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THE CELLULAR RESPONSE OF THE SPLEEN AND ITS RELATIONSHIP TO THE CIRCULATING 19S AND 7S ANTIBODY IN THE ANTIGENICALLY STIMULATED RABBIT*

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IT has been reported that under appropriate conditions in a primary immunization to a given antigen, antibody of the γ_1 (19S) macroglobulin class appears first and that of the γ_2 7S type later (Stelos and Taliaferro, 1959; Bauer and Stavitsky, 1961; Benedict, Brown and Ayengar, 1962; LoSpalluto, Miller, Dorward and Fink, 1962; Fink, Miller, Dorward and LoSpalluto, 1962; Uhr and Finkelstein, 1963; Bauer, Mathies and Stavitsky, 1963). As the circulating titre of the 7S immune globulin rises, there is a concomitant fall in the 19S antibody titre. These findings suggest that different cells produce two different species of antibody or that the same cell produces 19S antibody first and then converts to the synthesis of the γ_2 7S antibody.

Although the general aspects of this phenomenon have been described in terms of the kind of antibody produced, there have been few reports that have considered this in relation to the histological and cytological responses that occur in the lymphoid tissues (Zucker-Franklin, Franklin and Cooper, 1962). The question of the production of different molecular species of antibody by different cells has recently been reviewed by Amano (1962). When complete Freund's adjuvant (water-in-oil emulsion of *Mycobacterium butyricum*) is injected intravenously, alone or in combination with other antigens, the response in the spleen and lymph nodes corresponds to a primary cellular reaction (Moore, Lamm, Lockman and Schoenberg, 1963) followed by an uninterrupted progression to the type of cellular change associated with a secondary response. Circulating antibody titres appear early, are generally elevated and prolonged. The intensity

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of the cellular reaction in the spleen and the type of cells that respond can be correlated with the time of appearance and the level of circulating antibody.

While these changes are continuous, there are certain patterns that suggest a relationship between the cellular events and the type of antibody produced. Using the Freund's adjuvant-diphtheria toxoid system, a study was made of the relationship between the type of circulating antibody and the type of cells that synthesize or perhaps store antibody.

MATERIALS AND METHODS

Albino rabbits weighing between 2·5 and 3·0 kg. were used. They were maintained on a standard laboratory diet with unrestricted access to tap water.

Complete Freund's adjuvant (Difco Products Co., Chicago, Ill.) (8·5 ml. Bayol F [Liquid Petrolatum USP], 1·5 ml. Arlacel and *Myco. butyricum* 5 mg./10 ml.) and both soluble and alum precipitated diphtheria toxoid (Lederle Laboratories Div., American Cyanamid Company, New York) were injected into the marginal ear vein. When given together the adjuvant was injected first followed by the toxoid through the same needle. The animals were bled at intervals to obtain sera for determinations of the antibody to *Myco. butyricum* and diphtheria toxoid.

The injection schedule and the time of killing of the rabbits were as follows :

1. Groups of four rabbits were killed after receiving a single injection, 1 ml. of adjuvant, on each of the following days after injection 1, 2, 3, 5, 7, 9, 11, 14, 18, 21, 28, 42, 56, 70 and 84.

2. Groups of six rabbits that received 1 ml. of adjuvant immediately followed by 0·5 ml. of diphtheria toxoid (80 Lf) were killed on days 1, 3, 5, 7, 9, 10, 11, 14, 21, 28 and 35 after injection.

3. Eight untreated rabbits were used as controls for the histological studies.

Antibody titres to *Myco. butyricum* were measured by complement fixation. Heat killed, desiccated *Myco. butyricum* were finely divided and suspended in either veronal or tris (triethanolamine) buffer at pH 7·2, ionic strength 0·15 to make a 1 per cent wt/v suspension. Since a 1 per cent suspension had some anticomplementary activity, the dilution of the suspension of *Myco. butyricum* used was 1/4 the concentration that was not anticomplementary toward 3 units of guinea-pig complement using a 100 per cent haemolytic end point. Almost uniformly a 1/16 dilution of the 1 per cent suspension was satisfactory. Antibody titres to *Myco. butyricum* were determined in the usual manner using a 50 per cent haemolytic end point. The antibody titres to diphtheria toxoid were measured by haemagglutination (Stavitsky, 1954).

