THE IMMUNE RESPONSE TO FOREIGN RED BLOOD CELLS AND THE PARTICIPATION OF SHORT-LIVED LYMPHOCYTES*

By C. G. CRADDOCK, M.D., A. WINKELSTEIN,‡ M.D., Y. MATSUYUKI,§ M.D., AND J. S. LAWRENCE M.D.

(From the Department of Medicine, University of California School of Medicine, Los Angeles, and Hematology Research Laboratory, Veterans Administration Center, Los Angeles, California)

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The immunological response to heterologous erythrocytes has been used by many investigators to study the initial phase of humoral antibody production. The recent development of techniques for determining the number of hemolytic plaque-forming units (PFU) in suspensions containing hemolysin-producing cells has permitted localization of the process at a cellular level and more precise quantitation of the primary hemolysin response (1, 2). The purpose of this study is to present morphological, immunological, and kinetic observations of some of the cells involved in the primary response to sheep red blood cells (SRBC). These include granulocytes, macrophages or monocytes and lymphocytes. Cortisol in large doses is known to exert a marked lympholytic effect and to interfere with the inflammatory response as well as the induction phase of antibody formation. Comparison of events in normal animals and animals given cortisol before receiving various types of cells has been used to study the interaction of different cells in the primary response. The rate of recovery of lymphopoiesis and volume of various components of the lymphatic system resulting from cortisol depletion has also been assessed. Proliferative activity at various times during the response to SRBC and during the recovery from cortisol depletion has been estimated by the uptake of tritiated thymidine (^{3}HT) into DNA. The results of these diverse approaches are presented in a single paper rather than in separate segments in order to correlate the findings.

Cortisol in large doses reduces the volume of all components of the lymphatic system but does not suppress hematopoieses or production of macrophages. The parts of the lymphatic system most seriously depleted and requiring the longest period for restoration after cessation of the hormone are the areas where production of lympho-

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‡ Instructor in Residence, Department of Medicine, Division of Hematology, UCLA School of Medicine, and USPHS Special Postdoctoral Fellow in Hematology.

§ Postgraduate Research Hematologist, Department of Medicine, UCLA School of Medicine, from Kyushu University, Japan.

cytes is normally the highest; i.e., the thymus cortex, the bone marrow lymphocytes, and the germinal centers of lymphoid follicles in the spleen and lymph nodes. These areas of active lymphopoiesis give rise to lymphocytes, the function of which is not defined. These cells are kinetically different from and produced independently of the long-lived small lymphocytes which comprise the majority of cells in the normal peripheral lymphatic tissue. Lymphocytes produced in these areas are denoted short-lived since the evidence is overwhelming that most of these cells die shortly after their production (3, 4, 5). The long-lived lymphocytes of the peripheral lymph nodes, blood, and spleen are the cells which manifest the capacity to transform and divide in response to certain nonspecific and antigenic mitogens, as will be discussed. The long-lived lymphocyte is believed the cell which expresses certain types of delayed hypersensitivity and antibody production.

The evidence to be presented in this paper indicates that in the adult animal germinal center lymphopoiesis does *not* give rise to long-lived lymphocytes and is *not* essential for recovery of lymphatic tissue after depletion. The development of germinal center activity following SRBC correlates well with hemolysin production and elimination of germinal centers by cortisol is associated with impairment of the immune respone. Our findings suggest that the development of new germinal centers in the spleen, with prominent phagocytic activity, occurs before the appearance of cells which produce hemolysin. However, hemolysin-producing cells are not the products of germinal center proliferation. The data suggest that short-lived lymphocytes such as those of the thymus and germinal centers enhance antibody production by improving the phagocytosis and processing of SRBC antigen rather than by providing cells which will evolve into hemolysin-producing cells. The major immunosuppressive effect of cortisol, in these experiments, appears to be impairment of the nonspecific inflammatory events which occur in the spleen prior to antibody production. The major cellular effect of cortisol is depletion of short-lived lymphocytes and their precursors.

Materials and Methods

All studies were performed in male Sprague-Dawley rats from Berkeley Pacific Laboratories, Berkeley, Calif., and housed in separate, temperature-controlled quarters. Animals were obtained at 75–100 g of weight (weanling) and were used after attaining 150–200 g at a normal growth rate. All animals had normal blood counts and were examined postmortem for infection; only those proved to be free of disease were accepted for results.

Rats receiving tritiated thymidine (^{3}HT) were given 0.5 μc/g body weight 1 hr before sacrifice. The methods of histological examination, ^{3}HT content in the tissue, measurement of the weights of thymus, spleen, and a standard lymph node mass, radioautography, and radiochemical measurements are similar to those previously reported (3, 5), except for the method of tissue preparation for histology and radioautography. Portions of each tissue were fixed in formaldehyde for paraffin embedding and sectioning at 6 μ and stained with hematoxylin and eosin (H and E). Other portions were fixed in glutaraldehyde for Epon embedding and sectioning at 1.0 μ with a glass microtome. These thin sections were stained with toluidine

blue pyronine (6). Radioautography was performed on thin sections with AR10 stripping film and exposed for 8 wk. Thin sections for autoradiography were stained with Giemsa (pH 5.7) for 30 min. Bone marrow preparations were painted on gelatin-coated slides with a sable brush dipped in 5% bovine albumin. Total marrow nucleated cell counts were determined in triplicate by dispersing a measured core volume of femoral marrow, obtained by forcing a polyethylene catheter into the femoral shaft. Cellularity could then be expressed as the number per cubic millimeter of marrow volume. Variation between normal animals was less than 10%. The number of small lymphocytes per cubic millimeter of marrow was calculated from the total cellularity and the differential count (500 cells). The uptake of ^{3}HT by marrow cells was determined by DNA extraction and calculation of ^{3}HDNA specific activity, rather than by the combustion technique. The results are expressed as counts per minute per microgram of DNA phosphorus, as described previously (3, 5). Animals receiving cortisol (hydrocortisone aqueous, 50 mg/ml, Upjohn Co., Kalamazoo, Mich.) were injected intramuscularly.

