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AN AUTORADIOGRAPHIC AND HISTOLOGIC
STUDY OF SPLEEN WHITE PULP GERMINAL
CENTERS DURING EARLY INTERVALS OF
THE PRIMARY IMMUNE RESPONSE

M. G. Hanna, Jr.

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BIOLOGY DIVISION

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PULP GERMINAL CENTERS DURING EARLY INTERVALS
OF THE PRIMARY IMMUNE RESPONSE

M. G. Hanna, Jr.

This report is based upon a thesis submitted to the University of Tennessee in partial fulfillment of the requirements for the doctoral degree. The report describes research carried out in the Institute of Radiation Biology at the University of Tennessee and in the Biology Division at the Oak Ridge National Laboratory. The research conducted at the Oak Ridge National Laboratory was supported by the Oak Ridge Graduate Fellowship Program of the Oak Ridge Institute of Nuclear Studies.

MAY 1964

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INTRODUCTION

The immune mechanism has been subdivided and studied at various levels. Thus far the results of these endeavors have brought into sharp focus several questions of paramount importance: How does a cell recognize foreignness? What are the functions of the various cells involved in the immune response and how are they carried out? What controls the proliferation of the antibody-forming cell? Further questions which are still not resolved and which seem to be of major importance in answering the above inquiries are the origin of these cells and their life history.

As related by Doan (1940), early studies of Metchnikoff demonstrated that the endothelium of blood vessels, and large numbers of cells in the spleen, liver, lymph nodes, bone marrow, and scattered throughout the body were capable of phagocytizing bacteria and other antigenic substances. This system of cells was later termed the "reticulo-endothelial (RE) system" (Ashoff and Kiyono, 1913; Doan, 1940). This designation implied that the definitive phagocytes of blood and tissues, other than the "microphages" (neutrophilic granulocytes) of Metchnikoff, could be derived either from connective tissue reticulum or from specialized endothelial cells, or from both, depending upon location and circumstance. Accordingly, it was assumed that these phagocytic cells (macrophages) were capable of forming antibody. This belief was encouraged by the finding that "blockade" of the RE system with certain inert particles cuts down antibody formation (Cannon et al., 1929). Sabin (1939) contributed to this view in a most lucid fashion.

On the basis of results obtained with colored azoproteins she postulated that phagocytes produced normal globulin, and that when they took up antigen the synthetic process was so modified as to lead to the appearance of specific antibody.

The postulation of a histologically diffuse phagocytic antibody-forming cell was challenged by Rich et al. (1939) on histologic grounds. They described a large mononuclear cell with basophilic cytoplasm, termed the "splenic tumor cell". It appeared in the red pulp of the enlarged spleens and in the medullary regions of lymph nodes of rabbits given multiple injections of sterile foreign proteins. They considered it a lymphoblast probably involved in mechanisms of immunity, and proposed that the cells observed by Sabin were actually large lymphocytes.

The idea of the lymphocyte as the antibody-forming cell gained impetus from the experiment of Ehrlich and Harris (1942). This study revealed that the first response in the lymph node draining the site of infection with typhoid bacillus was an hypertrophy accompanied by the outpouring of lymphocytes into the efferent lymph. Later, Harris et al. (1945) demonstrated that such efferent lymph incubated in vitro released antibody to the surrounding media. Since over 90 per cent of the cells in the efferent lymph were small lymphocytes, it was concluded that these were the antibody-forming cells.

Owing to the prominence of lymphocytes in the spleen and lymph nodes, the concept of the lymphocyte as the cellular origin of antibody condensed the site of antibody formation from a diffuse reticulo-endothelial function to a more localized organ response. This idea was

compatible with those from earlier investigations which indicated that the spleen and lymph nodes were the major organs in antibody production. Harris et al. (1948) found high titers of antibody in splenic extracts from rabbits injected intravenously with various antigens and low titers after subcutaneous injections, although the serum titers were high. This showed that the spleen, as well as extrasplenic regions of the body, were capable of forming antibody. Splenectomy following antigen stimulation also had shown a decreased antibody formation (Luckhardt and Becht, 1911). Swartzendruber et al. (1961) demonstrated the same response with splenectomy combined with lymphadenectomy prior to primary and secondary skin homografting. The obvious correlation of X-ray-induced reduction of lymphoid tissue and decreased antibody response (Taliaferro and Taliaferro, 1951; Keuning et al., 1963) also indicated that spleen and lymph nodes were the major sites of antibody synthesis.

While the above work establishing the lymphocyte as the chief cellular source of antibody was being carried out, other evidence was accumulating attributing the same role to the plasma cell. It should simply be pointed out here that after antigen injection large primitive cells appear predominantly in the spleen and lymph nodes. Conway (1937) termed these cells "monocytoid lymphocytes." Bloom (1938) called them "large lymphocytes." They have also been called "developing plasma cells" by Koluch et al. (1947), "transitional cells" by Fagraeus (1948), "plasmablasts" by Coons et al. (1955), and Marshall and White (1950), "splenic tumor cells" by Rich et al. (1939), "AB-forming cells" by Wissler (1957), and "primitive reticulum cells" by Mellors and Korngold (1963).

An important study correlating spleen morphology and the development of large primitive lymphatic tissue cells after antigen stimulation was performed by Fagraeus (1948). She observed a transformation of reticulum cells successively to transitional cells, to immature plasma cells, and finally to mature plasma cells in the red pulp of the spleens of rabbits which had been injected with various antigens two or three days previously. The technique consisted of culturing in vitro small pieces of spleen red pulp and white pulp separately. She found much antibody formed by the red pulp in tissue containing a correspondingly high number of plasma cells. The white pulp made up chiefly of lymphocytes yielded a relatively small amount of antibody.

A later study by Marshall and White (1950) incorporated a more critical histologic as well as cytologic evaluation of the lymphatic tissue response to antigenic stimulation. It was shown that following an intravenous injection of antigen, two types of cellular proliferation occur in the spleen and that these changes may be associated with cellular proliferation in other lymphoid organs. In both types of splenic reaction the initial change was a multiplication of reticular cells leading in one case to the formation of plasma cells in the red pulp, and in the second to the formation of lymphocytes in the white pulp germinal centers. It was also pointed out that although in the two reactions the end product was the formation of plasma cells and lymphocytes separately, the formation of antibody could not be the sole prerogative of either of the two cells in their mature state. This idea was proposed primarily because at the time of the first detection

of antibody maturation of cells in the proliferative phase had not progressed far beyond the stage of "activated reticulum cell" or plasma-blast. Therefore, it was suggested that one may not speak of the plasma cell or lymphocyte reaction resulting in antibody formation. This point of view, however, was immediately challenged by workers using newly developed techniques.

So far, the evidence involving the plasma cell in antibody formation had been mainly inferential. The first of a series of experiments which stabilized this concept was by Reiss et al. (1950). When draining lymph nodes were removed from animals immunized with killed Salmonella sp., it was demonstrated that certain cells in the nodes had the capacity to adsorb on their surfaces killed bacteria from the immunizing strain. Upon examination these cells turned out to be plasma cells in nearly every case. The interpretation of this bacterial adherence phenomenon was that the positive cells had on their surface, antibody which combined specifically with the bacterial surface antigens, the stable antigen-antibody complex being visible with phase microscopy as a corona of bacteria surrounding the cell. This technique was repeated by a number of investigators (Hayes and Dougherty, 1954; Mäkelä and Nossal, 1961) and the results were completely verified.

The fluorescent antibody technique was a most important discovery of modern immunology. This histochemical technique was originally introduced (Coons et al., 1942) for the localization of antigen in tissue. It found an even wider application in the ability to demonstrate the intracellular localization of antibody. The principle of

the detection of intracellular antibody uses a so-called "sandwich technique." A frozen section or a gently fixed smear is prepared from the organ (to be screened for antibody) which has previously been exposed in vivo to an antigen (preferably of a soluble nature). The preparation is then flooded with antigen until all of the antibody sites form an antigen-antibody union. The preparation is then gently washed and flooded with a fluorescent labeled antibody. This is antibody (prepared against the immunizing antigen) coupled with a fluorescent dye, usually fluorescein or rhodamine.

With the fluorescent antibody technique, Coons and collaborators (1955) were able to confirm the hierarchy of the plasma cell family previously established by Fagraeus (1948), and were also able to demonstrate that these cells actually contained antibody. The logical conclusion was once again that these were the antibody-forming cells.

Ortega and Mellors (1957) using the methods of Coons, prepared antihuman γ -globulin and studied the cellular sites of γ -globulin formation in lymphoid tissues taken from human subjects at operation. The fluorescent antibody flooded over these tissues became localized in the cytoplasm of three types of cells, two of which were immature and mature plasma cells. The third type consisted of cells in the germinal centers which the authors called "intrinsic" cells to differentiate them from medium or large lymphocytes, or from the primitive lymphatic tissue cells that occur elsewhere in the spleen. The intrinsic cells of the germinal centers appeared to form antibody only when they occurred as aggregates; thus, Ortega and Mellors regarded the germinal center as a

minute organ of internal secretion of γ -globulin. These findings were also demonstrated by White (1960).

In a study of the cellular origin of each of the three types of human immunoglobulins (γ_2^- , γ_{1m}^- , and γ_{1a}^-), Mellors and Korngold (1963) observed that two closely related families of cells form immunoglobulins in human lymphoid tissue: germinal center cells and plasma cells. However, immunoglobulins were also found in what they termed "primitive reticulum cells" which they described as resembling the cells of the germinal center but topographically separated from them.

Thus, the fluorescent microscopy technique has pointed to the germinal centers as functional structures in the immune response (Ortega and Mellors, 1957; White, 1960; and Mellors and Korngold, 1963). The histologic studies of the lymphatic germinal centers during the immune response are also consistent with these findings.

The lymphatic nodules of the spleen and the cortex of the lymph nodes have a characteristic histologic appearance which consists of an aggregated zone of large pale-staining cells distinguishable from a zone of dark-staining, closely packed cells, consisting mostly of small and medium lymphocytes. The former was named "keimzentrum" or germinal center by Flemming in 1885. Flemming observed the high mitotic activity of the germinal center and concluded that this area was actively forming lymphocytes. Since the original description of germinal centers, various ideas have been advanced in respect to their possible function. Some investigators supported Flemming's suggestion; however, a conflicting view was that the germinal center was primarily an area of

degeneration (Latta, 1922) or that it was a reaction center to noxious substances (Hellman, 1921). These views were proposed primarily because the centers could often be observed to contain macrophages with pyknotic nuclear debris, which Flemming had termed "tingible korper" or tingible bodies.

Conway (1937) attempted to combine the opposing view of Flemming and those of Latta and Hellman by suggesting that the differences reported were due to observations of the germinal centers in different cyclic phases that she described in the lymphatic nodules of normal and antigen-stimulated animals. Later observations of Ringertz and Adamson (1950) supported the opinion that the role of the center in antigen-exposed animals was indeed germinal and that the phenomena of cell disintegration and resulting phagocytosis which appears in the active type of germinal center are either a result of the intense cellular proliferation or a toxic cellular response. These investigators suggested that the role of the germinal centers was to produce immature lymphocytes which, upon antigenic stimulation, would become converted into antibody-producing plasma cells instead of, as is normal, developing into mature lymphocytes.

In describing the early lymphatic tissue changes of normal mice given foreign bone marrow, Congdon et al. (1960) proposed that the cellular system that leads to antibody formation is the germinal center. On proper stimulation the germinal centers were described as losing their architectural arrangement (germinal center dissociation) resulting from the migration of cells from the center, probably into the red pulp

of the spleen and lymph node medulla. This histologic observation suggested that the germinal center is the source of the large primitive cells observed in the antigen-stimulated lymphatic tissue, and considered by Fagraeus (1948) and Coons (1942) as the plasmacyte stem cell. It was also proposed by Congdon that failure of this cell to respond to antigen resulted in its transformation into a mature lymphocyte.