Fractionation of the rabbit sera was done on DEAE cellulose columns using a concave pH gradient with tris-phosphate buffer. Antibody titres to *Myco. butyricum* were determined for each of the peaks and shoulders. The fractions were lyophilized and the nature of the proteins determined by filter paper electrophoresis, using veronal buffer, pH 8·6, ionic strength 0·05. Staining for protein and carbohydrate was by the Bromphenol blue (BPB) procedure and the periodic acid Schiff reaction (PAS) respectively.

The diphtheria toxoid-Freund's adjuvant system was studied by the sucrose density gradient centrifugation method, using a 10–40 per cent sucrose gradient (19 hr. in a swinging bucket rotor at 39,500 r.p.m. Spinco Model E).

At the appropriate time the animals were anaesthetized with sodium pentobarbital (Veterinary Nembutal, Abbot Laboratories, Chicago, Ill.) and the visceral organs removed. This report considers only studies on the spleen. Imprints of the cut surface of the spleens were made on glass slides and samples of tissue were frozen for the detection of γ-globulin and antibody by the immunofluorescent procedures.

Sheep anti-rabbit γ-globulin globulin conjugated with fluorescein isothiocyanate was used with and without prior treatment of the specimens with unconjugated antibody.

Since the sheep anti-rabbit γ-globulin globulin could not distinguish between the antibody to *Myco. butyricum* and diphtheria toxoid, another series of experiments was done in the following fashion. Sections from the spleens of animals that received adjuvant and diphtheria toxoid were incubated with soluble diphtheria toxoid for 1 hr. at room temperature, thoroughly washed in phosphate buffer, pH 7·2, ionic strength 0·15 and then layered

with fluorescein isothiocyanate conjugated rabbit anti-diphtheria toxoid γ-globulin obtained from hyperimmunized rabbits that had not received Freund's adjuvant.

The remainder of the spleens was fixed in 10 per cent neutral buffered formalin, embedded in paraffin, sectioned in the usual manner and stained with haematoxylin and eosin and with toluidine blue with and without ribonuclease digestion for the qualitative estimation of RNA content.

RESULTS

Antibody titres to Myco. butyricum

Low levels (1 : 2 to 1 : 4) of complement fixing circulating antibodies to *Myco. butyricum* were generally detected 7–10 days after the injection of complete Freund's adjuvant alone or with diphtheria toxoid (Table I). The circulating

TABLE I.—*Average Circulating, Complement Fixing Antibody Titres to* Myco. butyricum *and Types of Antibody and Cells Present*

Days after injection of adjuvant	1–6	7	10	14	21	28	35	42	51	63	71	84
Antibody titres to *Myco. butyricum*	0	2–4	16–24	32	192	192	192	128	80	80	32	24
Per cent antibody in 19S fraction*	—	†	†	100	100	75	50	25	25	25	†	—
Per cent antibody in 7S fraction*	—	—	—	0	0	25	50	75	75	75	100	†
Large cells with gamma globulin	0	+	2+	3+	4+	4+	3+	2+	2+	+	+	+
Plasma cells with gamma globulin	0	0	+	2+	4+	+	+	±	±	±	±	±

* This is expressed in terms of the percentage of recoverable antibody.
† Titre in fractions too low for determination.

antibody titre remained low (1 : 16 to 1 : 24) for the next 4 days after which there was a sharp rise in the titre that reached its peak value between the 18th and 21st days (1 : 128 to 1 : 256). This level remained unchanged for an additional 2 weeks after which there was a steady but slow decline that paralleled the gradual return of the spleen to its normal histological counterpart.

From the time that measurable circulating antibody first appeared in the serum and up to 21 to 28 days, the complement fixing antibody towards *Myco. butyricum* was of the macroglobulin (19S) class in the fractions obtained from the DEAE cellulose columns (Table I); it is possible that 7S γ_2 antibody globulin was present in a concentration too low to be detected by the complement fixing method. From approximately 28 days onward complement fixing antibody was also found in fractions containing 7S γ_2 globulins.