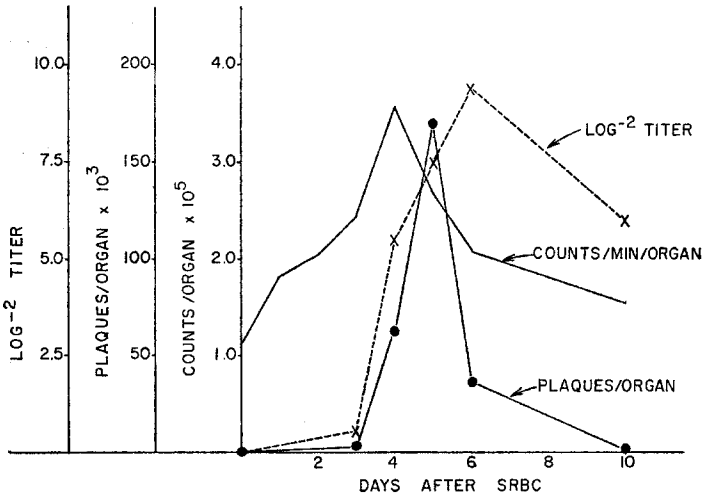
The observation of cells adhering to glass cover slips inserted subcutaneously (skin windows) was performed as described by Volkman and Gowans (7, 8). The morphology of cells and their quantitation in the normal peritoneal cavity and after the intraperitoneal injection of 5 ml beef heart infusion broth with 10% proteose-peptone No. 3 was accomplished as follows. The ether-anesthetized rat's abdomen was opened by a small lateral incision. 10 ml of of saline was introduced in two aliquots with gentle kneading of the abdominal contents after each injection. The abdomen was then opened widely and all visible fluid aspirated. The cell suspension was centrifuged, the supernate removed, and the cell button resuspended in 1 ml of TC199. Cell counts were made in triplicate and aliquots mounted on slides for staining and radioautography. Those animals injected intraperitoneally with beef broth were examined at the particular time interval noted after the injection. In skin window and peritoneal fluid preparations 500 cell differential counts were made. Five hundred cells were counted on autoradiographs for determination of the percentage of labeled cells and the mean grain count of labeled cells. Cells with three or more grains above background were scored, as described previously.

The response to a standard intravenous dose of 0.25 ml of a 4% solution of washed SRBC (approximately 17×10^7 cells) was measured by serial determinations of serum hemolytic antibody (9) and by single determinations of the number of hemolytic plaques in suspensions of spleen cells on pour plates containing these cells and SRBC, as described by Jerne et al. (1). The value for each rat spleen is the average of five separate pour plates. Quantitation in terms of the number of plaques per 10^6 spleen cells, the calculated plaques per organ, and the number of plaques per 100 mg of spleen yielded parallel results. The standard number of SRBC per plate was 45×10^7 and of spleen cells 7.5×10^6 or 15×10^6, depending on the concentration of plaque-forming cells. The normal rat spleen contains the maximum number of hemolysin-producing cells on days 5 and 6 after a single injection of the antigen. In all instances, except where indicated, determinations were carried out on day 6 after antigen (day +6). In those instances where the thymus or bone marrow was examined for hemolysin-forming cells these tissues were handled in the same manner as the spleen. Background numbers of PFU in various lymphoid organs of normal rats not given SRBC were less than 1 per 10^6 cells.

Animals receiving cortisol were given the steroid at the indicated time intervals with respect to SRBC injection (day 0). SRBC were given 12 hr after the last dose of cortisol. Rats receiving cortisol are referred to as "cortisonized" for the sake of brevity. Cortisonized animals receiving lymph node, marrow, thymus, or peritoneal cells were injected intravenously with fresh cell suspensions at the same time that SRBC were injected.

RESULTS

Hemolysin Production in Normal and Cortisonized Rats.—The production of hemolysin by normal rats following a single intravenous injection of SRBC occurs almost entirely in the spleen. PFU in significant numbers above background were not found in bone marrow, liver, thymus, or lymph nodes. Administration of antigen by other routes (intraperitoneally and in the foot pads) elicited only a few PFU in the thymus and lymph nodes. Animals splenectomized prior to SRBC administration showed significant numbers of PFU in

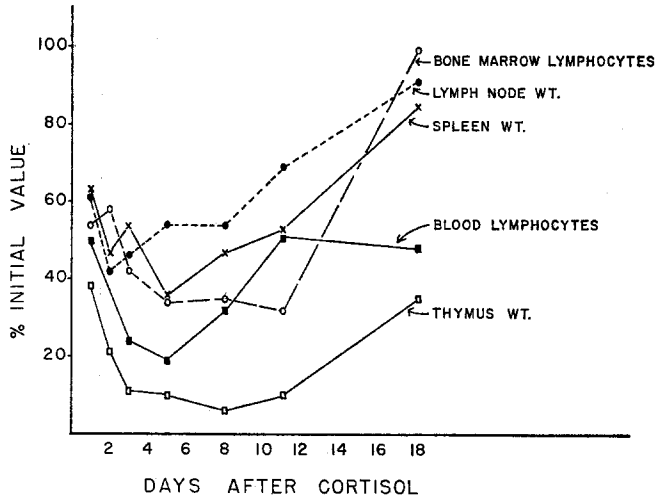


TEXT-FIG. 1. Serial determinations of spleen proliferative activity, number of hemolytic plaques, and serum hemolysin titer in normal rats sacrificed at intervals after 17×10^7 SRBC and 1 hr after 0.5 μc ^{3}H thymidine per gram body weight. Uptake of ^{3}HT is expressed as counts per minute in the entire spleen. Each point represents the average values for two to ten animals.

lymph nodes and thymus on repeated secondary challenge. However, the titer of serum hemolysin in these animals was markedly diminished even after multiple booster injections.

