follicular white pulp of the spleen contained antidiphtheria toxoid and the rabbits' sera had both γM- and γG-globulin hemagglutinating antidiphtheria toxoid. It was, therefore, tentatively concluded that the mononuclear cells produced γM-globulin antitoxin and the plasma cells synthesized γG-globulin antitoxoid.

The present experiments took advantage of the previous findings (4, 5) that the injection of diphtheria toxoid and Freund's adjuvant resulted in an in-

* Supported by Grants AM 07161 (MDS and RDM) and E-1865 (ABS) from the United States Public Health Service.

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[1] This nomenclature conforms to that recently proposed by a Committee which met at a World Health Organization meeting on Nomenclature of Human Immunoglobulins on May 29–30, 1964, in Prague (3). γM formerly was called γ1M, γ2M, 19S, or macroglobulin. γG formerly was designated γ, 7S γ, 6.6S γ, γ_2, or γSS.

[2] Preliminary reports of these experiments were presented to the IVth International Symposium on Reticuloendothelial System, Otsu-Kyoto, Japan, May–June, 1964 and to the International Symposium on Molecular and Cellular Basis of Antibody Formation, Prague, Czechoslovakia, June, 1964.

creased proliferation of cells usually associated with antibody synthesis; an accelerated, enhanced, and prolonged synthesis of antibody; and a lengthened interval between the appearance of γM- and γG-hemagglutinating antibodies in the circulation. The molecular species of antibodies that were synthesized by fragments of the spleen in culture were determined since antibodies in the serum probably represent a contribution of other organs in addition to the spleen. Incorporation of labeled amino acids into antibody by splenic fragments *in vitro* (6) was employed as a more rigorous criterion of active antibody synthesis by this organ than immunofluorescence, since the demonstration of antibody by the latter method does not necessarily reflect current synthesis of antibody.

Under the conditions of these experiments it appears that in the spleen nonphagocytic mononuclear cells are a major source of γM-globulins and antibodies and that plasma cells are a major source of γG-globulins and antibodies.

Materials and Methods

Antigens.—The following antigens were employed: Purogenated® soluble diphtheria toxoid and alum-precipitated diphtheria toxoid both containing 130 Lf units/ml, Lederle Laboratories, Pearl River, New York; egg albumin, 5 times crystallized, Pentex Inc., Kankakee, Illinois; diphtheria toxin, 5 times crystallized, containing 3000 to 3200 Lf units/mg protein N, courtesy of Dr. C. G. Pope, Wellcome Research Laboratories, Beckenham, England; complete Freund's adjuvant, containing 8.5 ml bayol F, 1.5 ml of arlacel and *Mycobacterium butyricum*, 5 mg/10 ml, Difco Laboratories, Inc., Detroit, Michigan; and a soluble antigen prepared by extracting a suspension of heat-killed, dried *Mycobacterium butyricum* with tris or phosphate buffer, pH 7.0 and ionic strength 0.15. The suspension was kept at 4°C for 1 week with intermittent stirring. It was then centrifuged and the supernatant was used.

Semipurified macroglobulins were prepared from normal rabbit serum by sequential precipitation with 12 and 18 per cent Na_2SO_4, followed by precipitation of the euglobulins by dialysis against distilled water. The euglobulin fractions, either with or without multiple recycling and selection of heavier components by ultracentrifugation, were purified further by one or more cycles of filtration through sephadex G-200. Details of the fractionation scheme will be published (7). Rabbit globulins were prepared by precipitation from normal rabbit serum at 50 per cent saturation with $(NH_4)_2SO_4$. Rabbit γG-globulins were purified from normal rabbit serum by chromatography on DEAE cellulose (8). Cohn fractions II and III from rabbit serum were purchased from Pentex Inc.

Antisera.—Albino rabbits of both sexes, weighing 2.5 to 3.0 kg, were maintained on a standard laboratory diet and tap water. After immunization periodic bleedings were made from the marginal ear vein and final bleedings by cardiac puncture. Serum was removed from the clotted blood and refrigerated.

Rabbit antidiphtheria toxoid was prepared by repeated injections of 40 to 80 Lf units of alum-precipitated diphtheria toxoid without Freund's adjuvant into the hindfoot-pads and intravenously. Rabbit antiegg albumin sera were prepared by repeated intravenous injections of 1 to 3 mg of alum-precipitated egg albumin. Rabbit anti-*Mycobacterium butyricum* sera were made by repeated intravenous injections of complete Freund's adjuvant. Sheep anti-rabbit γ-globulin, sheep and goat anti-rabbit γG-, and goat anti-rabbit γM-globulin sera were prepared by repeated subcutaneous injections of the antigens in complete Freund's adjuvant.