The chromatographic fractions were examined by zone and immuno-electrophoresis. The macroglobulin fraction containing complement fixing antibody migrated in the pre-γ_2 or β_2M region. In addition there was a fast moving β_2A component and one in the α_2 region that did not contain antibody. Absorption with *Myco. butyricum* substantially reduced the β_2M component and affected the others to only a small extent. After 28 days, complement fixing antibody was also found in the fractions that were electrophoretically identical to the γ_2 7S globulin. Sucrose density gradient determinations on selected samples confirmed these observations.

Antibody titres to diphtheria toxoid

When either soluble or alum precipitated diphtheria toxoid was injected with complete Freund's adjuvant, haemagglutinating antibody to diphtheria toxoid

appeared earlier, was 10 to 20 times higher and maintained for longer times than when diphtheria toxoid was given by itself (Table II). Measurable titres

TABLE II.—*Average Circulating Haemagglutinating Antibody Titres to Diphtheria Toxoid and Types of Antibody and Cells Present*

Days after injection	1–4	5	7	11	14	21	28	35
Antibody titre to diph. toxoid	0	20	160–320	640–1280	1280	1280–2560	640	80
Per cent antibody in 19S fraction*	0	†	100	100	80	70	30	†
Per cent antibody in 7S fraction*	—	—	0	0	20	30	70	100
Large cells with antibody to diph. toxoid	0	+	2+	4+	4+	+	±	±
Plasma cells with antibody to diph. toxoid	0	0	0	+	2+	4+	+	±

* This is expressed in terms of the percentage of recoverable antibody.
† Titre in fractions too low for determination.

were found in some animals by the 5th day and rapidly reached a maximum by the 14th to 21st day. They declined after this period, so that by 35 days the circulating haemagglutinating titre was in the order of 5–10 per cent of the maximum.

In all the animals, only heavy antibody (19S) toward diphtheria toxoid was found up to the 11th day after immunization (Table II). After this, haemagglutinating antibody associated with the 7S fraction was found as well as that associated with the 19S fraction. By the 21st day, antibody of the γ_2 type was approximately one-third of the total measurable antibody recoverable by the sucrose density gradient procedure; this total recoverable being at best 20–30 per cent of that in the starting material. By 28 days the ratio was inverted so that the antibody associated with the γ_2 fraction comprised two-thirds of the measurable antibody. Electrophoretic analysis confirmed these observations.

Histology

The histological descriptions in this report are limited to the changes in the spleens from those animals that received adjuvant or adjuvant and diphtheria toxoid. The histological and cytological changes in the spleen are the same for both groups of animals and are reported as a single observation. They have been described in detail previously and are summarized here (Moore *et al.*, 1963).

There was a rapid cellular alteration in the red pulp of the spleen. Within 24 hr. there was a small but definite increment in the larger forms of the lymphocytic series throughout the red pulp and a marked decrease in small forms. The large lymphocytes continued to increase to 28 days (Fig. 1), after which they slowly returned to a population comparable to that in the spleens of untreated animals. The populations of small lymphocytes returned to normal during this period. There were a few large mononuclear cells with deeply basophilic cytoplasm by 5 days (toluidine blue staining) (Fig. 2). Digestion with RNAase prior to staining markedly diminished the basophilia. These large mononuclear cells increased during the first 4 weeks after immunization and then gradually disappeared so that they were rare by 10 weeks.

With the indirect staining procedure (diphtheria toxoid), some of the large cells with basophilic cytoplasm had a faint diffuse fluorescence in their cytoplasm

by 5–7 days (Fig. 3). There was a sharp rise in the number of these cells that fluoresced and in the intensity of fluorescence of individual cells from 7 to 11 days (Fig. 4). This persisted for several days and then declined sharply (Fig. 5). This corresponds to the time when 19S γ–globulin antibody first is found in the serum (7 days) and approaches its maximum (11 days) (Table II).