Text-fig. 1 shows the correlation of increased proliferative activity in the spleens of rats injected with SRBC and the appearance of PFU. The uptake of ^{3}HT by the spleen increases within 24 hr. There is a subsequent marked increase, associated with an increase in splenic lymphoid tissue, peaking at day +4. It is of interest to contrast this response with that observed by Jandl et al. (10) in normal rats given heat-damaged autologous erythrocytes. These workers showed that a significant increase in total splenic DNA synthesis occurred as a result of the macrophage proliferation provoked by intravenous injection of nonantigenic material. The clearing of damaged red cells from the circulation

by splenic phagocytes evokes increased proliferative activity by percursors of these cells. This type of proliferative stimulus involves the replacement of macrophages mobilized into the areas of particle trapping, some of which are presumably consumed or destroyed in the "inflammatory" reaction. The maximum increment in ^{3}HT uptake by the spleen in Jandl's experiments occurred at an earlier point (1 to 2 days) after injection of damaged autologous erythrocytes than in the present experiments with SRBC. It is clear, therefore, that the curve shown in Text-fig. 1 for ^{3}HT uptake is a composite one including



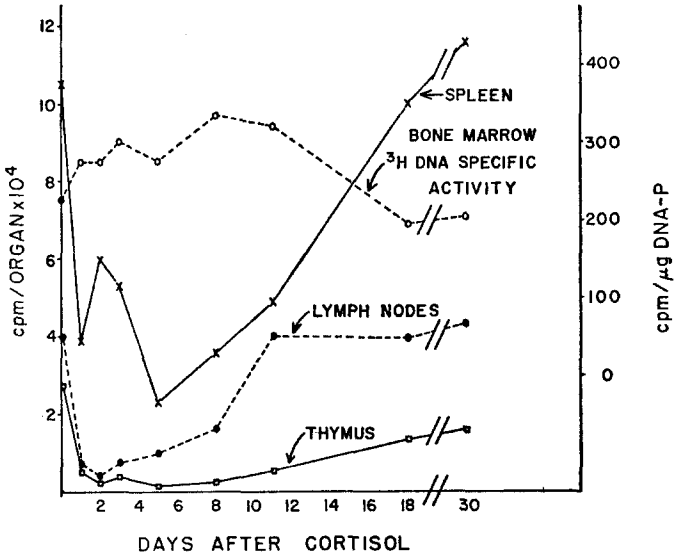
TEXT-FIG. 2. Per cent change in lymphoid tissue after cortisol (10 mg/100 g). Effect of a single dose of cortisol on the weight and cellularity of various components of the lymphatic system. Note that recovery of lymph node and spleen weight and blood lymphocytes begins sooner than the recovery of thymus weight and bone marrow lymphocytes. Each point is the mean for four animals. Normal values are based on 38 animals.

the nonspecific early proliferative activity by myeloid and macrophage precursor cells and the later lymphoproliferative response of immunologically responsive cells induced by the antigenic qualities of the SRBC. There is a close correlation between the time of appearance of PFU and the peak of increased DNA synthesis.

The effect of a single intramuscular injection of cortisol, 10 mg per 100 g body weight, on the volume of lymphatic tissue in various areas of the body is shown in Text-fig. 2. Proliferative activity (1 hr uptake of ^{3}HT) is shown in Text-fig. 3. Recovery of lymphatic tissue in terms of volume and proliferative activity begins sooner in lymph nodes and spleen than in the thymus. Myelopoiesis and erythropoiesis in the bone marrow was not depressed by cortisol (Text-fig. 3). The results of pretreating rats with four daily doses of 5 mg/100 g

of cortisol gave qualitatively the same results, although the degree of lymphoid depression was more marked and the recovery more delayed.

Text-fig. 4 shows the effect of various doses of cortisol given before SRBC on the serum hemolysin response and on the number of PFU in the spleens of animals assessed at day +6 after SRBC. The regimen of 5 mg per 100 g body



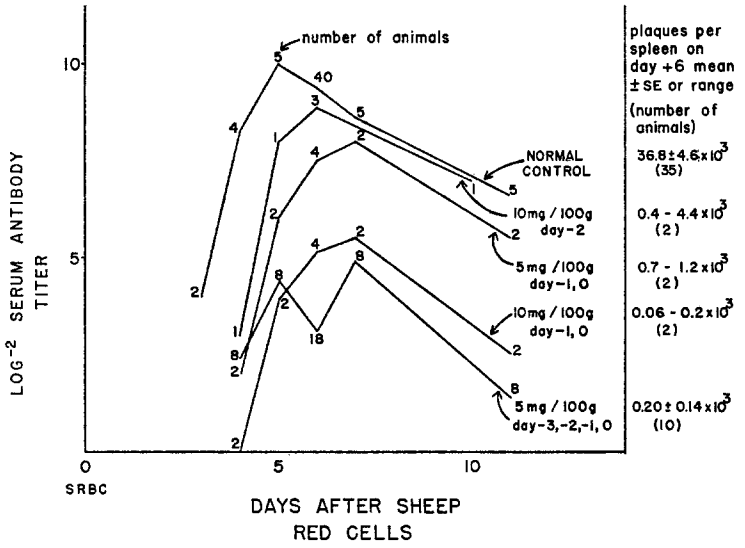
TEXT-FIG. 3. ^{3}HT uptake by lymphoid organs after cortisol (10 mg/100g). The effect of a single dose of cortisol on proliferative activity of various components of the lymphatic system of normal rats, as estimated by the incorporation of ^{3}H thymidine into DNA 1 hr after administration of 0.5 μc/g of ^{3}HT. The radioactivity of thymus, lymph nodes, and spleen is expressed as total counts per organ; that of the bone marrow as specific activity of extracted DNA (counts per minute per microgram of DNA phosphorus). Each value is the mean of three to six animals. It will be noted that increased incorporation of ^{3}HT occurs sooner in lymph nodes and spleen than in the thymus. Marrow erythropoiesis and myelopoiesis is unchanged and the increase in marrow uptake of ^{3}HT is due to the elimination of the small lymphocyte population.

weight on each of 4 days prior to SRBC, the last dose being given 12 hr before SRBC, was selected for maximum suppression of antibody production and reduction in body lymphatic tissue (Text-fig. 5).