Thus, the early histologic studies of Ringertz and Adamson (1950) and Congdon et al. (1960) suggest that the site of origin of the immunogenically active primitive lymphatic tissue cells is the white pulp germinal center. However, both of these investigations consider an alternative pathway of the germinal center cell is to the small lymphocyte. Considerable doubt has been placed on this divergent transformation hypothesis by recent studies of lymphocyte depletion of the lymphatic tissue. Waksman et al. (1962) demonstrated that when rats were thymectomized at birth there was a profound depletion of small lymphocytes in various lymphatic organs. In the spleen these cells were completely lacking in the white pulp; however, in both spleen and lymph nodes the germinal centers were normal in size, number, and cellular make-up. Similar results were obtained in studies of Cottier et al. (1963) where lymphocyte depletion was achieved in calves by extracorporeal irradiation. Enhanced mitotic activity was not observed in the germinal centers.

Recent histologic studies of Congdon et al. (1961, 1962) on lymphatic tissue changes in mice during the primary and secondary immune response continue to stress the possibility of the germinal centers as

the source of primitive lymphatic tissue cells. A most important aspect of these studies is the time interval between antigenic injection and tissue examination. The dissociation of the germinal centers was described during the first 24 hours after antigen injection, and preceded the proliferative phase of the primitive lymphatic tissue cells described in earlier studies (Conway, 1938; Rich et al., 1939; Marshall and White, 1950). Although the presence and general significance of the primitive lymphatic tissue cells for reaction to antigen material had been recognized for some time, the studies of Congdon were the first to directly relate their appearance in lymphatic tissue with dissociative migration of germinal center cells.

Thus far in this review it has been shown that the reticulo-endothelial phagocytic cells were the first to be identified with antibody production. This hypothesis later yielded to one accepting the lymphocyte and then, after more critical observations, to the plasma cell hypothesis. The obvious reason for the confusion that existed was the lack of knowledge of the hematological cytology and the genesis and life cycles of the various cells mentioned. The question then arises: Where do the plasmablasts themselves come from? Which of the normally occurring cells in the lymphoid tissues are capable of being stimulated by antigen? Answers to these questions depend upon subjective interpretations of a series of complex static pictures (sections or cell smears). It is accepted that the important changes are the early ones, i.e., the transformation of some cell or cells into plasmablasts; and that histologic observations can never give more than suggestive evidence of this subtle alteration.

At present, histologic studies indicate that the precursor to the plasma cell is a primitive lymphatic tissue cell. Microscopic observations made at early intervals of the primary and secondary immune response relate the increase in number of primitive lymphatic tissue cells to germinal center dissociation. However, no attempt has been made to establish quantitatively that the primitive lymphatic tissue cells concentrated in germinal centers do migrate out to surrounding areas in normal or immunized animals.

This study is concerned with the quantitative and histologic aspects of germinal center dissociation and the migration and fate of germinal center cells in the spleen of normal and antigenically stimulated animals during the primary immune response. Studies were made on the spleen white pulp since it is the only lymphatic tissue receiving a large direct exposure to the antigen after intravenous injection, thus permitting a synchronized cellular response.

Autoradiographic labeling techniques were used in this study and were predicated on the uptake of tritiated (H^3 -) thymidine as an index of deoxyribonucleic acid (DNA) synthesis (Reichard and Estborn, 1951). The validity of the conclusions made from the present investigations depends on a number of premises which are widely held but are still the subject of discussion (Stohlman, 1959). The premises most directly relevant to these experiments are: (a) the tritium label on thymidine is stable and does not exchange, nor does the thymine on DNA exchange after it is incorporated; (b) DNA itself is metabolically stable and turnover is insignificant in mammalian cells; (c) H^3 -thymidine injected

intravenously is rapidly distributed throughout the body, and either promptly incorporated into DNA or eliminated; (d) thymidine or H^3 -thymidine does not affect the normal behavior of cells that have taken it up. The last premise has recently been challenged (Greulich et al., 1961).

As demonstrated by Yoffey et al. (1961), the incidence of labeling of germinal center cells is very high shortly after the injection of H^3 -thymidine. By systematically following the labeled germinal center cells at close intervals, data could be obtained on their activity and migration pattern. It was imperative in these autoradiographic studies that the quantitation be performed on tissue sections to maintain anatomical relationships. A further prerequisite was that the cells not be so heavily labeled as to prevent identification. In addition, since the germinal center cells have a relatively short generation time (to be demonstrated in these studies), a pulse label could not be followed for more than a 10-hour period. A further consideration was the variation in nuclear diameter which would be obtained in a cell population as a consequence of random sectioning. Thus, the scoring of labeling-intensity of cells was made on a per-unit-area basis.

Investigations were also made of changes of germinal center volume during the primary immune response with concurrent measurements of cellular changes in the peripheral blood. The fate of germinal center cells was interpreted from autoradiographic preparations of spleen imprints.

MATERIALS AND METHODS

Animals

Male LAF_1 ($\text{C}_{57\text{L}}^{\text{Cum}}$ x XA/HeCum) F_1 mice, 15 to 16 weeks of age were used. These were obtained from Cumberland View Farms. The animals weighed about 25 grams at the start of the experiments. An important consideration in studies of the immune response in mammals is that the animals have a minimum background infection. A significant infection of any nature prior to induced immunization is capable of masking the early lymphatic tissue changes of the primary response. Consequently, the animals used in these studies were subjected to the routine animal health program carried out at the Biology Division of the Oak Ridge National Laboratory.

Upon receipt from the breeder the animals were subjected to an 18 day quarantine. During this interval the mice were tested for Pseudomonas sp. and Salmonella sp. and spot checked for external and internal parasites. All positive animals were killed. The animals were then transferred to the Biology Division animal facility and kept 10 to a cage in a room automatically maintained at a temperature of 76 degrees F. and 55 per cent humidity. The cages were routinely changed twice weekly and the water bottles changed three times per week. The mice were given Purina Mouse Chow and water (containing 10 parts/ 10^6 chlorine) ad libitum.

Preparation and Injection of the Antigen

Sheep blood was collected fresh in a small amount of heparin.

This was centrifuged at 2000 rpm for 20 minutes at 5 degrees C. and the plasma, buffy coat, and top 2 to 3 ml. of packed cells removed. This procedure was repeated five times and the blood cells were resuspended each time in Difco Tyrode's solution. After the final washing, the packed cells were diluted to a 10 per cent suspension in Tyrode's solution and stored in the cold for 24 hours. No white blood cells could be detected in the final preparation by the conventional Neubauer counting chamber method (Todd et al., 1953). A preparation of sheep red blood cells (SRBC), if kept in the cold, remained intact (without an appreciable amount of cell lysis as determined visually) for four to five days; consequently, each lot was used within this time interval.

Prior to antigen injection the animals were pooled in one or two cages and heated for fifteen minutes with a table lamp. This gentle heating dilated the blood vessels of the tail and facilitated intravenous injections. The mice were then randomly selected and individually placed into a ventilated lucite mouse holder with the tail exposed. The tail was wiped with a gauze saturated with 70 per cent ethyl alcohol and the injections were generally made into the right lateral vein. Each animal received one cc. of the 10 per cent suspension (about 2.0×10^9 SRBC).

Isotope Preparation and Injection

Tritiated (H^3 -) thymidine with a specific activity of 0.9 c/mM. was obtained from New England Nuclear Incorporated. The H^3 -thymidine was diluted to 100 μ c/ml. with Tyrode's solution and stored in the cold. At all times the labeled thymidine was used within two weeks of the time

it was received.

The procedure for the injection of H^3 -thymidine was the same as that discussed for antigen, except that the labeled thymidine was injected into the opposite tail vein (generally the left lateral vein). All animals receiving H^3 -thymidine were injected with 50 μ c. (about 2.0 μ c./gram body weight) and were kept four or five to a cage in disposable plastic cages.

Blood Clearance of Tritium Activity

To determine the rate of clearance of tritium activity from serum, 24 animals were injected with 50 μ c. of H^3 -thymidine. Four animals were killed at 1, 5, 10, 15, 30, and 60 minutes after isotope injection. Blood was drawn from the ventral tail vein and allowed to clot. The blood samples were then centrifuged at 2000 rpm for 20 minutes in the cold (5 degrees C.) and 200 λ of serum was removed and placed in a standard 20-ml. glass counting vial.

To each glass counting vial was added 5 ml. of scintillation mixture of the following composition: 1 liter 1,4, Dioxane; 175 grams Naphthalene; 7 grams 2,5-diphenyloxazol; and .375 grams 1,4,-Bis-2-(4-methyl-5-phenyloxazolyl)-benzene. This scintillation mixture had been previously demonstrated to correct for 200 λ of water with 92-93 per cent efficiency (MacDonald and Odell, 1963). The tritium activity was counted in a Liquid Scintillation Spectrometer Model 3214 (Packard Instrument Company). Each sample was counted three times for a one-minute interval, and background determined from a scintillation mixture-blank was subtracted from the final count.

Experimental Procedure

The general plan consisted of three main experiments: Experiment I was an autoradiographic study of the dissociative proliferation of spleen white pulp germinal centers of normal and primary immunized animals during the first 48 hours of the immune response. Experiment II was a histologic study of the spleen lymphatic tissue during the primary immune response with particular attention given to the changes in germinal center number and volume. A concurrent study of the cellular changes in peripheral blood was also performed. Experiment III was an autoradiographic study of the cellular changes in spleens between the first and fourth days of the primary immune response as interpreted by spleen imprints.

Experiment I: General Procedure

Five groups of animals were used in this experiment. In group A, 20 mice were injected with 1 cc. of the antigen intravenously, and another 20 were given an equal volume of Tyrode's solution. One hour later, all animals received H^3 -thymidine. Four SRBC-stimulated and four control animals were killed at each of 2, 4, 6, 8, and 10 hours after receiving the antigen or Tyrode's solution respectively.

The same experimental procedure was followed for groups B and C. In group B, the isotope was injected 9 hours after the antigen or Tyrode's solution, and the animals were killed at 10, 12, 14, and 16 hours. In group C, H^3 -thymidine was injected 17 hours after antigen and Tyrode's solution, and the animals were killed at 18, 20, 22, and 24 hours.

Groups D and E both consisted of 24 animals which were injected with 1 cc. of antigen. Twenty-five hours after antigen injection all animals in group D were injected with the standard amount of H^3 -thymidine and killed at 26, 28, 30, 32, 34, and 36 hours after antigen. The same procedure was followed for group E, except that the H^3 -thymidine was injected at 37 hours after antigen and the animals were killed at 38, 40, 42, 44, 46, and 48 hours after antigen.

Autoradiographic Preparations of Tissue Sections

The mice were killed with ether anesthesia. Spleen, thymus, Peyer's patches, mesenteric lymph node, and liver were removed and fixed in Bouin's Fluid (Mallory, 1938), dehydrated in ethanol, and after paraffin embedding were cut at 5 μ . All tissue sections to be used for autoradiography were put on slides subbed with a thin layer of dried gelatin solution (5 grams of gelatin in 1000 ml. distilled water). The tissue was deparaffinized through xylol and progressively hydrated through alcohols.

Kodak NTB-2 emulsion, melted at 45 degrees C. was used for all autoradiography. Test slides of the emulsion were checked for clarity and background immediately prior to its use. The tissue was dipped into the emulsion, allowed to dry for one hour, and stored in the presence of Drierite (W. A. Hammond Drierite Co.) in the cold for 14 days. The autoradiograms were developed in Kodak D-19 developer, rinsed in water, fixed in Kodak acid-fixer (all solutions at 17 degrees C.), and washed in running water for 15 minutes. The developed autoradiograms were stained in Harris hematoxylin and eosin (Mallory,

1938), and mounted. After development, the background count was frequently low (below one grain/1000 μ^2); slides with background greater than one grain/50 μ^2 were discarded.

Histological Classification

The primitive lymphatic tissue cells concentrated in germinal centers and scattered throughout the surrounding lymphocyte mass and red pulp have a large vesicular nucleus (at least 6.5 μ in diameter), and a basophilic cytoplasm. They are further characterized by their nucleoli, of which there are at least two. One of these nucleoli is extremely large and occupies a considerable portion of the nucleus. In the germinal centers (GC) these cells were classified according to their topographic location. Those in the densely populated (DP) or dark-staining side, which is closely associated with the lymphocyte mass and is nearest the central artery, are designated C_1 . Those GC cells that were observed in the more thinly populated (TP) or light-staining side, nearest the red pulp, are classified as C_2 . The morphologically identical cells located in the lymphocyte mass outside the germinal center are called C_3 . Reticular cells and medium-sized lymphocytes were infrequently observed in the germinal centers and were not included in this study. The "tingible body" macrophages, generally associated with the DP side of the germinal center, were classified as GC cells because of the morphologic similarity of their nuclei. The nuclei of the macrophages never were observed to be labeled.