Compared with this, the spleens from animals that received Freund's adjuvant alone showed a much slower rise in the number of these large basophilic cells that fluoresced using sheep anti-rabbit γ–globulin globulin. The number of cells that showed fluorescence reached their peak between 21 and 28 days and remained numerous for another week. They declined in number after this period but a few could be identified at the termination of the experiment (Table I). Here again a parallel can be drawn between the circulating 19S antibody species and the number of cells fluorescing.

When the spleens from animals receiving adjuvant and diphtheria toxoid were stained with the sheep anti-rabbit γ–globulin globulin there was an additive effect and consequently more of the large cells fluoresced.

Beginning at about 9 days after immunization, mature plasma cells (Fig. 6) were found with increasing frequency in the intersinusoidal tissue, particularly in the non-follicular white pulp. They gradually reached a maximum at approximately 21 days (Fig. 7) and then rapidly diminished, so that by 28 days the number of plasma cells was comparable to that in the spleens of untreated rabbits. Initially the plasma cells had a basophilic cytoplasm (toluidine blue staining) which was sensitive to RNAase. From 11 days onward there was a gradual diminution in basophilia and a transition towards an eosinophilic cytoplasm.

With both the indirect staining procedure for antibody to diphtheria toxoid and the direct procedure for γ–globulin, the general pattern of fluorescence of the plasma cells was the same (Figs 5 and 8). There were a few plasma cells that fluoresced by 9 days after immunization and with both systems reached their peak by 21 days and fell precipitously soon after.

In those animals that received diphtheria toxoid, the fluorescence of the plasma cells did not parallel that of the large basophilic mononuclear cells that contained antibody or the level of circulating 19S antibody but did correspond to the appearance and rise of circulating 7S γ_2 antibody (Table II). It should be pointed out that when only 19S γ_1 antibody was found in the circulation, plasma cells containing antibody to diphtheria toxoid were scarce. When Freund's adjuvant was used alone this separation was not clear. The fluorescence of the large basophilic cells and plasma cells (direct method) reached their maximum almost at the same time. The fluorescence of the large basophilic cells persisted for some time in this case as did the 19S γ_1 antibody in the circulation.

When the spleens from animals receiving adjuvant and diphtheria toxoid were stained directly with sheep anti-rabbit γ–globulin globulin there was an additive effect and consequently there were more plasma cells that fluoresced than with adjuvant alone or when the sections were stained for antibody to diphtheria toxoid (by the indirect method). The fluorescence of these cells could always be blocked with the appropriate unconjugated antibody.

The reticulo-endothelial cells lining the sinusoids and the histiocytes in the intersinusoidal areas were larger and more numerous throughout most of the experiment. Toluidine blue staining of the cytoplasm was faint and sensitive to RNAase. Phagocytosed red blood cells and neutrophils were common in the

histiocytes. After high titres of antibody were found in the circulation, some of the histiocytes showed small packs of γ–globulin and antibody to diphtheria toxoid in their cytoplasm with the immunofluorescent procedures, probably as a result of phagocytosis.

Small clusters of RE cells and histiocytes were present in the walls of the sinusoids and marginal zones by 5 days. They increased in number and size to 4 weeks and gradually regressed after 8 weeks. A few persisted at the termination of the experiment. Toluidine blue staining was similar to that of the other RE cells and histiocytes. These cells did not contain any material that stained with the immunofluorescent methods.

Mature polymorphonuclear leucocytes were more common throughout the red pulp in the first 24 hr. after the injection of the adjuvant. They always fluoresced intensely and the fluorescence could not be altered using unconjugated antibody.

Germinal centres were more common during the first few weeks after immunization, increasing in size to 11 days. After 14 days they began to regress, and the population of reticulum cells comprising the germinal centres was supplanted by large and medium-sized lymphocytes. These cells stained faintly with toluidine blue and there was no evidence of γ–globulin or antibody to diphtheria toxoid.