Morphological and Radioautographic Changes in the Spleen Following SRBC.—

Normal rats: The nodular collections of lymphocytes in the spleen of the normal rat occur as sheaths of densely packed cells, mostly small lymphocytes, surrounding arterioles. The area bordering the lymphoid cells is highly vascular with concentric rows of poorly defined vascular sinusoids visible on cross section. These vascular channels extend throughout the nodule but are usually

not visible to light microscopy because of the numerous small lymphocytes in and around the vascular spaces. Some of the larger follicles have an eccentrically placed extension of lymphoid cells extending into the red pulp and these occasionally contain a central reactive zone or germinal center. These are sites of very active cellular proliferation and frequent mitoses are seen as well as mmature lymphocytes which stain more heavily with pyronine than other mmature cells. There is also a considerable amount of debris and occasional iarge macrophages with pale, poorly defined nuclei and cell outlines containing



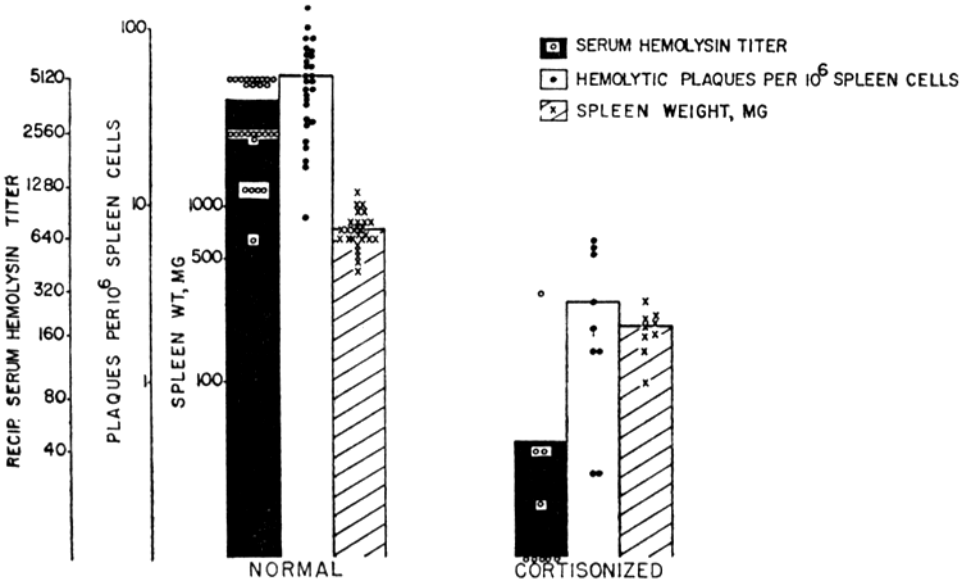
TEXT-FIG. 4. The effect of various doses and schedules of cortisol on serum hemolysin leve and the number of hemolytic plaques per spleen. 17 × 10^7 SRBC were given intravenously on day 0. The values for the number of hemolytic plaques on day +6 (number of animals in parentheses) are given on the right side of the figure.

many engulfed particles. These particles are known as tingible bodies and the large cells loaded with these particles are denoted tingible body cells.

Radioautography (RAG) of spleen and lymph node tissue embedded in plastic 1 hr after the animal has received 0.5 μc/g of ^{3}HT show large cells in DNA synthesis with a high grain count per cell scattered through the dense collections of lymphocytes (Fig. 1 *a*). There are many lymphoblasts which incorporate the DNA label in and near the germinal center. As shown in Fig. 1 *b*, the labeling of lymphoid cells in the germinal center is less intense per cell, although a higher percentage of cells in the germinal center is labeled than in the surrounding cuff of lymphocytes. This pattern of low uptake of ^{3}HT by each cell in DNA synthesis is similar to that seen in the thymus and is due to

reutilization of the DNA thymidylic acid in these areas of active cell breakdown (3).

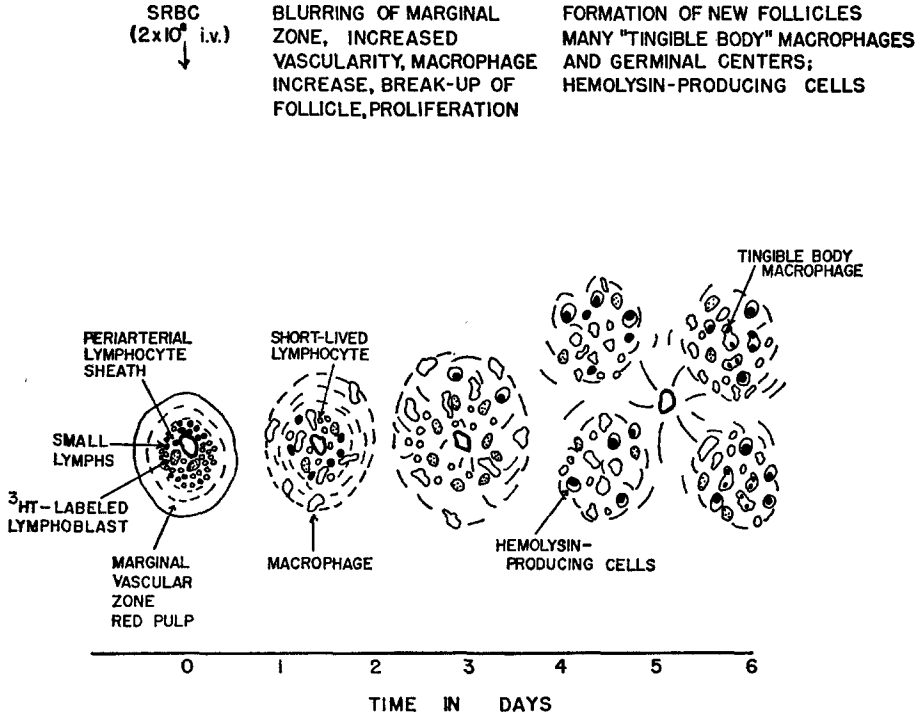
Following the injection of SRBC there is a striking sequence of changes in the rat spleen, similar to those described by Congdon and Makinodan in the mouse (11). 1 day after SRBC there is "pitting" of the lymphoid follicles which sheath the arterioles. These areas, which appear as irregular clear spaces on the paraffin-embedded sections stained with H and E are due to distention of vascular sinuses, loss of small lymphocytes in these areas, and the appearance of



TEXT-FIG. 5. The effect of cortisol, 5 mg/g body weight on day −3, −2, −1, and 0, on serum hemolysin titer, concentration of hemolytic plaques in the spleen, and spleen weight on day +6 after 17×10^7 SRBC, intravenous.