Scoring of Autoradiograms

When possible, four germinal centers and associated lymphocyte masses were scored in each autoradiogram. The germinal centers were selected for their clear demonstration of the two topographic areas previously discussed.

Three parameters were measured: the percentage of labeled cells in the densely and the thinly populated areas of the germinal centers, and the grain counts per unit area of nucleus in the labeled C_1 , C_2 , and C_3 cells. The third parameter measured was the percentage of labeled mitotic figures in the germinal centers. Scoring grain counts per unit area was accomplished by placing a small hole in an aluminum planchet, which was then inserted into one of the oculars of the microscope. This provided under oil immersion, a microscopic field of $11.3 \mu^2$.

In an attempt to obtain an objective estimate of labeling in cells, only those cells were scored which demonstrated three or more grains over the nucleus.

Experiment II: General Procedure

Fifteen animals were used at each interval studied. Five animals were selected randomly and injected intravenously with 1 cc. of 10 per cent SRBC, while another five were selected randomly and injected intravenously with 1 cc. of Tyrode's solution. The remaining five animals were used as normal controls.

The groups of 15 mice were killed at hours 1, 1.5, 2, 4, 6, 8,

10, 12, 14, 16, 18, 20, 22, 24, and days 1.5, 2, 2.5, 3, 3.5, and 4 following antigen injection. The 2-hour postinjection interval was later repeated and therefore includes 10 experimental, 10 Tyrode's injected control, and 10 normal control animals.

Hematological Studies

Peripheral blood studies were made on all animals. Total white (WBC) and red cell (RBC) counts were determined (using a Fisher Autocytometer, Model 6-246V1) on blood drawn from the ventral tail vein. A previously determined threshold setting of 55 was used for both white blood cells and red blood cells (Ingersoll, 1962). Twenty lambda blood samples were diluted with Unopettes (Becton, Dickenson and Company) containing 5 ml. of filtered diluent. The dilutions were 1:250 in 3 per cent acetic acid for WBC and 1:62,500 in normal saline for RBC.

Peripheral blood smears were prepared, air dried, fixed 15 minutes in absolute methanol, and stained with a modified Wolbach's Giemsa stain (Mallory, 1938). A differential count of 100 cells was made from each peripheral blood smear.

Histological Studies

All animals were killed by ether. The spleen was removed for study and was longitudinally bisected. One half was fixed in Bouin's Fluid and stained with hematoxylin and eosin, while the other half was fixed in Carnoy's fixative (Mallory, 1938) and stained with methyl green-pyronin (Kurnich, 1952).

In addition to the general histologic observations two

determinations were made from each hematoxylin and eosin-stained section: the number of germinal centers per longitudinal section of spleen and the longest diameter of each germinal center. The mean longest-diameter of the germinal centers in each longitudinal section was used to estimate the mean volume by the following formula:

$$V^{\text{sphere}} = \frac{4}{3} \pi r^3$$

Experiment III: General Procedure

Three groups of animals were used in this study. In group 1, 15 animals were injected with 1 cc. of antigen intravenously and another 15 were given an equal volume of Tyrode's solution. Twenty-three hours later, all animals received H^3 -thymidine. Five SRBC-stimulated and 5 control animals were killed at 24, 36, and 48 hours after the antigen or Tyrode's solution.

The same experimental procedure was followed for groups 2 and 3. In group 2, the isotope was injected at 47 hours after antigen and Tyrode's solution and the animals were killed at 48, 60, and 72 hours. In group 3, H^3 -thymidine was injected at 71 hours after antigen and Tyrode's solution and the animals were killed at 72, 84, and 96 hours.

Authoradiographic Preparations of Spleen Imprints

Spleen imprints were made by cutting the dissected organ with a razor along the median ridge so as to provide an evenly faced surface. The cut surface was touched lightly and evenly to a cleaned, subbed slide. They were then air dried overnight and fixed and dehydrated for

15 minutes in absolute methyl alcohol.

The preparation of the spleen imprint autoradiograms was the same as that discussed for tissue sections except that the former were exposed for 10 days and stained with a modified Wolbach's Giemsa stain (Murray, 1938).

Scoring of Imprint Autoradiograms

The percentage distribution of cells was determined by differential identification of 1000 to 1500 nucleated cells on all spleen imprint autoradiograms. In a carefully made imprint preparation of the spleen it is possible to distinguish white pulp and red pulp by the cell types present in various areas. Dense accumulations of lymphocytes can be interpreted as lymphatic nodules, while the areas which appear less dense and contain histiocytes and cells of the erythrocytic series can be interpreted as red pulp. Thus, to insure a random sample of the percentage distribution of cell types the identification of all cells in at least seven randomly selected high power fields were made.

A second parameter measured in the imprint autoradiograms was the percentage labeled cells in each category of nucleated cell types. In an attempt to obtain an objective estimate of labeling in cells, only those cells were scored which demonstrated three or more grains over the nucleus.

The nucleated cells of the spleen imprints were classified into eight major categories.

Primitive Lymphatic Tissue Cells (PLTC). This group of cells includes all germinal center cells and C_3 cells. In imprints this cell is at least $20\ \mu$ in diameter and has a round or oval nucleus which is very large and is stained a transparent, pale rose color. The chromatin structure is barely distinguishable as delicate interlacing strands. One large nucleolus is occasionally observed in these cells.

The cytoplasm of the PLTC is sharply limited. It does not extend far beyond the nucleus and forms a narrow perinuclear band. Its structure is uniform, often staining more basophilic along its edge.

Large Lymphocyte (LL). This cell measures at least $13\ \mu$ in diameter. The nucleus, always central and round, is stained a deeper rose color than the nucleus of the PLTC. The chromatin is quite distinct and irregularly blocked. The nucleus usually contains one or two small round nucleoli.

The cytoplasm of the large lymphocyte is usually round, hyaline, and clear blue in color. It has a sharply demarcated edge.

Small and Medium Lymphocytes (S&ML). These cells have a diameter of $6\ \mu$ to $12\ \mu$. The nucleus sometimes is oval in shape, but more generally round, and is stained a deep violet. It is composed of very dense chromatin blocks surrounding a main central chromatin mass.

The cytoplasm is very reduced and is often scarcely visible. The edges are stained a sky blue, and the cytoplasm often appears on one side of the nucleus.

Granulocytes. This category of cells included all stages of development of the neutrophilic and eosinophilic granulocytes. The morphology of these cells has been described by Dunn (1954).

Erythrocytes. This series is made up of a succession of cells which start with the rubriblast and end with the erythrocyte or red cell. In the normal mouse the cells of this series are found in the red bone marrow, and an extramedullary source is the spleen. The classification and nomenclature described by Diggs et al. (1954) were accepted for this series.

Histiocytes. The histiocytic series in a strict sense is considered to give rise to histiocytes which as mature cells are phagocytic in nature, and a certain number enter the circulation to be known as "monocytes" (Bessis, 1956). The characteristics and classification discussed by Bessis (1956) were used in the establishment of this category.

Plasma Cells. Plasma cells are generally characterized by an eccentric nucleus, strongly basophilic cytoplasm, and often, although not always, a perinuclear clear zone. In this study the nomenclature established by Nossal (1959) was accepted, and the plasma cells were subdivided into two classes; (a) immature plasma cells, and (b) mature plasma cells.

Immature Plasma Cells (IPC). This term is applied to cells which resemble the PLTC, but have a hyperbasophilic cytoplasm. The

chromatin structure of the nucleus is denser and more distinct. The measurements of this cell range from 16 μ to 20 μ in diameter.

Mature Plasma Cells (MPC). This cell is generally 8 μ to 15 μ in diameter. The nucleus always lies to one side of the cytoplasm. The chromatin structure of the nucleus forms dense blocks which are very distinct.

The cytoplasm is stained an intense blue color which decreases in intensity toward the center of the cell. The cytoplasm generally is oval in shape.

RESULTS

Blood Clearance of Tritium Activity

Table I shows the counts per minute of tritium activity in the blood serum at various intervals after H^3 -thymidine injection. These data are minimum values due to inherent counting error. An exponential loss of tritium from blood serum occurred during the first thirty minutes after H^3 -thymidine injection (Table I and Fig. 1). At the thirty-minute interval the counts per minute represented approximately 25 per cent of the activity measured one minute after injection. Between the thirty- and sixty-minute postinjection intervals a less dramatic decrease in tritium activity occurred. The residual counts per minute remaining in the serum at sixty minutes represented 20 per cent of the initial activity measured one minute postinjection.

Experiment I: Percentage of Labeled Germinal Center Cells

Among the group A animals at one hour after H^3 -thymidine injection there were approximately 19 per cent of the C_1 cells labeled in control, while there were 30 per cent of these cells labeled in SRBC-stimulated animals. After two hours there were 23.6 and 43.3 per cent of the C_1 cells labeled in the control and stimulated animals respectively (Table II and Fig. 2). In the TP area (C_2 cells) of the antigen exposed animals a more gradual increase occurred which eventually surpassed the percentage of labeled C_1 cells in the adjacent region (DP) at the nine-hour postinjection interval.

In the control animals, a progressive increase in percentage

TABLE I
BLOOD CLEARANCE OF TRITIUM ACTIVITY (COUNTS PER MINUTE)

Animal	Time after H ³ -thymidine injection*					
	1 min	5 min	10 min	15 min	30 min	60 min
1	316,701	225,965	214,591	183,374	70,168	60,961
2	327,633	236,028	170,318	182,959	77,853	62,113
3	319,058	236,613	202,653	160,419	78,984	61,030
4	285,423	210,356	187,959	188,103	88,222	69,249
$\bar{X}$	312,454	227,241	193,880	178,714	78,804	63,338
$\pm s$	18,466	12,247	19,131	12,450	7,348	4,000

*All means based on observations on four animals.

$$s = \left[\sum_{i=1}^n (x_i - \bar{x})^2 / n(n-1) \right]^{1/2} = \text{standard deviation of the mean.}$$

Figure 1. Clearance of tritium activity in blood serum at various times after H^3 -thymidine injection. All means based on observations of four animals. Perpendicular bars represent one standard deviation from the mean.

TABLE II
PERCENTAGE OF LABELED CELLS IN GERMINAL CENTERS

Group	Cell Type	Hours after H ³ -thymidine*									
		1		3		5		7		9	
		$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$
A Control	C ₁	19.1	3.7	23.6	6.9	28.7	5.3	31.0	5.0	17.8	3.0
	C ₂	13.6	5.6	19.7	6.6	16.5	1.8	23.4	3.0	14.8	1.0
	SRBC-stimulated										
	C ₁	29.9	4.9	43.3	12.0	29.9	7.3	14.6	5.0	11.5	2.6
	C ₂	13.7	2.8	22.1	4.0	23.4	4.1	17.0	5.1	16.6	2.8
B Control	C ₁	16.2	2.6	21.5	2.1	28.7	5.0	27.3	3.6		
	C ₂	10.8	2.6	13.4	0.5	22.1	2.8	19.8	2.5		
C Control	C ₁	16.8	2.1	20.3	1.3	28.0	4.5	29.4	4.1		
	C ₂	15.9	2.5	14.5	2.0	16.5	1.5	21.8	1.6		

*See footnote, Table I.

Figure 2. Percentage labeled C₁ and C₂ cells in the germinal centers of control and SRBC-stimulated animals at various times after H³-thymidine injection. All means based on observations of four animals. O, 2- to 10-hour control (Group A) C₁ cells; ◇, 10- to 16-hour control (Group B) C₁ cells; □, 18- to 24-hour control (Group C) C₁ cells; ●, 2- to 10-hour control (Group A) C₂ cells; ◆, 10- to 16-hour control C₂ cells; ■, 18- to 24-hour control (Group C) C₂ cells; ○, 2- to 10-hour SRBC-stimulated (Group A) C₁ cells; ●, 2- to 10-hour SRBC-stimulated (Group A) C₂ cells.

labeled cells in both areas of the germinal center was demonstrated up to seven hours after H^3 -thymidine injection. An abrupt decrease followed, which possibly, was partially caused by dilution of the label.