The small and medium-sized lymphocytes of the primary centres and marginal zones were enlarged within 24 hr., and by 2 days there were many mitotic figures and an increase in the number of cells in the marginal zones. These changes persisted to 28 days and then there was a gradual return to normal by 10 weeks. The cytoplasm of the lymphocytes stained faintly with toluidine blue and there

EXPLANATION OF PLATES

FIG. 1.—Representative area of the red pulp of the spleen from a rabbit killed 11 days after receiving 1 ml. of adjuvant and 0·5 ml. diphtheria toxoid. There are large mononuclear cells with basophilic cytoplasm and various sized forms of the lymphocyte. There are also nuclear forms similar to those of RE cells and histiocytes. H. and E. ×500.

FIG. 2.—Cells with basophilic cytoplasm and a nucleus with a prominent chromatin pattern designated as the large mononuclear cells in the text. These may be a form of the lymphocyte. ×1000.

FIG. 3.—Faint diffuse fluorescence of the cytoplasm of large mononuclear cells in the red pulp 7 days after the rabbit received 1 ml. of adjuvant and 0·5 ml. of diphtheria toxoid. There are several neutrophils present that show more intense fluorescence. Indirect immunofluorescence. ×600.

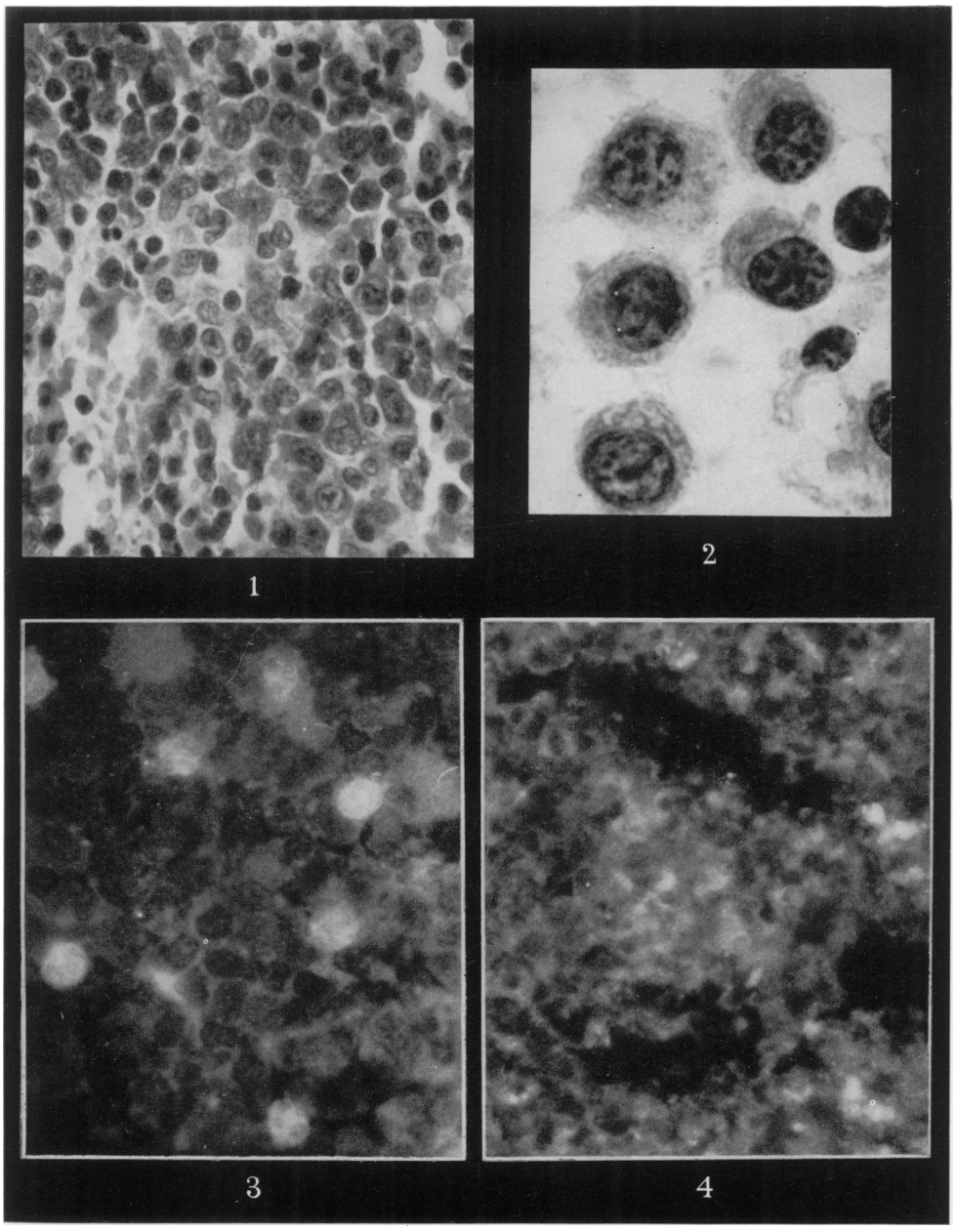
FIG. 4.—Red pulp of spleen from a rabbit killed 11 days after receiving Freund's adjuvant and diphtheria toxoid. The number of large cells that contain antibody to diphtheria toxoid and the intensity of fluorescence reaches its maximum with the indirect staining procedure for antibody to diphtheria toxoid at this time. Indirect immunofluorescence. ×300.