large pale cells, many of which are phagocytic, as indicated by ingested material in the cells (Fig. 2 *a* and Fig. 2 *b*). The appearance of these large phagocytic cells in the lymphoid follicle occurs at the time when increased ^{3}HT uptake begins and reflects macrophage production in response to nonspecific stimuli, as described by Jandl et al. (10). At the same time there is blurring of the margin between the lymphocyte aggregates and the red pulp of the spleen because of both a diminished number of small lymphocytes and the appearance of many endothelial and phagocytic cells in the marginal areas. The lymphoid mass of the spleen, which is now less discrete, appears to expand rapidly during day +3 and +4 after SRBC. This occurs at a time when ^{3}HT uptake by the spleen is reaching a peak and is that portion of the proliferative response which

is followed by the appearance of hemolysin-producing cells. The relative numbers of small lymphocytes is diminished and the number of larger pyroninophilic lymphocytes is increased; many of these cells are in DNA synthesis as indicated by the incorporation of ^{3}HT. These loosely arranged clusters of proliferating lymphocytes are often grouped in or near a small vascular sinus



TEXT-FIG. 6. Diagrammatic representation of the cytological changes in splenic lymphoid tissue at various times after intravenous injection of SRBC. For orientation, a central arteriole is placed at the center of each of the phases of the progression from a "resting" lymphoid nodule to the formation of multiple new aggregates of lymphocytes and macrophages and the appearance of hemolysin-producing cells.

and many macrophages are in the area (Fig. 3 *a* and Fig. 3 *b*). These clusters of lymphocytes and macrophages appear to organize themselves into new reactive centers and by day +5 and +6 the red pulp of the spleen appears compressed between these expanding lymphoid aggregations. Many of these lymphoid cells are pyroninophilic and morphologically resemble plasma cells or Russell body cells. The number of tingible body cells reaches a peak by day +5. The changes are depicted diagrammatically in Text-fig. 6.

These results, when correlated with the sequence of hemolysin production,

suggest that appearance of hemolysin-producing cells in the spleen coincides with or shortly follows the increase in proliferation by lymphocytes. This is accompanied by the development of multiple areas of mixed phagocytes and lymphopoiesis in and around existing lymphoid aggregations of the spleen (11). In view of the evidence that small lymphocytes have the capacity to develop into lymphoblasts (12, 13, 14) under suitable stimulation it may be presumed that one reason for the decrease in small lymphocytes is because some of these cells are transformed into larger proliferating cells in response to the antigenic stimulation. There is no alteration in the volume, proliferative activity, or histology of lymph nodes, thymus, or marrow, as might be expected if these areas were providing the spleen with many cells. The proliferative response by macrophages and lymphocytes in the spleen appears to involve cells which are localized in the spleen at the time of antigenic stimulation.

Cortisonized rats: Cortisol causes a marked depletion of lymphoid cells throughout the body. However, small collections of proliferating lymphocytes remain in the perivascular sheaths of the spleen and the lymph nodes, although germinal centers are totally eliminated (Fig. 4 *a–c*). The thymus cortex and bone marrow are virtually effaced of lymphocytes and the only viable (nonpyknotic) cells which remain in the thymus after massive amounts of cortisol are the stromal cells of the supporting tissue, scattered myeloid cells, and many mast cells (Fig. 5 *a* and Fig. 5 *b*). Mast cells are normally present along the stromal bands which divide the thymus cortex into separate lobules. Their apparent increase in the cortisonized thymus may be due to collapse of the fibrous network of the tissue. There is no evident increase in proliferative activity in these cells in the cortisonized animal. Proliferative activity of lymphocytes returns very early in the lymph nodes and spleen follicles and is especially striking on RAG of these areas (Fig. 4 *b* and Fig. 4 *c*). There is a much higher than normal percentage of the cells which incorporate ^{3}HT. Little proliferation of lymphoid cells is evident in the thymus until considerably later. However, the medullary area of the thymus shows active myelopoiesis at various times after cortisol depletion. Myelopoiesis and erythropoiesis are also active in the marrow and spleen.

The spleens of cortisonized rats receiving SRBC fail to show the sequence of changes described for normal animals. Nevertheless, they showed increased lymphoproliferative activity and a gradual return toward a normal volume of lymphoid tissue. Germinal centers appear considerably later. The most notable effect of the cortisol, aside from reduction in volume of lymphoid tissue, was elimination of the early pitting of lymphoid follicles by phagocytic cells, lack of development of tingible body cells, and the absence of germinal centers in lymph follicles during the 3 wk period of observation. This was true in rats whether or not they received SRBC.

Effect of Cortisol on Macrophage Production and Mobilization.—An important part of the response to SRBC occurs prior to hemolysin production. It is during this so-called induction period that the vascular changes and pitting of lymphoid follicles by macrophages is most evident. The question arises, therefore, as to whether one effect of cortisol is to depress the number of macrophages by some sort of lytic effect similar to that exerted on lymphocytes. The actual number of macrophages in the spleen at various times after cortisol is difficult to assess. Presumably these cells originate from fixed reticuloendothelial elements in the

TABLE I

Effect of Cortisol on Macrophage Response to Intraperitoneal Inflammation and to Subcutaneous Insertion of Glass Cover Slips in Normal and Cortisonized Rats

Peritoneal fluid						Skin window					
No. of rats	Time after broth i.p.	Cortisol		Macrophages		No. of rats	Time after cover slip	Cortisol		Macrophages	
		Dose	Time*	Total No. $\times 10^7$	Labeled‡ %			Dose	Time*	Per cent of cells	Labeled‡ %
	hr	*mg/100 g*	*hr*	*Avg* ± SD	*Avg* ± SD		*hr*	*mg/100 g*	*hr*	*Avg.* ± SD	*Avg.* ± SD
5	0			1.7 ± 0.4	6 ± 1						
6	6			1.3 ± 0.8	54 ± 11	6	6			8 ± 3	32 ± 6
5	24			9.0 ± 1.2	59 ± 8	6	24			85 ± 11	75 ± 9
6	48			8.2 ± 2.1	41 ± 8	4	48			54 ± 8	16 ± 6
3	0	10	−24	1.3 ± 0.5	6 ± 2	2	24	10	0	98, 98	
4	24	5	−96, −72, −48, −24	3.5 ± 0.8	40 ± 3	3	24	10	−48, −24	22 ± 12	
2	48	5	−96, −72 −48, −24	5.0, 5.3		4	24	5	−96, −72, −48, −24	12 ± 15	52 ± 7
4	24	20	+12	5.9 ± 0.9		3	24	20	+12	86 ± 8	

* Time of cortisol administration before (−) or after (+) intraperitoneal injection of 5 ml beef heart infusion with 10% proteose-peptone No. 3 or subcutaneous insertion of glass cover slips.