Germinal Center Cell Proliferation

An analysis of variance was performed on these data of group A (Appendix I). One hour after isotope injection, no significant difference at the 5 per cent level existed in mean grain count per unit area of cell type C_1 , C_2 , and C_3 between the control and experimental animals (Table III and Fig. 3). When the control or experimental subgroups were examined individually one hour after isotope injection, there was no significant difference at the 5 per cent level between C_2 and C_3 cells while such a difference was found to exist between the paired cell type (C_2, C_3) and C_1 .

At three hours after H^3 -thymidine injection, the mean grain counts decreased in the C_1 and C_2 groups of cells. The C_2 cells of both control and stimulated animals demonstrated a two-cell population. One with a higher grain count corresponds more closely with the C_2 cells scored in the previous one-hour interval. The second population could possibly represent daughter cells of either the original C_1 or C_2 cell population. At five hours, the mean grain counts further decreased and there is evidence for a more rapid dilution of the label in the C_1 and C_2 groups than in the C_3 cell population. Between five and seven hours, C_1 and C_2 cells in both the stimulated and control animals are not significantly different on the basis of mean grain count per unit area,

TABLE III
MEAN GRAIN COUNT/11.3 μ^2

Group	Cell Type	Hours after H ³ -thymidine*										
		1		3		5		7		9		
		$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	
A	Control	C ₁	6.0	1.3	4.3	1.0	3.7	0.8	3.6	0.5	2.9	0.5
		C ₂	6.9	1.4	4.0	1.2	3.3	1.0	3.3	1.4	3.0	0.2
		C ₃	7.2	0.6	7.8	1.0	7.0	1.4	6.6	0.3	4.9	0.3
	SRBC-stimulated	C ₁	6.2	0.3	4.0	0.2	3.3	0.2	2.7	0.2	2.6	0.3
		C ₂	7.2	1.0	4.2	0.8	3.3	0.2	2.7	0.8	2.5	0.2
		C ₃	7.9	0.8	7.1	0.2	6.7	0.2	4.2	0.6	3.8	0.1
B	Control	C ₁	6.8	0.2	4.9	0.3	4.0	0.2	3.4	0.2		
		C ₂	6.9	0.1	4.7	1.0	3.7	0.3	3.1	0.3		
		C ₃	7.3	0.2	7.5	0.1	6.7	0.2	5.8	0.5		
	SRBC-stimulated	C ₃	8.6	1.4	7.8	0.7	6.6	0.7	5.2	0.1		
C	Control	C ₁	6.5	0.3	4.8	0.1	3.7	0.6	3.5	0.4		
		C ₂	7.5	1.0	4.6	1.1	3.6	0.3	3.2	0.1		
		C ₃	7.9	0.8	7.2	0.5	6.2	0.4	6.1	1.0		
	SRBC-stimulated	C ₃	7.8	0.3	7.1	0.1	5.7	0.3	4.8	0.4		

*See footnote, Table I.

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Figure 3. Frequency distribution of grain counts per $11.3 \mu^2$ of cell types C₁, C₂, and C₃ at various times after H³-thymidine. All frequency distributions based on 100 per cent. The number of cells scored at each interval is noted under the representative figure of each cell type.

while such a difference exists between the paired cell types (C_1, C_2) and C_3 . In the seven hour interval the label is further diluted in the three cell populations.

A continuation of this measurement after the nine-hour interval was impractical because of the dilution of the label. At twelve hours after SRBC-injection in the experimental animals, the germinal centers were dissociating, and the morphologic integrity of the centers was lost.

Among the control animals in the three groups, a good agreement exists in the mean grain counts of the three cell types. The proliferation in the stimulated germinal center cells (group A) prior to germinal center dissociation, is similar to that of the three control groups. It is reasonable to assume that in a fairly homogeneous population of cells the time taken for the mean grain count to be halved should give an indication of the doubling time (synthesis + G2 + prophase + metaphase + anaphase) of these cells. According to these data, the dividing C_1 cells have a doubling time of five to seven hours (Fig. 4). The mean grain counts of the C_1 cells at the nine hour interval are not considered reliable because of the small number of labeled cells present in the DP area. Moreover, the inherent error resulting from reutilization of the H^3 -thymidine becomes more pronounced when the amount of radioactivity in the cells is low (less than 3 grains/11.3 μ^2). Applying the same criterion to the C_3 cells, the doubling time is demonstrated to be about nine hours in duration (Fig. 5).

Table IV and Fig. 6 show a study of the percentage labeled mitotic

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Figure 4. Mean grain count per $11.3 \mu^2$ over C_1 cells in densely populated side of SRBC-stimulated and control germinal centers at various times after H^3 -thymidine. All means based on observations on four animals. ○, 2- to 10-hour control (Group A); ◇, 10- to 16-hour control (Group B); □, 18- to 24-hour control (Group C); ●, 2- to 10-hour SRBC-stimulated animals (Group A).

Figure 5. Mean grain count per $11.3 \mu^2$ over C_3 cells in lymphatic nodules of SRBC-stimulated and control animals at various times after H^3 -thymidine. O, 2- to 10-hour control (Group A); $\diamond$, 10- to 16-hour control (Group B); $\square$, 18- to 24-hour control (Group C); $\bullet$, 2- to 10-hour SRBC-stimulated (Group A); $\blacklozenge$, 10- to 16-hour SRBC-stimulated (Group B); $\blacksquare$, 18- to 24-hour SRBC-stimulated (Group C).

TABLE IV
PERCENTAGE LABELED MITOTIC FIGURES IN GROUP A*

Group	Stage	Hours after H ³ -thymidine**							
		1		3		5		7	
		$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$
Control	Prophase and Metaphase	14.7	4.0	74.8	8.7	37.7	6.2	36.6	3.8
	Anaphase and Telophase	16.9	2.2	73.1	14.8	57.4	16.0	32.3	17.0
SRBC-stimulated	Prophase and Metaphase	24.4	8.0	84.5	7.9	38.3	14.8	10.9	6.4
	Anaphase and Telophase	16.0	9.3	76.4	5.6	50.0	22.4	26.3	14.1

*Fifty to one hundred mitotic figures were scored per animal.

**See footnote, Table I.

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Figure 6. Percentage labeled mitotic figures in control (right) and SRBC-stimulated animals (left). O, prophase and metaphase; ●, anaphase and telophase.

figures in the germinal center during the first ten hours after injection of SRBC or Tyrode's solution. Estimations of the time sequences of the generation cycle were based on a theoretical evaluation of the percentage of labeled mitotic figures following a pulse label (Cronkite et al., 1959). The synthesis (S) period is between one- and five-hours after H^3 -thymidine injection or about four hours in duration. The 20 per cent difference that exists in labeled prophase and metaphase stages at seven hours, between the control and stimulated animals may be partly caused by a second wave of division in the control animals, thus the generation time of these cells is no longer than seven hours in duration.

An extended study of the C_3 cell population was considered desirable due to the increased number of these cells throughout the spleen between days one and two. These data show that the doubling time of the C_3 cells during this interval is between nine and ten hours (Table V and Fig. 7).

A study of the percentage labeled mitotic figures of the C_3 cell population estimates that the S period is between the one- and seven-hour postinjection interval or about six hours in duration (Table VI and Fig. 8). The total generation time is estimated to be no longer than ten hours in duration.

Experiment II: Peripheral Blood Studies

An immediate leucocytosis was present in the SRBC-stimulated animals (Table VII and Fig. 9). This was first detected at one hour and

TABLE V
MEAN GRAIN COUNT/11.3 μ^3 OF C_3 CELLS BETWEEN DAYS ONE AND TWO*

Group	Type	Hours after H^3 -thymidine**											
		1		3		5		7		9		11	
		$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$
D SRBC- stimulated (26-36 hr.)	C_3	7.6	.3	6.9	.6	6.2	.1	5.3	.6	4.4	.5	3.6	.4
E SRBC- stimulated (38-48 hr.)	C_3	7.3	.4	6.9	.4	5.8	.1	4.8	.3	4.9	.7	4.1	.1

* 200 cells were scored per animal.

** See footnote, Table I.

Figure 7. Mean grain count per $11.3 \mu^2$ over C_3 cells in lymphatic nodules of SRBC-stimulated animals at various times after H^3 -thymidine. All means based on observations on four animals. 0, 26- to 36-hours after SRBC-stimulation (Group C); $\diamond$, 38- to 48-hours after SRBC-stimulation (Group E)

TABLE VI
PERCENTAGE LABELED MITOTIC FIGURES IN GROUPS D AND E*

Group	Stage	Hours after H ³ -thymidine **											
		1		3		5		7		9		11	
		$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$
D	Prophase and Metaphase	47.7	5.9	90.0	2.5	91.8	1.4	60.7	12.2	43.1	2.8	52.8	8.2
	Anaphase and Telophase	43.3	5.9	81.7	1.0	86.1	3.0	60.5	10.5	50.6	9.3	46.6	8.9
E	Prophase and Metaphase	42.9	6.2	87.3	3.0	89.3	2.5	67.8	11.7	43.4	3.0	55.6	5.0
	Anaphase and Telophase	24.6	6.3	73.8	5.9	78.6	9.1	72.8	14.3	47.7	6.2	58.6	11.1

* 100 mitotic figures scored per animal.

**See Footnote, Table I.

Figure 8. Percentage labeled mitotic figures in C₃ cells of SRBC-stimulated animals. 0, 26- to 36-hour prophase and metaphase; ◇, 38- to 48-hour prophase and metaphase; ●, 26- to 36-hour anaphase and telophase; ◆, 38- to 48-hour anaphase and telophase.

TABLE VII

PERIPHERAL BLOOD CELL COUNTS AND GERMINAL CENTER NUMBER AND VOLUME

Time Post- Inject.	SRBC-Stimulated Animals *							
	Total White		Total Red		GC Volume		GC Number	
	Counts x 10 ³		Counts x 10 ⁶		x 10 ⁴			
	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$
1 hour	20.9	5.7	11.5	.66	40.1	5.5	19.6	7.3
1.5	17.1	3.7	10.3	.51	45.7	20.3	23.0	8.8
2	18.8	4.1	9.7	.36	60.7	14.0	30.8	5.0
4	13.1	3.6	10.2	.90	47.9	12.6	18.0	9.6
6	13.4	1.7	8.9	.52	42.2	14.7	13.8	5.0
8	13.0	3.3	9.5	.69	16.4	3.5	5.4	2.3
10	10.3	1.0	9.7	.44	23.3	9.6	10.0	3.4
12	9.4	.9	9.8	.45	21.4	5.1	7.8	3.7
14	8.9	1.0	10.0	.52	19.9	3.4	6.0	2.5
16	10.3	2.0	9.7	.66	15.0	13.9	5.4	4.9
18	10.8	3.1	9.9	.55	3.1	7.0	.9	1.7
20	9.3	.8	9.8	.44	16.3	3.7	4.2	2.5
22	10.1	.5	9.8	.37	11.4	.1	1.0	1.0
24	6.5	1.2	9.8	.26	11.9	6.4	3.2	1.4
1.5 day	8.1	1.2	9.8	.42	33.3	16.2	9.4	2.5
2.0	7.8	1.0	9.9	.20	25.5	7.8	6.6	3.6
2.5	6.6	2.0	9.6	.59	39.8	14.8	9.2	3.9
3.0	8.0	1.2	9.7	.49	50.7	7.0	8.6	1.7
3.5	5.8	2.0	9.7	1.1	93.2	9.4	15.8	3.9
4.0	10.0	2.0	10.2	.4	134.4	16.5	24.0	2.0
All Controls	8.3	2.1	9.7	.9	34.6	11.0	16.3	7.5

*All means based on observations on five animals except the two hour interval in which each mean consists of ten animals.

$$s = \left[\sum_{i=1}^n (x_i - \bar{x})^2 / n(n-1) \right]^{1/2} = \text{standard deviation of the mean.}$$

Figure 9. White blood cell count per mm³ peripheral blood. ●, SRBC-stimulated animals; ○, Tyrode's control animals; ◇, normal control animals. All means based on observations on 5 animals except at the 2-hour interval, where each mean is based on observations on 10 animals. Perpendicular bars represent one standard deviation from the mean.

persisted until the eight-hour postinjection interval. WBC counts of both independent control groups showed no significant difference at the 5 per cent level or the four-day interval studied. The mean leucocyte count of the control animals was 8,310 cells per mm^3 .