FIG. 5.—Aggregates of plasma cells in the spleen from a rabbit that received Freund's adjuvant and diphtheria toxoid 14 days before. There is also a noticeable diminution in the fluorescence of the large mononuclear cells of the red pulp. Indirect immunofluorescence. ×300.

FIG. 6.—Mature plasma cells. ×1000.

FIG. 7.—Spleen from a rabbit killed 21 days after receiving 1 ml. of adjuvant and 0·5 ml. of diphtheria toxoid. Representative cluster of plasma cells as seen in the pulp of the spleen. H. and E. ×500.

FIG. 8.—Aggregate of plasma cells in the spleen showing antibody to diphtheria toxoid 21 days after the injection of adjuvant and diphtheria toxoid. At this time plasma cells are most numerous and the fluorescence of their cytoplasm most prominent. Indirect immunofluorescence. ×300.



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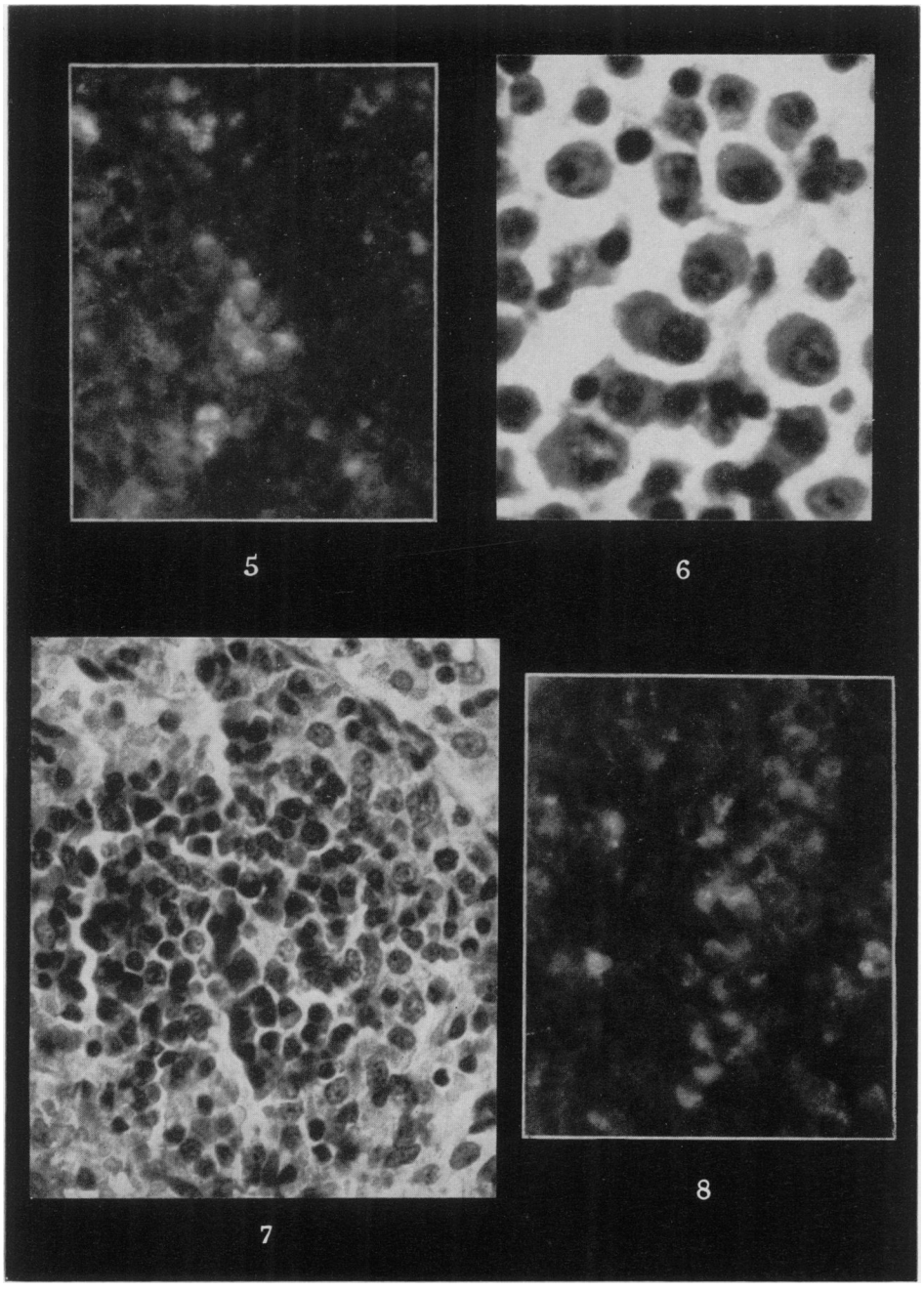