‡ Rats receiving ^{3}HT were given 1 μc/g 21 hr before beef broth i.p., or insertion of skin window, or examination of unstimulated peritoneal fluid.

spleen and other lymphoid areas. These precursors are not obviously damaged by cortisol; indeed, they became more prominent when the lymphoid cells are depleted. Quantitative studies were made of the effect of cortisol on the number of macrophages in the peritoneal fluid and in inflammatory peritoneal exudates. Table I gives the results. These were considered more definitive than similar studies of the attachment of macrophages to subcutaneously implanted glass cover slips ("skin windows") since the latter observation presumably depends in part upon the ability of the macrophage to attach itself to a foreign surface and since cell numbers cannot be determined on cover slips. The effect of cortisol on the rate of production of macrophages was estimated by observing the percentage of ^{3}HT-labeled macrophages which appeared in these areas, the ^{3}HT

having been given 21 hr before induction of inflammation or examination of macrophages in the unstimulated peritoneal fluid. Presumably, any macrophages containing the DNA label in their nuclei were derived from precursor cells which incorporated the ^{3}HT during the brief time it was available ("flash" labeling). Labeled macrophages in the inflammatory sites were, therefore, considered as produced during the interval between ^{3}HT administration and examination, as was done by Volkman and Gowans (7, 8). The possibility that some of the labeled macrophages became labeled by phagocytosis of labeled nuclear material from other cells breaking down within macrophages or in their environment was not assessed. However, this could be an important factor in the interpretation of such data. In spite of this limitation and the lack of assurance that macrophages in skin windows or peritoneal fluid are identical with those participating in the splenic response to SRBC, these observations were made in an effort to assess the effect of cortisol on macrophage viability and production.

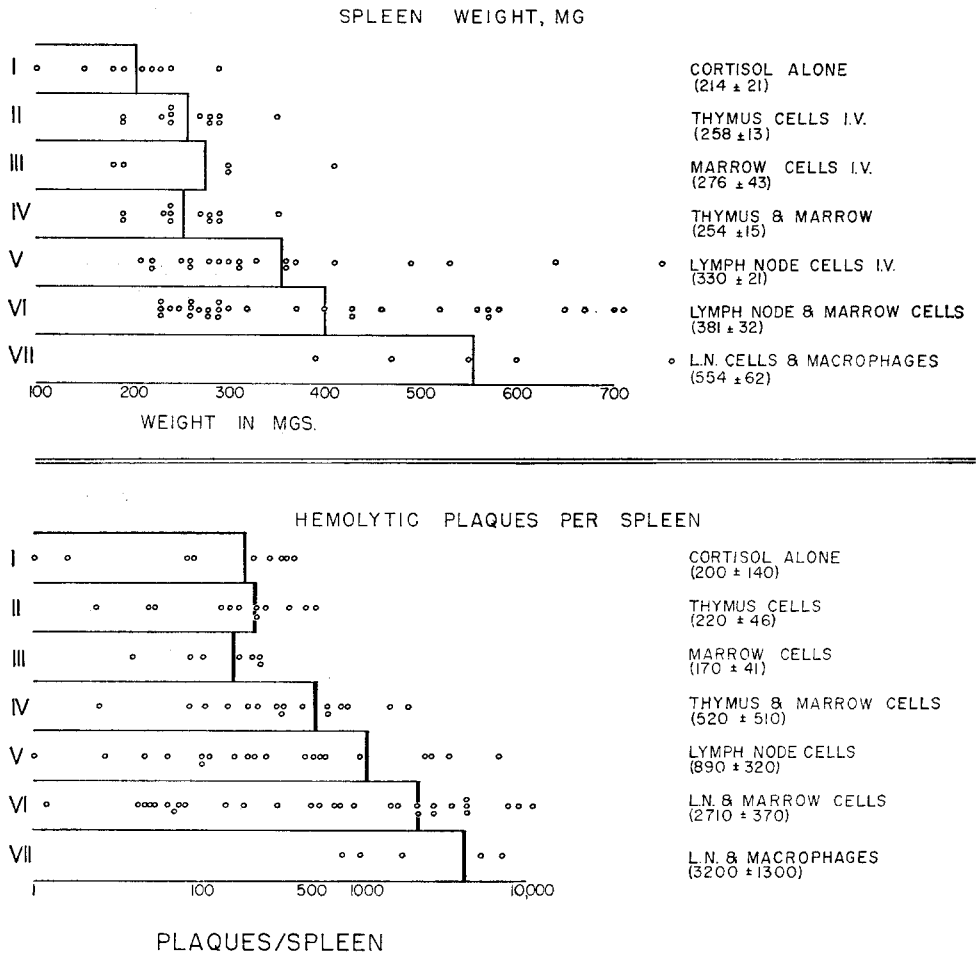
It will be noted that large repeated doses of cortisol failed to entirely suppress the appearance of macrophages at these inflammatory sites, although the total number appearing in the peritoneum was reduced. The phagocytic capacity of these macrophages was not tested, but it may be assumed from the work of others that it was impaired (15). The more marked effect of cortisol on the attachment of these cells to the subcutaneously inserted glass cover slips than on their appearance in the peritoneal exudate may be related to this effect of cortisol. It is also evident, from the percentage of macrophages which contained the ^{3}HT label and hence presumably were produced during and after the period of cortisol treatment, that the production of these cells is not curtailed by cortisol. Furthermore, it is clear that large amounts of cortisol do not bring about lysis or disintegration of mature macrophages already present in the peritoneal fluid or attached to skin windows (last row, Table I). Therefore, the reduction in numbers of macrophages appearing in the peritoneal exudate must be explained by inhibition of their mobilization by the cortisol treatment.

Lymphocytes, on the other hand, were entirely absent from the inflammatory reactions manifested in all animals which received cortisol in any amount given and at any time interval relative to the induction of inflammation. It would appear that macrophages are produced by a progenitor population which, unlike the lymphocytes and some lymphocytic precursors and like the myelocyte population, is insensitive to suppression by cortisol. The findings fail to support the view, held by many workers over the years (16), that macrophages evolve from lymphocytes. The findings of a rapid replacement rate of macrophages at inflammatory sites (as indicated by the high percentile labeling after ^{3}HT with the aforementioned limitations of the meaning of ^{3}HT labeling) and resistence to destruction by cortisol are of interest in view of the findings of Volkman and Gowans that large doses of whole body irradiation are required to curtail macro-

phage production (7, 8). These workers showed that the marrow is a major site of production of these phagocytic cells and provided additional evidence that lymphoid cells are not progenitors of macrophages. Such studies as that of Whitelaw in the rat (17) indicate that blood monocytes are a rapidly renewed system with kinetics similar to neutrophilic granulocytes.