In the SRBC-stimulated animals the leucocytosis up to two hours postinjection was predominantly due to a significant increase in small and medium lymphocytes, while both granulocytes and lymphocytes constituted the remainder of the leucocytosis (Fig. 10). Although the total number of small and medium lymphocytes recovered to normal levels during the first twelve hours, the granulocytes remained higher until twenty-four hours postinjection.

Except for a slight granulocytosis in the first two hours in the control group receiving Tyrode's solution, no difference was found in the distribution of cells per mm^3 , in the two control groups (Figs. 11a and b).

An increase in RBC count was present in the experimental animals one hour after SRBC-stimulation (Fig. 10). This response had subsided by two hours postinjection, and the RBC counts of the experimental animals remained normal throughout the remaining intervals.

No significant difference in the total number of large lymphocytes occurred in the experimental or control animals at any of the intervals studied (Figs. 10 and 11). The number of peripheral blood monocytes remained consistently below 500/ mm^3 in both the experimental and control animals with no significant difference in total number in either the experimental or control animals during the first four days after antigen injection.

Figure 10. Total differential count per mm^3 peripheral blood in animals injected with 1 ml 10 per cent SRBC. ●, RBC x 10^6 ; ○, small and medium lymphocytes x 10^3 ; □, granulocytes x 10^3 ; △, large lymphocytes x 10^3 . All means based on observations on 5 animals except at the 2-hour interval, where each mean is based on observations on 10 animals.

(a) Normal Control.

(b) Tyrode's Control.

Figure 11. Total differential count per mm^3 peripheral blood in control animals. ●, RBC $\times 10^6$; ○, small and medium lymphocytes $\times 10^3$; □, granulocytes $\times 10^3$; △, large lymphocytes $\times 10^3$. All means based on observations on 5 animals except at the 2-hour interval, where each mean is based on observations on 10 animals.

Normal Histology of Spleen

The general appearance of the normal mouse spleen as seen in sectioned material stained with hematoxylin and eosin is that of a collagenous framework within which is suspended a reticular framework. The collagenous framework consists of a capsule, which is thickened at the hilus of the organ. Branching and anastomosing continuations of the capsule, called trabeculae, penetrate the organ.

Between the trabeculae is the reticular framework which together with the cells present, forms the splenic pulp. This is composed of a typical lymphatic (white pulp) and an atypical lymphatic tissue (red pulp).

The lymphatic areas or white pulp of the spleen surround the splenic arterioles. The reticular framework of the white pulp is predominantly filled with free lymphocytes of various sized distributed to form nodules of lymphatic tissue, each of which is surrounded by a marginal zone of reticular cells. Generally, at the periphery of the nodules are one or two secondary nodules called germinal centers. Topographically these consist of a densely populated cellular side facing the center artery of the lymphatic nodule and a thinly populated cellular side facing the red pulp (Fig. 12).

The densely populated side of the centers are usually demarcated from the lymphocyte mass by a reticular border. This border does not appear to surround the germinal center completely and may be continuous with the marginal zone. The germinal centers appear paler than the surrounding mass of small lymphocytes with their small dark staining

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Figure 12. Spleen longitudinal section: C = capsule, RP = red pulp, T = trabeculae, GC = germinal center, CA = central artery, LN = lymphatic nodule.

nuclei. The centers also contain more mitotic figures in the densely populated side than are found in any other portion of the lymphatic nodule.

Scattered throughout the germinal centers are large primitive cells. These germinal center cells as seen in spleen sections are distinct in that they possess a large nucleus (at least $6.5\ \mu$ in diameter) which is stained pale violet. The chromatin is indistinct except for a large central dark-staining nucleolus with small interlacing strands attached to the periphery of the nucleus. The cytoplasm of these cells is basophilic and smooth. Cells of identical morphology could occasionally be observed in the lymphatic nodules and red pulp.

The red pulp is composed of a framework of reticular fibers (Billroth cords). At the boundary of the red and white pulp fibers of the former continue into those of the latter. Located in the meshes of the framework of the red pulp are all the elements of circulating blood. The cells found in the largest quantity are mature red cells. Among the nucleated cells present are predominantly lymphocytes and nucleated red cells, with granulocytes, histiocytes, megakaryocytes and occasionally mast cells and plasma cells.

Histology of Red Pulp of Primary Immunized Animals

The predominant change at one hour after SRBC injection was a diffuse RBC congestion (Figs. 13a and b). Coinciding with this was a marked depletion of small and medium lymphocytes in the red pulp. The congestion increased through the two-hour interval to the point that the marginal zones of the lymphatic nodules were almost completely masked

(a) SRBC, one hour after injection, X 250.
Note RBC congestion.

(b) Normal control animal, X 250.

(c) SRBC-stimulated, 24-hours after injection, X 400.

Figure 13. Spleen red pulp changes during the primary immune response.

by red blood cells. Granulocyte infiltration into the red pulp sinus was also evident at the two-hour postinjection interval.

Six hours after SRBC the diffuse pattern of red pulp congestion began to subside, and the red blood cells predominantly appeared in clumps. Accompanying the clumping of red cells was an increased number of lymphocytes in the red pulp. This pattern persisted during the first twenty-four hours postinjection. Active phagocytosis of red blood cells by reticular macrophages was evident throughout the red pulp and the congestion had significantly decreased by twenty-four hours after injection of the sheep red blood cells.

At twenty-four hours after injection of the antigen a number of primitive lymphatic tissue cells were detected in the red pulp (Fig. 13c), closely associated with the marginal zones of the lymphatic nodules. A difference in the number of these primitive cells in the red pulp as compared to the normal animals was evident. A moderate amount of mitosis among these cells could also be observed.

Between days one and two the most striking change in the spleen red pulp of SRBC-stimulated animals was a progressive increase in the number of primitive lymphatic tissue cells. Although they could still be observed closely associated with the marginal zones of lymphatic nodules they were also diffusely scattered in other areas of the red pulp. Concurrent with the increase in number was an increase in detectable mitosis among these cells. A contrasting morphological difference in these cells at the two day interval as observed in the hematoxylin and eosin stained preparations was an increased basophilia of the smooth, irregular cytoplasm.

By the middle of the third day clusters of primitive lymphatic tissue cells were isolated in the Billroth cords and along trabeculae of the red pulp and upon closer inspection were found to be in approximation with immature and mature plasma cells. Although these plasmacytic islands were small at the 2.5 day interval, they increased in size and by the fourth day postinjection large sheaths of plasma cells occupied the majority of the red pulp (Fig. 14). Characteristic of the sheaths at this time were mature cells containing small pyknotic nuclei and deeply pyroninophilic or basophilic cytoplasm.

No congestion or increased plasmacytogenesis was observed at any time in the spleen of control animals.

Histology of White Pulp of Primary Immunized Animals

In the SRBC-stimulated animals no histologic changes were observed in the lymphatic nodules until two hours postinjection, at which time the germinal centers were well defined and larger in mean surface area (estimate) than those of control animals. Figure 15a shows a germinal center in a stimulated animal two hours after SRBC-injection. At all periods, the germinal centers contained predominantly pyronin-positive cells. At the two hour interval, cells in the germinal centers of the stimulated animals appeared to be more intensely stained by the pyronin than those in animals given Tyrode's solution or in normal control animals.

In the lymphatic nodules of the animals stimulated six hours previously, an edema-like change was observed around the central artery, accompanied in this region by a decrease in the number of lymphocytes

(a) Spleen red pulp, 2.5 days after SRBC-stimulation,
X 400.

(b) Spleen red pulp, 4 days after SRBC-stimulation,
X 400.

Figure 14. Plasmacytopoiesis in the spleen red pulp of
SRBC-stimulated animals.

(a) Spleen white pulp germinal center 2-hours after SRBC-stimulation: GC = germinal center, MZ = marginal zone, LM = lymphocyte mass, X 250.

(b) Spleen white pulp germinal center 16-hours after SRBC-stimulation: ELA = edema-like area, RGC = remnant of germinal center, X 400.

Figure 15. Spleen white pulp germinal center changes during early intervals of the primary immune response.

per unit area and in increased prominence of fixed reticular cells and reticular macrophages. The area of the edema-like change increased by eight hours postinjection, and at ten hours was surrounded by a dense border of cells, predominantly composed of small and medium lymphocytes (Fig. 16a). By twenty-four hours after SRBC injection almost all lymphatic nodules of the spleen were affected by the edema-like change (Fig. 16b). During the development of this condition a small but obvious amount of phagocytosis was observed occurring in this area of the lymphatic nodules. Clumps of red cells and small pyknotic nuclear debris were observed in the reticular macrophages.

In the hematoxylin and eosin-stained sections of spleen from SRBC-stimulated animals the germinal centers were large and prominent at two hours. Measurement of the mean longest-diameter, expressed as mean volume, demonstrated that during the first twenty-four hours the germinal centers attain maximum volume at two hours postinjection (Fig. 17). This coincides with the peak number of germinal centers counted per longitudinal section of spleen (Fig. 18). The volume of the germinal centers began to decrease at four hours after SRBC-injection and was less than that of the controls between sixteen and twenty-four hours. Figure 15b shows a remnant of a germinal center at sixteen hours after SRBC-stimulation (page 56). Concurrently the number of germinal centers per longitudinal spleen section in the stimulated animals declined.

Histologically the germinal centers at one day postinjection appeared to be depleted of the characteristic large cells and

(a) Edema-like area of spleen lymphatic nodule 10-hours after SRBC-stimulation: ELA = edema-like area, CA = central artery, GC = germinal center, X 250.

(b) Spleen lymphatic nodule, 24-hours after SRBC-stimulation, X 100.

Figure 16. Spleen lymphatic nodule changes during the first day of a primary immune response.

Figure 17. Mean germinal center volume $\times 10^4 \mu^3$: O, SRBC-stimulated animals; ●, Tyrode's control animals; Δ, normal control animals. All means based on observations on 5 animals except at the 2-hour interval, where each mean is based on observations on 10 animals. Perpendicular bars represent one standard deviation from the mean.

Figure 18. Mean number of germinal centers per longitudinal section of spleen: ○, SRBC-stimulated animals; ●, Tyrode's control animals; △, normal control animals. All means based on observations on 5 animals except at the 2-hour interval, where each mean is based on observations on 10 animals. Perpendicular bars represent one standard deviation from the mean.

demonstrated a residual reticular stroma. Generally observed within the residual stroma were medium and small lymphocytes, macrophages and occasionally granulocytes.

A recovery of the germinal center volume occurred between days one and three postinjection. By day four the mean germinal center volume of the experimental animals demonstrated an approximate four fold increase over the control animals. Concurrently the number of germinal centers per longitudinal spleen section in the experimental animals began to increase between days one and three postinjection; however, at day four they only reached the upper limit of the control animals.

The histologic observations of the germinal centers during these intervals suggested an increase in the number of germinal center cells with an increased number of mitotic figures. By day four the germinal centers were extremely hyperplastic and the number of germinal center cells had increased to the point that it was difficult to distinguish the densely populated from the thinly populated region.

Analysis of variance of the two independent control groups showed no significant difference at the 5 per cent level either in germinal center volume or number per longitudinal section. A significant difference at the 5 per cent level did exist between the over-all response of the experimental and control animals.