The globulin fractions of the rabbit antitoxoid and anti-mycobacterial sera were prepared by precipitation at 50 per cent saturation of ammonium sulfate. The crude antiserum to rabbit

γM-globulins was absorbed with rabbit Cohn Fr. III and chromatographically purified rabbit γG-globulin. Antibodies to other globulins were then absorbed by addition of rabbit Cohn Fr. II to the antiserum. By immunoelectrophoretic and Ouchterlony analyses the absorbed anti-γM-serum contained antibodies to γM-, γA-, and α-globulins, but not to γG-globulins. By the technique of radioimmunoelectrophoresis (9) a trace of anti-γG- and a moderate amount of anti-γA-like globulin antibodies were detected.

Antibody Assays.—Antibodies to diphtheria toxoid were determined by hemagglutination (10). Antibody titers to *M. butyricum* were determined by complement fixation as described previously (4). The active synthesis of antibodies to diphtheria toxoid was assayed by determining the extent of incorporation of C_{14} glycine into the antitoxoid found in the culture media (11). After removal of tissue and cells by low speed centrifugation sufficient egg albumin and rabbit antiegg albumin were added to yield about 500 μg of immune precipitate in order to coprecipitate non-specific radioactivity which would be removed by any antigen-antibody precipitate. The precipitate was removed by centrifugation and discarded. A second egg albumin–antiegg albumin precipitate was prepared in the supernate from the first precipitate to provide the background activity. Finally, a specific diphtheria toxin–antitoxin precipitate was prepared in the supernatant from the second non-specific precipitate to bind the antitoxoid in the medium. The second non-specific and the specific precipitates were separately washed, plated, and counted as previously described (11). The counts in the non-specific precipitate were subtracted from the counts associated with the specific precipitate. The resulting figure was used as an index of antitoxoid synthesis. Previous studies had showed that this figure is a reliable measure of the extent of *de novo* synthesis of antibody (12).

The antigen binding assay (13) employed the 5 times crystallized diphtheria toxin. The toxin was labeled with S_{35} by the method of Garvey and Campbell (14) starting with S_{35}-sulfanilic acid (Radiochemical Center, Amersham, England) and diazotizing the sulfanilic acid before coupling it to the toxin. The material which was tested for antibody was mixed with S_{35}-labeled toxin and incubated for 2 hours at 5°C. Sufficient sheep anti-rabbit γ-globulin serum was added to precipitate all of the rabbit γ-globulin in the reaction mixture. Incubation was carried out for 1 to 2 hours at 37°C, followed by storage for 1 to 2 days at 5°C to complete precipitation. The resulting precipitate was treated in the same manner as the immune precipitates in the incorporation assay for washing, plating, and counting. The controls consisted of labeled antigen mixed with normal rabbit serum, heterologous antiserum, or fractions of these sera isolated by centrifugation in a sucrose gradient. The resulting complex was isolated and treated in the same manner as the complexes resulting from mixing the specific antibody or gradient fractions. The control counts were subtracted as blanks from the counts obtained with the specific antisera or fractions.

Tissue Culture.—The spleens were cut into cubes about 2 mm^3 and 6 fragments were attached to 60 to 80 mesh stainless steel grids with sterile 0.8 per cent Ionagar (Colab Laboratories, Inc., Chicago Heights, Illinois). The grids were placed in 13 x 100 mm tubes containing 1 ml of Eagle's medium with 20 per cent rabbit serum and 1 μc of glycine-1-C_{14} (16 mc/mmole). Ten to 20 tubes were prepared from each spleen. The tubes were stoppered and spun on a roller drum at 37°C for 18 hours. The media from the cultures of each spleen were pooled, cleared by centrifugation, concentrated by pervaporation, and used for the various antibody assays.