If these findings can be related to the splenic response to SRBC, the effect of cortisol in suppressing the appearance of phagocytic cells and germinal center formation in the spleens of rats given SRBC is probably not attributable to direct elimination of macrophages by the steroid. Rather, the cortisol effect would appear to be an indirect one of interference with chemotaxis and phagocytosis (15). Equally striking in the cortisonized rats (with and without SRBC administration) was the absence of tingible body macrophages. These phagocytes containing cytoplasmic material which has the staining characteristics of nuclei are always prominent in germinal centers, especially when the animal is responding to certain immunological challenges. Apparently the cellular breakdown which is such a prominent feature of germinal center activity is necessary for the formation of these distinctive cells. The most likely mobile, nucleated cellular candidates for such phagocytosis are short-lived cells of the granulocytic or lymphocytic series. Short-lived lymphocytes and their progenitors are prominent in germinal centers and both are transiently eliminated by cortisol. Myelopoiesis and polymorphonuclear leukocytes are not detectably suppressed by cortisol. It is, therefore, tempting to conclude that short-lived lymphocytes contribute most of the nuclear material engulfed by macrophages in reactive germinal centers. However, cortisol is known to affect the distribution of granulocytes (especially eosinophils) within vascular and extravascular compartments and to suppress their migration into inflammatory sites. These observations fail to define which cell type provides the nuclear material incorporated by macrophages.

Effect of Various Cell Suspensions on Hemolysin Production by the Cortisonized Rat.—Finally, an attempt was made to see if the suppressive effect of cortisol could be partially overcome by the administration of various types of cell suspensions given simultaneously with the SRBC. The results, as shown in Text-figs. 7 and 8, indicate that lymph node cells alone provided highly variable degrees of improvement in hemolysin production. Although hemolysin production was markedly enhanced in some cortisonized animals receiving lymph node cells, there was no significant improvement in the group as a whole. There was a significant increase in spleen weight over that of animals receiving cortisol alone and histological examination of the spleens of these animals showed more lymphoid tissue relative to red pulp at day +6 after SRBC. Marrow cells, containing large numbers of phagocytic cells and their precursors, but which did not appear to augment hemolysin production when given alone, improved hemolysin production when given together with lymph node suspensions (group VI vs.



TEXT-FIG. 7. Spleen weight (linear scale) and the number of PFU (log scale) in spleens of rats receiving cortisol, 5 mg per 100 g body weight, on day -3, -2, -1, and 0 and given various types of normal cells intravenously at the same time as 17×10^7 SRBC on day 0. The numbers in parentheses are the mean and one standard error. Numbers of nucleated cells given were as follows: thymus $500–1000 \times 10^6$, marrow $300–500 \times 10^6$, lymph node $200–500 \times 10^6$, and macrophages $30–60 \times 10^6$. The significance of difference between these various groups is given in Text-fig. 8.

group I, $p < 0.05$, >0.02). A few animals given peritoneal macrophages along with lymph node suspensions showed striking improvement in hemolysin production. Those rats receiving macrophages were given cells from the unstimulated peritoneal cavities of four or five normal donor rats, the suspension con-

taining about 80% macrophages. Similar improvement in hemolysin production (not shown) was observed in six of eight rats receiving combinations of lymph node suspensions and thymus cells. However, thymus cells alone or in combination with marrow cells and given in larger numbers than lymph node suspen-

P VALUES

SPLEEN WEIGHT GROUPS

HEMOLYTIC PLAQUES/SPLEEN GROUPS	I	II	III	IV	V	VI	VII
I		N.S.	N.S.	N.S.	< 0.01	< 0.01	< 0.001
II	N.S.		N.S.	N.S.	N.S.	< 0.01	< 0.001
III	N.S.	N.S.		N.S.	N.S.	N.S.	< 0.01
IV	N.S.	< 0.02	N.S.		< 0.001	< 0.01	< 0.001
V	N.S.	N.S.	N.S.	N.S.		N.S.	< 0.01
VI	< 0.05	< 0.02	N.S.	< 0.02	< 0.05		< 0.02
VII	< 0.01	< 0.01	< 0.05	< 0.05	< 0.02	N.S.	

N.S. NOT SIGNIFICANT ($P > 0.05$)

TEXT-FIG. 8. Levels of significance of difference between the mean values for spleen weight (above and to right of diagonal empty boxes) and number of PFU per spleen (left and below) in the seven groups shown in Text-fig. 7.

sions, appeared to have less effect on spleen weight and hemolysin production than did lymph node cells (group IV vs. group I, not significant).

It is impossible to know whether the effect of such cell suspensions is attributable to participation of the injected cells in the steps involved in hemolysin production or to nonspecific influences on the SRBC or on the residual tissue in the cortisonized animal. Nevertheless, it is evident that a major effect of the cell suspensions was on the induction phase of the process. The lymph node cell suspensions, rich in cells which can produce antibody, were augmented in their effect in these immunologically crippled animals by the addition of tissues which display little inherent capacity for hemolysin production in the normal animal.

DISCUSSION

Various aspects of the type of immune response presented in this paper have been investigated by many other workers. Major emphasis in recent studies has been on the proliferative events involving lymphocytes or plasma cells which precede the appearance of antibody-producing cells. For example, Kennedy et al. (18) have shown that production of hemolysin-producing cells in the spleens of mice given SRBC, after a finite lag period, increases exponentially with an estimated cell generation time of 6 to 7 hr. The final quantity of several million PFU appears to evolve from serial divisions of a very few, about 1000, responsive lymphoid cells in the spleen. The present study has focused more upon the coordination of cellular events which precede this remarkable lymphoproliferative response. Whereas many aspects of the results are confirmatory of previous findings by others, they emphasize certain features which require further definition. Chief among these is the true relationship of the germinal center to lymphopoiesis and the production of antibody.