In both stimulated and control animals the large primitive lymphatic tissue cells were initially concentrated in the germinal centers, and a small but constant number of them were present in the lymphatic nodules, and red pulp during the early intervals of this study. An

increase in these cells was detected in the lymphatic nodules of the stimulated animals at sixteen hours after SRBC injection. At this time they were predominantly associated with the lymphocyte band surrounding the edema-like area, although some were also observed in the region of the nodule where the edema-like change was developing. A steady increase in the number of these primitive cells occurred throughout the nodule during the remaining intervals. Figure 19 shows the primitive lymphatic tissue cells in the spleen lymphatic nodules of antigen-stimulated animals at 16 and 24 hours after antigen injection. In the stimulated animals the increase in the large lymphatic tissue cells in the nodules coincided closely with the decrease in germinal center volume. The decrease in germinal center volume thus is a result of cellular emigration.

Experiment III: Spleen Imprint Autoradiography

In the animals injected with H^3 -thymidine twenty-three hours after having been stimulated with SRBC, a significant increase at the 5 per cent level occurred in the PLTC cells in comparison with the controls (Table VIII). This response was measured at all three intervals scored between days one and two after SRBC-stimulation. Approximately 77 per cent of the PLTC cells were initially labeled and the percentage labeling decreased only slightly within the first twenty-four hour interval. A significant increase also occurred in the percentage distribution of immature plasma cells between days one and two post-antigen injection. Although the percentage of labeled cells in this group was

(a) Primitive lymphatic tissue cells in spleen lymphatic nodule 16-hours after SRBC-stimulation, X 1000.

(b) Primitive lymphatic tissue cells in spleen lymphatic nodule 24-hours after SRBC-stimulation, X 400.

Figure 19. Increase in primitive lymphatic tissue cells in the splenic lymphatic nodules during the primary immune response.

TABLE VIII

PERCENTAGE DISTRIBUTION AND PERCENTAGE LABELING OF CELLS IN SPLEEN IMPRINTS

Cell Type	H ³ -thymidine Time After Antigen	Time after H ³ -thymidine*											
		1 hour				13 hours				25 hours			
		% Cells		% Labeled		% Cells		% Labeled		% Cells		% Labeled	
		$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$
PLTC	23 hr	1.1	0.3	76.8	7.1	1.3	0.1	67.1	11.7	2.3	0.5	65.0	10.0
LL		1.4	0.4	25.4	10.2	1.1	0.3	37.4	5.8	1.6	0.3	32.7	7.6
S&ML		87.2	1.5	0.4	0.1	88.8	1.4	0.8	0.1	85.8	1.6	0.9	0.1
Gran. .		2.6	1.0	0.0	0.0	1.2	0.1	1.4	1.2	1.9	0.2	0.0	0.0
Eryth.		5.1	0.7	30.1	20.5	5.8	1.5	38.6	4.8	6.1	0.5	44.3	15.8
Hist.		2.7	1.1	14.4	3.9	1.3	0.2	27.6	9.2	1.3	0.1	21.5	7.6
IPC		0.1	0.1	13.3	29.9	0.5	0.1	81.2	15.2	1.4	0.4	86.5	9.3
MPC		0.0	0.0	0.0	0.0	0.2	0.1	0.0	0.0	0.2	0.1	26.4	40.1
PLTC		2.1	0.4	79.8	6.1	1.4	0.2	71.7	4.2	0.6	0.1	54.6	8.5
LL		1.1	0.1	21.5	6.4	0.9	0.1	41.3	5.3	1.0	0.1	36.8	6.4

TABLE VIII, CONTINUED

Cell Type		H ³ -thymidine Time After Antigen	Time after H ³ -thymidine*											
			1 hour				13 hours				25 hours			
			% Cells		% Labeled		% Cells		% Labeled		% Cells		% Labeled	
			$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$
S&ML	47 hr.	86.3	1.3	0.5	0.1	87.8	1.4	0.9	0.2	82.8	2.6	0.6	0.1	
Gran.		1.5	0.4	0.0	0.0	1.1	0.2	0.0	0.0	1.4	0.1	0.0	0.0	
Eryth.		6.2	0.8	35.9	10.4	4.8	0.5	40.0	6.6	7.2	3.1	48.8	9.2	
Histio.		1.2	0.1	15.2	5.3	1.0	0.2	18.7	4.5	1.1	0.2	23.4	5.1	
IPC		1.5	0.3	78.6	5.1	2.6	0.4	86.0	5.1	2.3	0.6	74.7	5.3	
MPC		0.4	0.1	6.8	9.5	0.7	0.1	64.2	18.2	3.6	1.1	66.2	7.5	
PLTC	71 hr.	0.4	0.1	84.0	8.3	0.7	0.2	49.6	4.8	0.4	0.2	42.1	14.6	
LL		0.7	0.2	31.9	10.6	1.0	0.1	47.0	4.5	0.7	0.1	33.3	6.5	
S&ML		84.5	1.3	1.0	0.4	79.2	2.1	0.9	0.2	76.1	1.5	1.5	0.3	
Gran.		1.5	0.2	0.0	0.0	0.9	0.2	0.0	0.0	1.1	0.1	0.0	0.0	
Eryth.		4.1	0.4	30.0	7.4	5.3	1.1	40.8	3.9	6.6	0.5	50.2	4.6	
Hist.		1.1	0.2	27.5	12.4	0.9	0.2	30.7	11.6	0.9	0.1	27.9	8.1	
IPC		3.2	1.0	88.5	8.5	6.2	0.9	83.9	5.9	6.0	0.4	67.6	5.3	
MPC		4.5	0.8	16.7	10.6	4.1	0.6	75.0	2.2	9.1	0.4	69.6	1.6	

TABLE VIII, CONTINUED

Cell Type	H ³ -thymidine Time After Antigen	Time after H ³ -thymidine*											
		1 hour				13 hours				25 hours			
		% Cells		% Labeled		% Cells		% Labeled		% Cells		% Labeled	
		$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$	$\bar{X}$	$\pm s$
PLTC		0.3	0.1	79.8	21.2	0.2	0.1	69.4	24.3	0.3	0.1	56.6	22.3
LL		1.2	0.2	33.5	14.6	0.7	0.2	46.6	19.4	0.7	0.1	45.5	21.4
S&ML		90.9	2.4	0.5	0.1	92.1	2.5	0.7	0.1	92.8	0.2	0.8	0.2
Gran.	All controls: 23, 47, 71 hours	2.3	0.8	0.6	1.7	0.9	0.2	1.6	4.7	1.4	0.2	0.0	0.0
Eryth.		4.5	1.7	29.2	10.6	5.3	2.0	42.0	5.3	3.8	0.2	45.6	21.1
Hist.		1.0	0.2	18.5	9.6	0.7	0.2	20.4	16.0	0.7	0.2	23.6	8.6
IPC		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
MPC		0.1	0.2	0.0	0.0	0.1	0.1	0.0	0.0	0.1	0.1	0.0	0.0

*All means based on observations on five animals.

$$s = \left[\sum_{i=1}^n (x_i - \bar{x})^2 / n(n-1) \right]^{1/2} = \text{standard deviation of the mean.}$$

not high one hour after H^3 -thymidine, more than 85 per cent were labeled twenty-five hours later. No difference in the percentage distribution of other cell types was recorded in the spleens of stimulated and control animals during the first twenty-five hour interval.

A morphologic difference could be observed in the PLTC during the first two days following antigen stimulation (Figs. 20a and b). The cytoplasm was distinctly becoming more basophilic and the chromatin texture was more defined. The nuclei were stained more intensely and the chromatin blocking resembled that of a plasmablast. Although the nuclear-cytoplasmic ratio of these cells appeared not to have changed, due to the morphologic alteration these cells were considered intermediary between a C_3 or dispersed germinal center cells and immature cells of the plasma cell family.

In the animals injected with H^3 -thymidine forty-eight hours after antigen a decrease in the percentage distribution of primitive lymphatic tissue cells occurred during the following twenty-five hours with a concurrent increase in immature plasma cells. Mature plasma cells also increased during this interval and, although they were not significantly labeled at forty-eight hours, more than 65 per cent of them were labeled at seventy-two hours. Between days three and four postinjection of antigen, the number of primitive lymphatic tissue cells remained at about 0.5 per cent of the total cells while the percentage labeling of these cells decreased by one half. The immature plasma cells continued to increase with a concurrent decrease in percentage labeled cells, while the mature plasma cells increased with a concurrent increase in

(a) PLTC, 24-nours after
SRBC-stimulation
X 1000.

(b) PLTC, 48 hours
after SRBC-
stimulation
X 1000

(c) Plasmacytogenesis 4-days after SRBC-stimulation:
IPC = immature plasma cells, MPC = mature plasma
cells, X 400.

Figure 20. Primitive lymphatic tissue cell transformation during the primary immune response.

percentage labeled cells. Figure 20c shows a plasmacytic island in the red pulp of a SRBC-stimulated spleen four days after injection (page 68).

DISCUSSION

Blood Clearance of Tritium Activity

Rubini et al. (1958) have studied the plasma and body clearance of H^3 -thymidine in man. The plasma is almost completely cleared of H^3 -thymidine in thirty minutes. Nonvolatile tritium activity, other than thymidine, begins to appear in the plasma during the same thirty-minute interval, but diminishes between one-half hour and one hour, at which time it is negligible. These workers suggest that a large portion of the labeled nonvolatile compound is beta aminoisobutyric acid. The concentration of tritiated water reaches a maximum within forty minutes after thymidine administration, and subsequently demonstrates a nine-day half life in the body.

The results of the present study indicate an exponential decrease in tritium activity of plasma during the first thirty minutes after H^3 -thymidine administration. Based on the studies of Rubini et al., this is interpreted as being representative of the decrease in H^3 -thymidine. At the thirty-minute postinjection interval the counts per minute represented approximately 25 per cent of the activity measured one minute after injection and then decreased an additional 5 per cent during the following thirty minutes. This phase of the tritium activity is more closely related to the previously demonstrated clearance of nonvolatile products and tritiated water. Thus, it can be assumed that whereas tritiated thymidine activity decreases in the plasma in an exponential manner during the first thirty minutes post-injection, simultaneously an increase in the activity of other

tritiated nonvolatile products and water occurs. The latter is cleared at a less rapid rate.

Percentages of Labeled Germinal Center Cells

It is clear that the flux of cells has been distinctly altered in the germinal centers of SRBC-stimulated animals. At two and four hours after primary immunization, a 10 and 20 per cent increase, respectively, in the percentage of labeled cells is observed in the C_1 cells of the germinal centers in contrast to the controls. This difference in the early intervals can be accounted for in two ways: a stimulation by antigen for increased mitotic activity in the DP region of the germinal center, and/or an increase in migration from the DP to TP region of cells that were not labeled. It is quite reasonable that both of these possibilities could be operative at the same time.

The results presented here favor the idea that, as a direct or indirect result of the antigen, an increased number of C_1 cells were stimulated to divide in the DP side of the germinal center, and these cells emigrated from this area.

In the TP side of the germinal center of SRBC-stimulated animals, a slight increase in percentage of labeled cells occurs in the early intervals, followed by a decrease at the seven- and nine-hour intervals. However, the mean percentage of labeled cells is higher than in the adjacent DP area at these intervals. This observation substantiates the idea that the cells in the DP region are precursors of the cells in the TP region (C_2). Support for the postulate that the C_2 cells

then migrate from the germinal center is obtained from the difference in expected and observed percentage of labeled C_2 cells (see Table II).

In the control animals, a progressive increase in the percentage of labeled cells in both areas of the germinal center is demonstrated up to seven and nine hours after H^3 -thymidine. An abrupt decrease follows which, in all probability, is partially caused by dilution of the label. These data indicate a steady state condition in the controls in which cells are entering and leaving each compartment at a relatively low but constant rate. This observation is consistent with recent reports of Fliedner et al. (1963) which suggest a steady state compartmentalization of cells in the splenic germinal centers of normal rats.

Germinal Center Cell Proliferation

Any attempt to determine the kinetics of cellular proliferation in germinal centers is complicated by the fact that these are not closed systems. A generalized scheme of an open system is that the size of the primary compartment at any given time is a function of the output rate and the generation time of the cells, while the size of the intermediate compartments is a function of the output rate, the generation time of the cells, and the rate of migration from the preceding compartments. A further consideration that must be made in evaluating these data is the reutilization of the labeled thymidine. Finally, any change in nuclear volume between interphase and telophase nuclei would be a factor in scoring grain density on a per unit area basis. However, it was considered that this variable would be minor

compared to those that would exist in counting total grains per nuclei in sectioned material.