Immunofluorescent Studies.—Cellular imprints and sections of fresh frozen tissue were fixed in acetone for 10 minutes at room temperature, dried, and stained by the following procedures. Total globulin was detected by the direct method (15) using sheep anti-rabbit γ-globulin conjugated with fluorescein isothiocyanate. Adjacent sections and imprints treated with unlabeled sheep anti-rabbit γ-globulin globulin were used as controls. The indirect method was used to identify specific antibody to diphtheria toxoid and to *M. butyricum*. Sections and cellular

imprints were flooded with soluble toxoid for 30 minutes, washed thoroughly with phosphate-saline buffer, and stained with fluorescein isothiocyanate–labeled rabbit antidiphtheria toxoid globulin with and without prior treatment of the specimens with unlabeled antibody. Additional sections and imprints were stained with the labeled antisera which had been absorbed with diphtheria toxoid. For detection of antibody to *M. butyricum* the sections and cellular imprints were flooded with the soluble *M. butyricum* antigen for 30 minutes, washed thoroughly with phosphate-saline buffer and stained with fluorescein isothiocyanate labeled rabbit anti-*M. butyricum* globulin with and without prior treatment with unlabeled antibody. Additional sections were stained with labeled antisera which had been absorbed with heat-killed desiccated *M. butyricum*.

The following additional immunofluorescent procedures were carried out on sections of spleen and cellular preparations from selected animals:

1. Treatment with goat anti-rabbit γM-globulin followed by soluble diphtheria toxoid and labeled rabbit antidiphtheria toxoid globulin.
2. Treatment with goat anti-rabbit γG-globulin followed by soluble diphtheria toxoid and labeled rabbit antidiphtheria toxoid globulin.
3. Treatment with goat anti-rabbit γ M-globulin, followed by soluble *M. butyricum* antigen and labeled rabbit anti-*M. butyricum* globulin.
4. Treatment with goat anti-γG-rabbit globulin, followed by soluble *M. butyricum* antigen and labeled rabbit anti-*M. butyricum* globulin.

It was expected that the interaction of the γM-intracellular antidiphtheria globulin with goat anti-rabbit γM-globulin would inhibit the reaction of the intracellular γM-antibody with diphtheria toxoid, but that treatment of γG-intracellular antibody with goat anti-rabbit γG-globulin would also specifically inhibit the reaction. Treatment of tissue containing anti-mycobacterial antibodies was also done with these reagents.

Histologic Preparations.—Portions of the spleens were fixed in 10 per cent neutral phosphate-buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Chromatography.—Sera were fractionated on DEAE cellulose columns using a concave pH gradient of decreasing pH and increasing molarity (8).

Density Gradient Ultracentrifugation.—The method of performing this procedure was described previously (2).

EXPERIMENTAL

Diphtheria toxoid and complete Freund's adjuvant was injected intravenously into rabbits. The adjuvant was injected first followed by the toxoid through the same needle. Rabbits were killed at 1, 5–8, 10, 11, 13 to 17 days. The rabbits were bled periodically *via* the marginal ear vein and at the time of killing by cardiac puncture to obtain sera for determination of circulating antibody to diphtheria toxoid and *M. butyricum*. The spleens were removed and samples used for tissue culture, immunofluorescence studies, and histologic examination. Of the 24 rabbits injected and studied, 9 were selected for intensive study. They were sacrificed on days 8 and 10 when only γM-hemagglutinating antibodies to diphtheria toxoid were found in the circulation, on day 13 when γM- and γG-antibodies were found, and at days 15 and 17 when γM-antibody to *M. butyricum* were also present, and γM-antibodies to toxoid were declining and γG-antibodies to the toxoid were increasing.

Characterization of Antibody Response to Diphtheria Toxoid.—

Nature of antibodies synthesized in vitro: Table I presents the C_{14} glycine incorporation and antigen-binding data obtained from the tissue culture media. The spleen from rabbit 1-3 did not produce antibody which was detectable by either method. Rabbits 2-0 and 2-1 synthesized labeled 19S antibody. Rabbit 1-4 produced labeled 7S and 19S antibody as well as labeled antibody activity which appeared in the uppermost fraction in a sucrose gradient. These findings in rabbit 1-4 were supported by the association of specific antigen-binding activity with each of the three sucrose density gradient fractions. Rabbits 3-1, 5-6, and 5-8 did not produce detectable labeled antibody, but did produce antigen-binding activity which was associated with each of the three ultracentrifugal fractions. Rabbits 5-1 and 5-7 synthesized only 7S labeled antibody.