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was never evidence of γ-globulin or antibody to diphtheria toxoid by the fluorescent procedures. In both normal and experimental animals there were always a few plasma cells and histiocytes in the primary centres and marginal zones. At 2 and 3 weeks in the immunized animals, some of these cells showed fluorescent material in their cytoplasm similar to that described for the plasma cells in the red pulp.

DISCUSSION

In the present study the choice of cells producing antibody in the spleen seems to be limited to a large mononuclear cell with cytoplasmic basophilia and the plasma cell. It would appear that the large basophilic cell is associated with the production of 19S antibody and the plasma cell with 7S antibody.

Whether Freund's adjuvant is used alone or in combination with diphtheria toxoid, there is a parallel between the number of large mononuclear cells, the immunofluorescence and basophilia of their cytoplasm and the time of appearance, rise and decline of heavy antibody in the circulation. The relationship between the circulating 19S antibody and the large basophilic cells is most clearly demonstrated when antibody production to diphtheria toxoid is considered. By 7 days after immunization, antibody specific for diphtheria toxoid can be readily demonstrated in the cytoplasm of some of these cells by the indirect immunofluorescent procedure. This corresponds to the time when only 19S antibody appears in the serum. At 11 days there is a sharp increase in the number of the large basophilic cells containing antibody to diphtheria toxoid and an increase in the amount of γ-globulin in their cytoplasm as demonstrated by an increase in the intensity of fluorescence. The increase in the number of large cells having these properties persists for an additional few days and then declines rapidly. The rapid rise of 19S antibody in the serum and its subsequent decline closely follows the increase and disappearance of these cells containing specific antibody to diphtheria toxoid. Concomitant with active protein synthesis these cells showed a qualitative increase in total RNA using the toluidine blue-RNAase system.

The plasma cell appears most likely as the source of 7S antibody from the fluorescent studies, particularly on the basis of observations with the diphtheria toxoid system. The sequence of changes in number of the plasma cells, the appearance of eosinophilia of their cytoplasm and their fluorescent staining correlates well with the circulating 7S antibody titres. An increment of plasma cells is first appreciated at 9 days at which time some of them contain antibody specific for diphtheria toxoid. The population of plasma cells gradually increases with a more pronounced fluorescence up to 21 days. After this there is a rapid decline of these cells so that they are difficult to find by 28 days. The transition of the staining of the plasma cells from a basophilic to an eosinophilic cytoplasm, the increase in numbers with fluorescent material and in the intensity of fluorescence followed by the rapid disappearance of these cells and the concomitant rise of 7S antibody in the circulation suggests a rapid synthesis, an initial storage of antibody, followed by its quick release. The decay in antibody titre is consistent with the half life of circulating 7S antibody.