The data presented in this study show that at two hours after the injection of SRBC or Tyrode's solution no significant difference in the mean grain count exists between the cells in the TP region of the germinal center and the morphologically identical cells in the lymphocyte mass. A statistically significant difference at the 5 per cent level does exist, however, between both of these cells and the cells of the DP side of the germinal center. The data also suggest a three- to four-hour difference in doubling time between cells of the DP side and the other two areas. If this is indeed the case, then the difference of mean grain counts one hour after H^3 -thymidine could be explained by the fact that some of the more heavily labeled C_1 cells would have reached prophase and would not have been scored.

The five- to seven-hour doubling time of dividing GC cells, which are primarily those cells of the DP side, substantiates the six to seven-hour estimate made by Fliedner et al. (1963). This assumes an approximate S period of four to five hours with a G_2 , M, and G_1 time of about one hour. In most mammalian tissues so far investigated, the length of the S period is five to ten hours (Lajtha, 1959). This indicates that the C_1 cells are approaching the lower limit in which any cell has thus far been shown to perform the required processes in preparation for division. A mitotic duration of less than one hour is consistent with measurements on several cell types in mice (Knowlton, and Widner, 1950).

The nine- to ten-hour doubling time of the C_2 and C_3 cell population, as measured in the first forty-eight hours in the primary immune response corresponds closely with the eight- to nine-hour estimate given by Mäkelä and Nossal (1962) for antibody forming cells in the first 24 hours of the secondary immune response and by Capalbo and Makinodan (1963) for transplanted antibody forming cells maintained in a closed system. These data also demonstrate that the C_3 cells have an S period of approximately six hours, and the total generation time is no longer than ten hours. These data are consistent with measurements of Sado (1963) for transplanted antibody forming blast cells of spleen which were maintained in a closed system.

Autoradiographic studies of GC cells shortly after H^3 -thymidine have demonstrated that the DP region nearest the central artery contains numerous dividing cells and is heavily labeled, while the TP region nearest the red pulp shows virtually no mitotic activity and is lightly labeled (Yoffey et al., 1961). Based on these studies and early histologic observations, it has been postulated that the cells in the DP region are precursors of the TP cells. The data obtained in these experiments on serial measurement of the mean grain counts of cells in the two topographical regions of the germinal center favor the proposed pattern of migration. Three hours after H^3 -thymidine, the C_2 cell population demonstrated two distinct modes based on mean grain counts. In both the stimulated and control animals, about 25 to 30 per cent of the labeled cells have mean grain counts comparable to those cells present in the TP region at one hour. The remaining 70

per cent correspond closely to daughter cells of both the previous C_1 or C_2 cell populations. As pointed out in this study, and as demonstrated by Yoffey et al. (1961), virtually all of the mitotic activity is restricted to the DP region of the germinal center; therefore, it is a natural conclusion that the daughter cells migrated into the TP side. In this region they undergo a second S period while waiting to pass out into the lymphocyte mass or red pulp. At seven to nine hours after the administration of H^3 -thymidine, the cells in both DP and TP regions are identical, thus indicating a complete turnover of the labeled cell population in the TP side of the germinal center.

Hematologic and Histologic Studies

According to the histologic findings of this study, the germinal centers increased in volume two hours after SRBC injection, and then progressively decreased in volume during the first twenty-four hour interval. There was also an increase in the number of germinal centers counted per longitudinal section during the first two hours after exposure to the antigen followed by a decrease during the first twenty-four hour interval. It is reasonable to assume that if the volume of each germinal center increased at two hours postinjection, the probability of sectioning through any one germinal center increases proportionately, assuming there is no increase in size of the spleen. The last assumption can be made based on studies of Congdon and Makinodan (1961) in which no increased weight in spleen was measured during the first twenty-four hours after a similar dose of SRBC in mice. Thus, a decrease in volume would result in a decreased number of germinal

centers counted per longitudinal section, the latter being relative rather than absolute.

The increase in the germinal center volume after SRBC injection can be accounted for in two ways: an increase in the size of the cells and/or an increase in the number of cells per germinal center. According to the previously discussed autoradiographic studies, the dividing cells of the germinal centers have a doubling time of five to seven hours. Also, in comparison with normal animals, increased proliferation has been observed in spleen white pulp germinal centers two hours after SRBC injection. Thus, an increase in the number of cells per germinal center is a reasonable explanation to account for the increased volume observed at two hours after SRBC stimulation.

This early hyperplasia of the germinal centers in the animals exposed to the antigen coincides with an apparent increase in generalized pyroninophilia, as demonstrated in the methyl green-pyronin preparations. No conclusions can be made regarding the relation of the hyperplasia to an increase in cytoplasmic RNA per germinal center cell. The increased pyroninophilia may simply be an artifact resulting from an increased density of the cell population.

The decrease in volume of germinal centers observed during the first twenty-four hours postinjection in hematoxylin and eosin stained preparations coincides closely with an observed increase in baophilic primitive lymphatic tissue cells in the lymphatic nodules. Histologically, the germinal centers during this interval appeared to be depleted of their characteristically large cells, and demonstrated a residual

reticular stroma. This observation lends further support to the idea that germinal center dissociation results in a migration of primitive lymphatic tissue cells from the germinal center to the associated lymphocyte mass.

The early lymphocytosis detected in the peripheral blood of SRBC-stimulated animals one hour after injection is a unique and interesting finding. Correlating this observation with those from the hematoxylin and eosin-stained sections of spleens taken at this interval, RBC congestion in the red pulp was maximal at the time when there was a marked depletion of small and medium lymphocytes from the same splenic region. Thus, a possible source of the peripheral blood lymphocytes at the one-hour postinjection interval is the splenic lymphocyte reserve that may have been forced out by the RBC congestion in the red pulp. It is not known what percentage of the red blood cells involved in the spleen congestion are heterologous; however, since a large number of RBC were injected ($\sim 2.0 \times 10^9$), it is assumed that some are of sheep origin. The granulocytosis observed in the peripheral blood of the experimental animals is consistent with previous observations (Conway, 1937).

An edema-like change was observed to form around the central artery of the lymphatic nodules of SRBC-stimulated animals at four hours after injection. This white pulp region was less dense than at the one-hour stage, and its reticular cells were more prominent. The development of this condition was present throughout the first twenty-four hour interval with a progressive increase in the edema-like area

until it occupied most of the nodule. Histologic observations also revealed a relationship between the increase in size of the edema-like change which was marked by a packing of cells (mostly small and medium lymphocytes) around the periphery of the nodule, and an increase in small and medium lymphocytes in the red pulp. Thus, it appeared that the lymphocytes generally observed in the periarterial lymphatic sheath were being forced out into the red pulp. This observation was also reported by Conway (1937) in guinea pigs after a bacterial infection.

The possibility was considered that the edema-like change of the white pulp of the spleen could be a stress response, as described by Dougherty and White (1945), resulting from cortisone action on lymphocytes. However, involution of the thymus, a consistent feature in the cortisone stress response in lymphatic tissue, was not observed in the present studies or in studies reported by Congdon (1962). A further response of cortisone, described in the studies of Dougherty and White, was the intense pyknosis and phagocytosis of small and medium lymphocytes in the lymphatic nodules of spleen and lymph node cortex. The present studies suggest a relocation of lymphocytes, but give very little evidence of their destruction, leading therefore to the view that the edema-like change is a local lymphatic tissue response more directly associated with antigenic stimulation.

Coinciding with the edema-like change in the white pulp of the SRBC-stimulated spleen was the dissociation of the cells constituting the germinal centers, and an increased number of primitive lymphatic tissue cells in the associated nodules resulting in a "blurring of the

white pulp." This observation is considered a consequence of proliferation in the associated lymphocyte mass of cells which imigrated from germinal centers.

Between days one and four after SRBC-administration, primitive lymphatic tissue cells were observed accumulating in the spleen red pulp. These cells were closely associated with plasmacytic islands between days two and four. By day four, large sheaths of plasma cells occupied the major portion of the red pulp. Thus, the "plasma cellular reaction" (Fagraeus, 1948) as observed in the experimental animals, is closely associated with the increased occurrence of the C_3 cells in the spleen red pulp.

In the lymphatic nodules between days one and four after SRBC-stimulation, an increase in the mean volume of germinal centers was measured. This secondary hyperplasia histologically suggested an increase in the number of GC cells with a concurrent increased number of mitotic figures. By day four the germinal centers were extremely hyperplastic and the number of GC cells had increased to the point that it was difficult to distinguish the densely populated from the thinly populated region. Coinciding with the increase in mean volume was a similar but less intense increase in the mean number of germinal centers per longitudinal section.

A gross increase in spleen volume was also observed during these intervals, which is consistent with a mean spleen weight increase between days one and four after SRBC reported by Congdon and Makinodan (1961). Thus, the measurements of both volume and number of germinal

centers per longitudinal section during this interval are relative to the total increase in spleen volume.

The combined results of experiments I and II suggest that germinal center cells respond to antigenic stimulation by proliferation which can be detected in two ways: (1) within intact germinal centers (hyperplasia), and (2) in a dissociated manner with migration. Both of these responses are considered characteristic of the inductive phase of the primary immune response, resulting in an accumulation of large numbers of primitive lymphatic tissue cells in the region of the lymphatic nodules and red pulp.

The hyperplasia of the intact germinal center is composed of a primary and secondary phase. The primary hyperplasia measured at two hours after antigen results in the migration of cells from the DP compartment to the TP compartment, and finally to the associated lymphocyte mass and red pulp (Fig. 21).

A conclusion relating to the primary hyperplasia and resulting dissociation of the germinal center is that there must be a primary compartment of the germinal center (which will contain C cells) not directly measured in these experiments. It is suggested by the data that this compartment must transport cells to the DP region of the germinal center resulting in a C to C_1 cell transformation. The input ($C \rightarrow C_1$) must be relatively constant, and for a period of time during the early intervals cannot keep up with the rapid emigration of C_1 and C_2 cells from the germinal center. Eventually the primary compartment produces enough cells to repopulate the germinal center, resulting in a secondary hyperplasia.

(a) Spleen white pulp germinal center 2-hours after SRBC-stimulation: RP = red pulp; TP = thinly populated; DP = densely populated; LM = lymphocyte mass; X 400.

(b) Schematic diagram of spleen white pulp germinal center. Arrows indicate emigration pattern.

Figure 21. A schematic illustration of the proposed pattern of migration of germinal center cells during the early intervals of the primary immune response.

Studies by Cottier et al. (1963) of the secondary immune response in mice have suggested the formation of new germinal centers in the popliteal lymph nodes draining the site of tetanus toxoid injection. This hypothesis was primarily based on the mean number of germinal centers counted per longitudinal section. Their incomplete data are consistent with those given in the present study. However, Cottier and collaborators assume that the initial increase immediately after stimulation and the later increase in number of germinal centers is a result of de novo formation of germinal centers in response to the antigen.

A failure of the study of Cottier et al., was that no measurements were made on changes in germinal center volume during the secondary immune response. Similarly, lack of measurements of the popliteal lymph node response between twelve and twenty-four hours after injection prevented the histologic observation of the residual germinal centers. Thus, merely counting the number of germinal centers per longitudinal section of tissue is not valid in establishing an absolute increase in number or de novo formation of germinal centers following antigenic stimulation.

The results of the present study confirm the idea of an intrinsic structure of lymphatic germinal centers (Ortega and Mellors, 1957). These data suggest that the lymphatic tissue response to antigen does not result in a change in absolute number of germinal centers but rather a change in mean volume of the existing germinal centers.

Spleen Imprint Autoradiography

The fate of the primitive lymphatic tissue cells was studied by systematically following the difference in percentage distribution and percentage of labeled nucleated cells of the spleen at various intervals after antigen injection. The results of these studies establish a significant difference in the percentage of PLTC in stimulated and control animals. An approximate threefold increase in these cells was measured at one day postinjection in the experimental animals. This difference more than doubled in the following twenty-four hours. Approximately 77 per cent of these cells were labeled one hour after H^3 -thymidine, and the percentage of the labeled cells decreased only slightly within the first twenty-four hour interval.