TABLE I

Species of Antidiphtheria Toxoid Synthesized by Spleen in Vitro

Rabbit No.	Days*	Incorporation, toxoid/EA			Antigen binding		
		1–3	4–6	7–10	1–3	4–6	7–10
		CPM	CPM	CPM	CPM	CPM	CPM
1-3	8	—	—	—	—	—	—
2-0	8	59/80 = 0.74	67/107 = 0.62	308/80 = 3.85	x	x	x
1-4	11	45/20 = 2.25	64/14 = 4.65	48/14 = 3.4	26	99	2
2-1	11	38/60 = 0.58	53/80 = 0.66	308/82 = 3.75	x	x	x
3-1	13	—	—	—	98	119	98
5-1	15	18 40 = 0.45	51.5/22 = 2.35	24/37 = 0.65	x	x	x
5-6	17	—	—	—	37	146	84
5-7	17	20/22 = 0.91	36/21 = 1.74	16.5/48 = 0.34	x	x	x
5-8	17	—	—	—	23	49	20

—, negative; x, not done.

1-3, 4-6, 7-10 are tube numbers, 1 the top, 10 the bottom after sucrose density gradient ultracentrifugation: 1-3, <7S; 4-6, 7S; 7-10, 19S. Incorporation-toxoid/EA in which toxoid is CPM in specific toxoid-antitoxoid precipitate and EA is CPM in second non-specific egg albumin-antiegg albumin precipitate.

* Days, time after injection when spleen removed.

Types of cells which contain γ-globulin and diphtheria antitoxoid: As found in previous studies (4, 5) two main types of cells were associated with the synthesis of antitoxoid in these experiments. Figs. 1 *a* and 1 *b* illustrate the appearance of the non-phagocytic mononuclear cells in the walls of the sinusoids of the red pulp by optical and fluorescent microscopy. Figs. 2 *a* and 2 *b* illustrate the appearance of the plasma cells in the non-follicular white pulp by the same methods.

Table II summarizes the data on the type of cell which contains γ-globulin and diphtheria antitoxoid and also summarizes the data from Table I so that

TABLE II

Types of Antibody Synthesized by Spleen and Types of Cells Containing Rabbit γ-Globulins and Diphtheria Antitoxin

Culture medium				Immunofluorescence data			
					Antidiphtheria toxoid		
Rabbit No.	Days	Incorporation	Antigen binding assay	γ-Globulin cell types	Cell types	19S block	7S block
1-3	8	–	–	L++, P+	L*	L, complete P, none	L, none P, complete
2-0	8	19S	X	L++, P+	L*	L, partial P, none	L, none P, complete
1-4	11	7S, 19S	7S	L++, P+	L*	L, complete P, none	L, none P, complete
2-1	11	19S	X	L++, P+	L*	L, partial P, none	L, none P, complete
3-1	13	–	7S, 19S	L++++, P++	L++, P+	L, complete P, none	L, none P, partial
5-1	15	7S	X	L+++, P++++	P+++	P, none	L, none P, partial
5-6	17	–	7S, 19S	L++++, P+++	L+, P++	L, complete P, none	L, none P, partial
5-7	17	7S	X	L++++, P+++	L+, P++	L, complete P, none	L, none P, partial
5-8	17	–	7S, 19S	L++++, P+++	L+, P++	L, complete P, none	L, none P, partial

–, neg; X, not done; L, mononuclear cell; P, plasma cell; ++, moderate number; +++, larger number, etc.

γ-Globulin cell types, cells fluorescing when treated with fluorescein-labeled sheep anti-rabbit γ-globulin.

Cell types, cells treated with soluble diphtheria toxoid, then with labeled rabbit anti-diphtheria toxoid; 19S Block, cells layered with goat anti-γM-serum, then with soluble diphtheria toxoid and, finally, with fluorescein-labeled rabbit antidiphtheria toxoid; 7S Block, cells layered with goat anti-γG-globulin serum, then with soluble diphtheria toxoid, and, finally, with fluorescein-labeled rabbit antidiphtheria toxoid.

* An occasional fluorescent plasma cell was seen.

the molecular species of γ-globulins and antibodies that were being synthesized by splenic fragments at the time of removal of the spleen can be recognized.