A similar situation, although not as readily appreciated, is found for the case of antibody production to *Myco. butyricum*. There is a parallel between the beginning appearance of 19S antibody and the increasing fluorescence of the

large basophilic cells of the red pulp. By 21 days it is difficult to correlate the cellular reaction and the type of circulating antibody. Both the large basophilic cell and the plasma cell are numerous and the circulating antibody reaches its peak at a time when other lymphoid tissues are responding.

It has been suggested that large lymphocytes are responsible for the synthesis of macroglobulin on the basis of somewhat different types of studies, and it is quite possible that the large basophilic cells of the spleen described in these studies represent such cells. A number of investigators have reported that large forms of the lymphocyte from patients with macroglobulinaemia stained with fluorescein conjugated antiserum to macroglobulin (Curtain and O'Dea, 1959; Dutcher and Fahey, 1960; Kritzman, Kunkel, McCarthy and Mellors, 1961). Staining of large lymphocytes in patients with rheumatoid arthritis has also been reported (Mellors, Heimer, Corcos and Korngold, 1959). Recently Zucker-Franklin *et al.* (1962) have reported the production of 19S macroglobulin in cultures of lymphocytes from lymph nodes from patients with macroglobulinaemia of Waldenström. There are reports demonstrating γ–globulin in the cytoplasm of various sizes of cells of the lymphocytic series in animals during the primary and secondary response (Coons, Leduc and Connolly, 1955) and in humans with malignancies (Ortega and Mellors, 1957). Nossal (1961) has shown the production of antibody by cells, either large lymphocytes or plasmablasts in single cell cultures.

The localization of 7S γ-globulin in plasma cells has been suggested with immunofluorescence (Ortega and Mellors, 1957) and the production of 7S globulin by myeloma cells has been reported (Sonnet, Gillant and Sokal, 1958). A strong argument in favour of the plasma cell as a source of 7S antibody is based on the cellular response seen as a result of a secondary stimulus. In such responses the plasma cells develop early and 7S antibody predominates in the circulation at this time (Bauer *et al.*, 1963).

Although the evidence presented is in favour of the production of two different species of antibody by cells of different appearance, the question remains whether the large basophilic cell in the red pulp of the spleen changes into a plasma cell and then produces 7S antibody in the primary response. If only the reaction to diphtheria toxoid is considered, this would seem a reasonable possibility. When the response to *Myco. butyricum* is examined, a sequential change from large basophilic cell to mature plasma cells seems unlikely. The large cells that contain γ–globulin in their cytoplasm remain elevated for many weeks as does the measurable circulating 19S antibody. The plasma cell population reverts to that of the untreated animals before there is evidence of a regression of the population of these large cells. The longer half life of the 7S antibody would explain its persistence in the circulation. The close parallel between the diminishing population of large cells and the decreasing 19S antibody is consistent with the short half life of this γ–globulin (Uhr, Finkelstein and Baumann, 1962). However, this does not rule out the origin of plasma cells from some other cell, particularly of the lymphocytic series. Finally it should be pointed out that others have described the presence of antibody in large cells by the immunofluorescence method using other antigens. These cells have been given a variety of names. It is likely that these are the same type of cell that is considered in this paper. However, there are no comparable studies with other antigens. In view of the evidence that many antigens give the same type of response with

respect to the kinds of circulating antibody that appear during successive periods after injection it would not be surprising that the cellular events described will be the same for other systems.

SUMMARY

From these results it can be tentatively concluded that in the spleen the plasma cell is associated with the production of 7S immune globulin and a large basophilic cell is associated with the production of 19S immune globulin. It is unlikely that these latter cells give rise to the mature plasma cell. This does not preclude the plasma cell from arising from lymphocytes, but a sequential change from a large cell containing antibody to a mature plasma cell containing antibody has not been established.

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