An apparent fourteenfold increase in the immature plasma cells occurred between days one and two after SRBC, and although these cells were not significantly labeled initially, more than 85 per cent of them were labeled by day two. These data suggest that the source of the immature plasma cells had to be a labeled cell population. Based on the changing morphology of the PLTC described between days one and two after antigen injection, it is reasonable to assume that these cells are precursors to the immature cells. Thus, the PLTC (C_3 cells), during their proliferation accompanying germinal center dissociation in the primary immune response, differentiate into immature plasma cells. This can also be measured by the retention of label in the plasmacyte.

Between days two and four, the percentage of PLTC decreases and the percentage of IPC increases. The immature plasma cells must also

be rapidly proliferating due to the initial percentage labeling. There is also an increase in mature plasma cells during the two and four day intervals. It can be observed that during the second and third day interval, the percentage of labeled mature plasma cells is extremely low one hour after H^3 -thymidine and increases very rapidly thirteen and twenty-four hours after H^3 -thymidine administration. This indicates that mature plasma cells are not actively synthesizing DNA, and are direct products of IPC. This is consistent with data presented by Mäkelä and Nossal (1962).

These data suggest that the PLTC (C_3 cells) transform into cells of the plasmacytic series during early intervals of the primary immune response. This interpretation is based on the relative changes in percentage distribution of cells, morphology, and percentage of labeled cells during these intervals. It must be pointed out that the data expressing the percentages of various cell types are not absolute. During the postinjection intervals of the study the spleen increased in size due apparently to an increased number of cells per unit volume.

General Discussion

There is evidence that all primary immune responses involve the induction of specific primitive cells in lymphatic tissue with subsequent proliferation and differentiation (Conway, 1937; Rich et al., 1939; Fagraeus, 1948; Ringertz and Adamson, 1950; Coons et al., 1955; Makinodan et al., 1954; Thorbecke and Keuning, 1955; Wissler et al., 1957; Ward et al., 1959). A most important problem remaining unanswered

is the origin and life history of these cells. This is true whether one agrees with the clonal selection model (Burnet and Fenner, 1949), or other existing models, in which the cells are multipotent and uncommitted until induced by the antigen (Schweet and Owen, 1957).

The results of the present studies suggest that in the spleen there is a series of at least four antigenically competent compartments of cells $[C_1 \rightarrow (C_2-C_3) \rightarrow IPC \rightarrow MPC]$, which originate in the white pulp germinal centers. The C_1 compartment originating in the DP region of the germinal center is the least differentiated of the series and possesses an unusually short generation time (~ 7 hours). The second compartment (C_2-C_3) is transient between the germinal center and other areas of the spleen, and is capable of transforming into cells of the plasmacytic family.

These studies further suggest that a primary compartment of the germinal center is also implicated in the series. This compartment apparently gives rise to the C_1 cell and could be termed the C-cell. The question of the origin of this cell has a special importance from an immunologic point of view. In the primary response the cell capable of this differentiation could possibly represent the type which either constructs or already has within it information necessary for antibody specificity. In the secondary antigenic response, the germinal center cell population is capable of undergoing a more rapid switch to plasmacytopoiesis, and presumably some members have "remembered" the antigen stimulus of the primary response. Perhaps the C-cell is the "memory cell" involved, which has remained fixed in the tissue since the primary

response, only waiting for the stimulation from further antigen to engage in a burst of plasmacytopoiesis.

On a histologic basis, the underlying reticular stroma of the germinal center is a good possible source of the C-cell. This would assume that primitive reticular cells forming the border between the DP side of the germinal center and lymphocyte mass could transform either directly or by division into C_1 cells. Although no other evidence can be provided to substantiate this hypothesis, the tissue autoradiograms have demonstrated incorporation of H^3 -thymidine in reticular cells of the marginal zone and residual stroma of germinal centers. Also, consideration must be given to the proposed transformation of the small lymphocyte into germinal center cells and primitive lymphatic tissue cells (Gowans, 1962; McGregor and Gowans, 1963).

SUMMARY

Autoradiographic studies using tritiated thymidine were performed on germinal centers in the spleens of primary sheep red blood cell (SRBC)-stimulated mice during the first 48 hours after intravenous injection. Mean grain counts and the percentage of labeled cells were determined for cells located in the densely populated (dark-staining) and thinly populated (light-staining) regions of the germinal centers. Mean grain counts were also made on the morphologically identical cells dispersed throughout the lymphocyte mass associated with the germinal centers.

The results suggest the following:

1. In SRBC-stimulated and control animals the cells of the densely populated side of the germinal centers are precursors of those in the adjoining thinly populated region, and the latter cells migrate into the lymphocyte mass and red pulp.
2. The doubling time of dividing cells in the germinal centers (those in the densely populated region) is 5 to 7 hours, while the doubling time of cells of the thinly populated region and those dispersed throughout the associated lymphocyte mass is 9 to 10 hours.
3. The germinal centers in control animals demonstrate a steady state system in which cells are entering and leaving each compartment at a constant rate. In SRBC-stimulated animals, evidence for disruption of this steady state is manifested by an increased number of dividing cells and an increase in the number of cells migrating into

the lymphocyte mass and red pulp. This response ultimately results in complete germinal center dissociation.

Histologic investigations were also performed on the lymphatic tissue and germinal centers of the mouse spleen with concurrent measurements of cellular changes in the peripheral blood during the first four days after a primary SRBC-stimulation.

The results of these studies suggest the following:

1. An increase in mean germinal center volume and mean number of centers per longitudinal section occurs two hours after SRBC-injection, followed by a rapid decrease in both parameters over the subsequent 22 hours. A recovery in both germinal center volume and number occurs between days one and four following injection. At day four a fourfold increase in germinal center volume was measured in the antigen stimulated group.

2. In the SRBC-stimulated animals the increase in the number of primitive lymphatic tissue cells in the lymphocyte mass coincides closely with germinal center dissociation. Topographically, the sequence of the increase in number of primitive lymphatic tissue cells is first in the area of the dissociated germinal center, second in the edema-like region of the lymphatic nodule, and third in the red pulp of the spleen.

3. An early lymphocytosis in the peripheral blood of the SRBC-stimulated animals one hour after injection coincides with a marked depletion of small and medium lymphocytes in the spleen red pulp resulting from RBC congestion in this area.

4. Germinal center cells respond to antigenic stimulation by proliferation which can be detected in two ways: (1) within intact germinal centers (hyperplasia), and (2) in a dispersive manner with migration. Both of these responses are considered characteristic of the inductive phase of the primary immune response, resulting in a local accumulation of large numbers of primitive lymphatic tissue cells in the spleen red pulp.

5. Coinciding with the increased number of primitive lymphatic tissue cells in the spleen red pulp was the formation of foci of plasma cells in which active plasmacytopoiesis could be observed.

The fate of primitive lymphatic tissue cells during early intervals of the primary immune response was interpreted from spleen imprint autoradiograms. These studies suggest that the cells (C_3) of the dissociated germinal centers transform into immature plasma cells, the transformation beginning between the first and second day after antigen injection.

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APPENDIX I

In establishing the model for the determination of the analysis of variance (Snedecor, 1956) the following components were incorporated:

1. The general mean grain count, μ .
2. The effect due to hours, H_i .
3. The effect due to treatment, G_j .
4. An interaction of treatment with hours, HG_{ij} .
5. An effect due to the animals within each hourly treatment, $A_{l(ij)}$.
6. An effect due to differences in germinal centers within each animal, within each hourly treatment, $D_{m[l(ij)]}$.
7. An effect due to different cell types, C_k .
8. An interaction between hourly groups and cell types, HC_{ik} .
9. An interaction between treatments and cell types, GC_{jk} .
10. An interaction between hours, treatments, and cell types HGC_{ijk} .
11. An interaction between cell types and animals within each hourly group, $AC_{kl(ij)}$.
12. An error factor termed an interaction between germinal centers and cells within each animal, within each hourly group, $CD_{km[l(ij)]}$.

In the model the following factors were assumed:

- (a) The effects due to treatment and cell types were fixed (selected for).

(b) The effects due to germinal centers and animals were random.

The following model (Kastenbaum, 1963), was used in the analysis of variance:

$$Y_{ijklm} = \mu + H_i + G_j + HG_{ij} + A_{l(ij)} + D_{m\ l(ij)} + C_k + HC_{ik} + GC_{jk} + HGC_{ijk} + AC_{kl(ij)} + CD_{km\ l(ij)}.$$

where $i = 1, 2, 3, 4, 5$, hours (fixed).

$j = 1, 2$, treatment groups (fixed).

$k = 1, 2, 3$, cell types (fixed).

$l = 1, 2, 3, 4$, animals in each hourly treatment group (random).

$m = 1, 2, 3, 4$, germinal centers in each animal (random).

On the basis of the assumptions the model suggests that the animal, by cell interaction (AC), be tested by the error-mean square defined above by the interaction of cells with germinal centers (CD). The model also suggests that the error-mean square attributed to the animal by cell interaction be used to test the hours by groups by cells interaction (HGC), the groups by cells interaction (GC), the hours by cells (HC) and the effect due to cells (C). The latter is testing the hypothesis of equal cell means.

The model also suggests that the mean square for germinal centers be used to test the hypothesis of equality of animal means (animal to animal variation between hourly groups). Furthermore, the mean square attributed to differences among animals should be used to test the hours by treatments (HG) and the main effect due to hours (H) and to groups (G). These results are summarized in Table IX.

TABLE IX

NUMERICAL AND EQUATIONAL VALUES OF THE STATISTICAL MODEL

Source	Degrees of freedom	Sum of squares	Mean square	Expected Mean* square	F value
Mean	1	10,656.6	--		
Hours (H)	4	719.4	179.85	$3s_D^2 + 12s_A^2 + 96s_H^2$	34.322**
Groups (G)	1	15.5	15.50	$3s_D^2 + 12s_A^2 + 240s_H^2$	2.891
Hours X Groups (HXG)	4	39.7	9.93	$3s_D^2 + 12s_A^2 + 48s_H^2$	1.895
Among animals in Groups (A)	30	157.2	5.24	$3s_D^2 + 12s_A^2$	5.758**
Among germinal centers in AHG (D)	112	101.9	.91	$3s_D^2$	
Cells (C)	2	572.8	286.40	$s^2 + 4s_{AC}^2 + 160s_C^2$	485.424**
Hours X Cells (HXC)	8	110.3	13.79	$s^2 + 4s_{AC}^2 + 32s_{HC}^2$	23.373**
Groups X Cells (GXC)	2	8.4	4.20	$s^2 + 4s_{AC}^2 + 80s_{HGC}^2$	7.119**
Hours X Groups X Cells (HXGXC)	8	13.4	1.68	$s^2 + 4s_{AC}^2 + 16s_{HGC}^2$	2.847**
Animals X Cells in hourly groups (AXC)	60	35.3	.59	$s^2 + 4s_{AC}^2$	1.255
Cells X Germinal Centers in AHG (CXD)	224	105.8	.47	s^2	
Total	456	12,536.2	--		

* Indicates F value which would occur less than one time in one hundred under the null hypothesis.

** At 10 hours only three germinal centers were observed in each animal. This fact is accounted for in the analysis. However, the expected mean squares are given as if the experiment were completely balanced with four germinal centers observed at all times.

GLOSSARY

<u>Abbreviations</u>	<u>Meaning</u>
SRBC	Sheep red blood cells
GC	Germinal center
DP	The densely populated (dark staining) region of the germinal center
TP	The thinly populated (light staining) region of the germinal center
C ₁ Cells	Germinal center cells located in densely populated region
C ₂ Cells	Germinal center cells located in the thinly popu- lated region
C ₃ Cells	The cells morphologically identical to cells in the germinal center but found in the lymphocyte mass.
PLTC	Primitive lymphatic tissue cells
LL	Large lymphocytes
S&ML	Small and medium lymphocytes
IPC	Immature plasma cells
MPC	Mature plasma cells

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