Some correlations are apparent from the data: in rabbits 1-3, 2-0, 1-4, and 2-1 γ-globulins and antitoxoid were found almost exclusively in the non-phagocytic mononuclear cells in the walls of the sinusoids of the red pulp. Since rabbits 2-0 and 2-1 produced only 19S labeled antitoxoid, this species of antibody was being made predominantly in these mononuclear cells of the spleen. The blocking of the fluorescent label with the anti-γM-serum also supports this conclusion. Rabbit 1-4 produced both 7S and 19S labeled antibodies. This rabbit's spleen contained antitoxoid predominantly in mononuclear cells, and it is possible that this cell was synthesizing both types of antibody, but a few plasma cells in the non-follicular white pulp contained γ-globulin and anti-

TABLE III

Serological and Immunofluorescent Studies of Response to M. butyricum Antigen

Rabbit No.	Days	Serum titer	Molecular species of antibody in serum	Immunofluorescence data			
				γ-Globulin cell types	*M. butyricum* antibody cell types	19S block	7S block
1-3	8	1/8–1/16	*	L++, P+	L+	L, complete	L, none
2-0	8	1/8	*	L++, P+	L+	L, complete	L, none
1-4	11	1/16–1/24	*	L++, P+	L+	L, complete	L, none
2-1	11	1/24	*	L++, P+	L+	L, complete	L, none
3-1	13	1/32	19S	L++++, P++	L++, P±	L, partial P, none	L, none P, complete
5-1	15	1/64	19S	L+++, P++++	L+++, P+	L, complete P, none	L, none P, complete
5-6	17	1/64	19S	L++++, P+++	L+++, P+	L, complete P, none	L, none P, complete
5-7	17	1/32	19S	L++++, P+++	L+++, P+	L, complete P, none	L, none P, complete
5-8	17	1/64	19S	L++++, P+++	L+++, P+	L, complete P, none	L, none P, complete

* Titer in fractions too low to determine.

diphtheria toxoid. The spleen from rabbit 3-1 had both mononuclear and plasma cells which contained γ-globulin and antitoxoid and synthesized both 7S and 19S antitoxoid. Only the reaction of mononuclear cells with toxoid was blocked by the anti-γM-serum, again suggesting that these cells are a major source of this species of antibody. Rabbit 5-1 synthesized 7S antibody *in vitro*. While both mononuclear and plasma cells contained rabbit γ-globulins only plasma cells contained antitoxoid, consistent with the conclusion that the plasma cell was a major source of the 7S antibody. Rabbits 5-6 and 5-8 made both 7S and 19S antibodies and mononuclear and plasma cells contained antitoxoid, supporting the idea that two different cells are involved in the synthesis of the two antibodies. The spleen from rabbit 5-7 synthesized predominantly 7S antibody and antitoxoid was observed mainly in plasma cells.

Characterization of Antibody Response to M. butyricum Antigen.—The response of the spleen to this antigen was not studied in tissue culture. Table III summarizes the results of serological and immunofluorescent studies on the 9 rabbits. The serum data, based on complement fixation titrations, indicate, as reported previously (5) that the synthesis of 19S antibody to *M. butyricum* antigens starts later but is more prolonged than the synthesis of 19S antitoxoid. It is, therefore, interesting that anti-mycobacterial antibody is found exclusively in mononuclear cells for 11 days and predominantly in these cells but in an increasing number of plasma cells through day 17. It was also found that the immunofluorescence in these mononuclear cells was blocked by prior treatment of the sections with anti-γM-antibody. Later plasma cells contained anti-mycobacterial antibodies whose reactivity with antigen could be blocked by goat anti-γG-antibody, suggesting that these cells produced γG-antibody.

DISCUSSION

Employing two additional assays, incorporation of radioactive precursors into antibody and binding of radioactive antigen by antibody, additional evidence is presented for the sequential synthesis of 19S and 7S antibodies during the primary response of rabbit spleen to diphtheria toxoid and to antigen(s) from *mycobacterium butyricum*. There is little doubt that the 19S antibody is γM-globulin. That the 7S antibody in serum from these rabbits is largely, if not exclusively, γG-globulin is indicated by the results of extensive studies by electrophoresis and chromatography and by its resistance to reductive cleavage with mercaptoethanol (1, 2). Further studies will have to be conducted to exclude the presence of some γA-globulin and γA-antibody in the culture medium.

The significance of the finding of antibody activity associated with the upper fractions derived from density gradient ultracentrifugation (Table I) is not clear. This activity may be an artifact representing the contamination of these fractions with 7S γ-globulin. Should this activity be associated with globulin of

a molecular weight less than 7S it may be due to fragments of the γ-globulin molecule derived from the breakdown of the intact 7S or 19S antibody molecules. Alternatively, these fragments may be synthesized directly and represent half molecules of γ-globulin (16). Experiments to distinguish among these possibilities are being devised.

Further evidence is presented for the participation of at least two cytologically distinct cells in the synthesis of γM- and γG-globulins and antibodies. Under the conditions of these experiments, non-phagocytic mononuclear cells in the walls of the sinusoids of the red pulp of the spleen are predominantly associated with the synthesis of γM-globulins, γM-antidiphtheria toxoid and γM-anti-*M. butyricum* antibody. Plasma cells in the non-follicular white pulp of the spleen are associated predominantly with the synthesis of γG-globulins, γG-globulin antidiphtheria toxoid, and γG-anti-*M. butyricum* antibody.

The role of the mononuclear cells in the synthesis of γM-globulin antibodies is supported by the observations of few cells of this type in spleens and low hemagglutinating titers of circulating γM-antibody in rabbits which were given alum-precipitated diphtheria toxoid (4) and the large numbers of these cells and high titers of circulating γM-antidiphtheria toxoid when toxoid and complete Freund's adjuvant were given (4, 5). The correlations of circulating γM-antitoxoid, anti-*M. butyricum* antibody, and intracellular antitoxoid and anti-*M. butyricum* antibody with active synthesis of γM-antibodies (rabbits 1-3, 2-0, 2-1 in Tables I to III) provides even more direct evidence. Further support is added by the blocking of fluorescence of intracellular γM-antibody in the mononuclear cells but not within plasma cells with goat anti-rabbit γM-globulin.

The response of the plasma cells lagged behind that of the mononuclear cells. The majority of the plasma cells developed in the non-follicular white pulp. Some rabbit spleens (No. 5-1, Table II) synthesized only 7S antitoxoid *in vitro* and contained only plasma cells with intracellular antitoxoid. Rabbit 5-7 spleen produced only 7S antitoxoid and contained mainly plasma cells with intracellular antitoxoid. Moreover, the goat anti-γG-antibody blocked or attenuated the fluorescence of the plasma cells, but did not affect the fluorescence of the mononuclear cells.

It has been suggested previously that large "lymphocytic" mononuclear cells synthesize macroglobulins and macroglobulin antibodies (5, 17, 18). Similar cells have been found to contain macroglobulin in studies of patients with macroglobulinemia (19) and rheumatoid arthritis (20). Anti–typhoid O antibodies (21), presumably γM-globulins, have been demonstrated in the sera of rabbits 4 to 8 days after primary immunization, at which time only mononuclear cells have increased in lymphoid organs and no increase in plasma cells was noted. Rabbit antidiphtheria toxoid was found in mononuclear cells as early as 4 days after a single injection of alum-precipitated diphtheria toxoid

at which time it would be expected that only γM-globulin antibody was synthesized. In a human dysgammaglobulinemia (22) characterized by inability to synthesize 7S globulins and antibodies but a capacity to synthesize γM-globulins and antibodies mononuclear cells contained γM-globulins. Recently Zucker-Franklin *et al.* (23) reported the synthesis of γM-macroglobulin in cultures of lymph nodes rich in large lymphocytic cells of patients with Waldenström's macroglobulinemia.

It has been presumed that the large mononuclear-type cell is a precursor of the mature plasma cell (24). However, Cruchaud *et al.* (22) observing the synthesis of exclusively 19S antibody by the mononuclear cells in the absence of plasma cells suggested that at least some of the mononuclear cells are mature cell forms rather than precursors of plasma cells. In their recent study Nossal *et al.* (24) also found certain large cells which continue to make 19S antibody even during the secondary response. In the present study no obvious connections or transitional forms between the mononuclear cells and plasma cells were observed. The mononuclear cells were present in the walls of the sinusoids of the red pulp, while the plasma cells developed in a geographically separate region in the non-follicular white pulp and appeared to arise from or to replace the lymphocytes.

The present data do not exclude the possibilities that mononuclear cells also synthesize γG-globulins and antibodies (*cf.* rabbit 1-4, Table II), that plasma cells also synthesize γM-antibodies (*cf.* rabbits 3-1, 5-6, 5-8, Table II) and that occasionally the same cell synthesizes both antibodies. Further evidence has been provided by Nossal *et al.* (24) who studied the antibodies in cultures of single lymph node cells derived at various times during the primary response of rats to a flagellar antigen of *Salmonella adelaide*. The rat synthesized 19S and then 7S antibodies. Some of the cells produced 19S antibodies, some 7S and some cells, removed when the rats were producing both 19S and 7S antibodies, appeared to synthesize both antibodies *in vitro*. No clear-cut correlation between cell maturity or morphology and 19S or 7S antibody production was observed. They suggested that many cells go through a sequence whereby each forms first 19S and later 7S antibody. However, only 144 cells were observed. Moreover, the authors recognized the possibility that the type(s) of antibody present in each culture does not necessarily reflect the type(s) of antibody the cell is currently synthesizing.

The apparent discrepancies between the results of Nossal *et al.* (24) and those of the present study may be due to one or more of the differences between these studies, including the species immunized, antigenic preparations, use of adjuvant, antibody assays, and the organs studied. The use in the present study of complete Freund's adjuvant with its capacity to stimulate the proliferation of non-phagocytic mononuclear cells (4, 5) which are involved in γM-globulin synthesis may be especially significant.

Our data do not answer the question whether the 19S and 7S responses are interdependent; *i.e.*, whether the 19S response must precede the 7S antibody synthesis. The data of Nossal *et al.* (24) indicate that some 19S cells may be converted into 7S cells. However, in a recent study from the same laboratory (25) it was found that the 7S globulin antibody response alone may be obtained in rats immunized with monomeric flagellin from *Salmonella*, implying that the γM-response is not always prerequisite. Lymph node fragments from primarily immunized rabbits synthesized 19S antihemocyanin antibody *in vitro* and did not shift to the synthesis of 7S antibody during prolonged culture, whereas the lymph node left *in situ* did make this shift (26).

Considering all of the data, we hypothesize that the 19S and 7S antibody responses evolved independently with the development of at least two different cell types; the mononuclear cell with a capacity for 19S gobulin and antibody synthesis evolved first, followed by the development of plasma cells with the capability of producing 7S antibodies. The capacity for the simultaneous synthesis of 19S and 7S antibodies by single cells of either morphologic type and the conversion of some 19S cells into 7S-synthesizing cells may have been a parallel or subsequent development. Data provided by recent studies on the phylogeny and ontogeny of the immune response are in accord with this general hypothesis. Thus it has been found that the lamprey (27), the sheep fetus (28), neonatal rabbits (29), and human infant (30) synthesize 19S antibodies in the absence of plasma cells.

If the γG- and γM-globulin antibody responses are separable and largely independent, it would be predicted that the γM-globulin antibody response might not always precede the γG response, but that all permutations of the sequential synthesis of these two antibody species would be seen, depending upon various factors which influence each of these syntheses. Recent studies in this laboratory are consistent with these predictions (31). If borne out, these new findings would provide a more complex, comprehensive, and realistic picture of the relationship of these two syntheses than provided by previous studies (1, 2, 32) which often indicated a clear-cut $\gamma M \rightarrow \gamma G$ pattern during the primary antibody response.

Better understanding of the types and interrelations of cells which synthesize various immune globulins and different species of antibody may contribute to the elucidation of the mechanisms whereby adjuvants affect antibody synthesis, the pathways of conversion from one cell type to another, genetic and other regulatory controls of antibody synthesis, of immunological memory, and the phylogenetic and ontogenetic development of immunologic competence.

SUMMARY

The present studies are based on previous observations that the intravenous injection of diphtheria toxoid and complete Freund's adjuvant into rabbits

resulted in an increased proliferation of cells associated with antibody synthesis; an accelerated, enhanced, and prolonged synthesis of antibody; and a lengthened interval between the appearance of γM- and γG-hemagglutinating antibodies in the circulation. The molecular species of antibodies that were synthesized by fragments of the spleens were determined by the incorporation of labeled amino acid into antibody and by binding of radioactive antigen by antibody. These studies were paralleled by determination of the presence and type of antibody within the cell by immunofluorescence. Evidence was obtained that non-phagocytic mononuclear cells in the walls of the sinusoids of the red pulp of the spleen are a major source of 19S γM-antibody and plasma cells in the non-follicular white pulp are a major source of γG-antibody. The data did not exclude the synthesis of γG-antibodies by the mononuclear cells, the synthesis of γM-antibodies by the plasma cells, or the synthesis of both antibodies by an occasional cell of either morphology. It was hypothesized that the 19S and 7S antibody responses evolved independently with the development of at least two different cell types, a mononuclear cell with capacity for 19S immunoglobulin synthesis and a plasma cell with capacity for 7S immunoglobulin synthesis.

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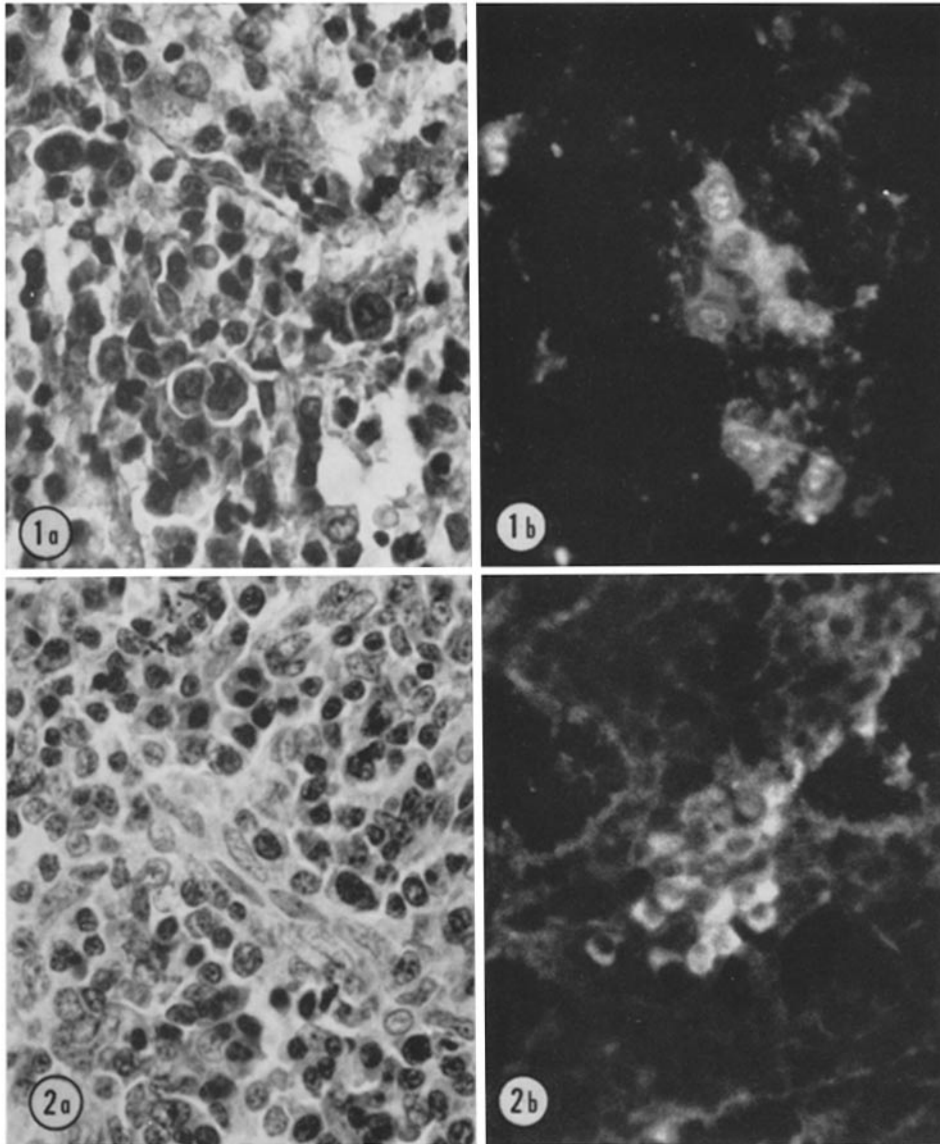
EXPLANATION OF PLATE 49

FIGS. 1 *a* and 1 *b*. Illustration of the appearance of the non-phagocytic mononuclear cells in the walls of the sinusoids of the red pulp of the spleen by optical (Fig. 1 *a*) and fluorescent (Fig. 1 *b*) microscopy. Fig. 1 *a*. Hematoxylin and eosin. × 640. Fig. 1 *b*. Indirect immunofluorescence for antidiphtheria toxoid. × 480.

FIGS. 2 *a* and 2 *b*. Illustration of the appearance of plasma cells in the non-follicular white pulp by optical (Fig. 2 *a*) and fluorescent (Fig. 2 *b*) microscopy. Fig. 2 *a*. Hematoxylin and eosin. × 640. Fig. 2 *b*. Indirect immunofluorescence for antidiphtheria toxoid. × 480.



(Schoenberg *et al.*: Cellular sites of antibody synthesis)