The term germinal center originally implied that these sites of active cellular proliferation were the chief sites of production of lymphocytes which are concentrated in the surrounding cuff of cells forming a lymphoid follicle. This view is no longer tenable for several reasons. First, kinetic studies by several groups have shown that germinal center proliferation does not give rise to cells which persist as labeled small lymphocytes outside of the germinal center (3, 19). Second, the long-lived small lymphocytes which comprise the majority of lymphoid cells in peripheral lymph nodes, spleen, and blood are themselves capable of enlarging, dividing, and producing more lymphocytes (3, 11, 12, 13). This behavior of small lymphocytes indicates that they are a self-sustaining population which does not require replacement from a rapidly renewing precursor pool, at least in the normal adult animal. The capacity of small lymphocytes to remain in prolonged interphase between successive divisions has been shown from the studies by Norman et al. (20) of women cured of carcinoma of the cervix by radiation therapy. Long-lived lymphocytes, which incurred recognizable chromosome changes during local pelvic irradiation, were forced to divide by short-term culture with phytohemagglutinin (PHA) at intervals after the irradiation. Chromosome changes which would have been lost had the cells divided in the interim were scored. These cells disappeared from the blood with a half-time of over 500 days. This study establishes the long-lived, recirculating small lymphocyte as the PHA-responsive cell in the peripheral blood, a cell which is capable of repeated divisions but which, in the normal environment of the body, may exist for long intervals in interphase. Nowell, in a similar study, showed that these long-lived cells divide in response to tuberculin when obtained from tuberculin-sensitive subjects (21). They are, therefore, cells which are responsive to antigenic challenge. This concept of antigen-related induction of proliferation by immunologically responsive cells ("immunocytes") fits well

with the phylogeny and ontogeny of the development of the peripheral lymphatic system. Its development requires antigenic experience (22). Its physiology, mobility, and sites of concentration are suited to cope with environmental antigen. Division by these cells can occur in the blood, lymph, tissues, or wherever conditions require. Germinal centers are not necessary for those cells to replicate.

The third reason for considering lymphopoiesis which produces long-lived immunocytes as occurring independently of germinal center proliferation is afforded by studies by Cottier et al. (23) and this laboratory (24) of the effects of extracorporeal irradiation of the blood (ECI). ECI will deplete the animal of long-lived, recirculating lymphocytes. Such depletion fails to diminish the size or proliferative activity of germinal centers, although the surrounding cuffs of small lymphocytes may be seriously reduced. Lymphopenia in such animals may persist for many months despite hypertrophy of germinal centers. For these reasons the concept that germinal center lymphopoiesis is responsible for production of long-lived small lymphocytes (immunocytes) in the adult animal must be rejected. In view of the evident phagocytosis and cell death and antigen trapping in these areas (25), the term reactive centers would seem more accurate.

The "Inflammatory Response" and Antibody Production.—The lymphoproliferative response which precedes the appearance of hemolysin-producing cells in the rat spleen following SRBC does not occur as an isolated event. A considerably increased proliferative activity occurs in the spleen before the lymphocyte mass increases in volume. This early proliferative activity is nonspecific (10); it will result from the clearing of a variety of nonantigenic materials from the circulation. This early increase in cell proliferation involves, in part, the production of increased numbers of macrophages in the spleen. In the present studies this early macrophage proliferation was accompanied by the appearance of phagocytic cells around and scattered throughout existing lymphoid nodules (with or without germinal centers) which represented the earliest detectable change in the normal spleen responding to SRBC.

The point cannot be overemphasized that the stimulus to increased production of macrophages is not antigen-related. It is clear from the report by Whitelaw that the kinetic behavior of blood monocytes is quite similar to that of neutrophilic granulocytes in the rat (17). In the present paper the blood monocyte is considered a mobile part of the body's macrophage system and analogous to the macrophages participating in the splenic response to SRBC. Blood monocytes and mobile spleen macrophages are probably rapidly renewed in the same sense that neutrophiles are rapidly renewed. Blood monocytes and neutrophiles are predominantly phagocytic cells with the lysosomal enzymatic equipment to attack certain pathogens. In the inflammatory response these cells are consumed in large numbers. Production control of neutrophiles is clearly

geared to peripheral utilization (26). Although the point remains to be established, it is likely that monocyte or macrophage production is geared to utilization. Production of long-lived lymphocytes (immunocytes), on the other hand, is dependent on antigenic experience (22).

In terms of the initial cytological changes in the rat spleen following SRBC injection, the increased production of macrophages and the appearance of these cells in lymphoid nodules is in many respects similar to the nonspecific inflammatory response. There is now ample experimental evidence, such as that provided by Fishman et al. (27, 28), that the mounting of a specific immune response by lymphoid cells requires the prior phagocytosis and "processing" of the antigen by phagocytic cells. It is this "inflammatory" aspect of the response to SRBC which is most obviously impaired by pretreatment with cortisol of whole body irradiation (15, 29–33). The series of cytological events in the rat spleen responding to SRBC is prevented by cortisol. These include the formation of new germinal centers and the striking development of tingible body macrophages seen in the normal animal. Lymphopoiesis during the period of antibody synthesis is not impaired and, indeed, may be greatly increased. Therefore, the failure of cortisonized rats to develop PFU is not due to suppression of lymphopoiesis. Pretreatment with cortisol impairs the inflammatory phase (antigen processing) of the response to SRBC to such an extent that immunocytes are not induced to proliferate in response to the SRBC antigen. Consequently, hemolysin-producing cells never comprised a significant fraction of the lymphatic mass recovering from cortisol injury.

This conclusion that the major effect of cortisol is to suppress the antigen-processing phase of the immune response to SRBC is supported by the results of administering various types of cells to cortisonized animals. Normal lymph node cells, consisting chiefly of small, long-lived lymphocytes, clearly augmented the total amount of lymphoid tissue in the spleens of cortisonized recipients. However, they failed to consistently improve hemolysin production. Only when lymph node cells were given along with cells with phagocytic capacity was there a significant increase in hemolysin production.

Correlation of the Immunosuppressive Effect of Cortisol with Changes in the Lymphoreticular Tissue.—The most obvious effect of large amounts of cortisol is the dramatic dissolution of lymphatic tissue. One might anticipate that recovery of lymphocyte mass would occur first in areas where cell production is highest, i.e., thymus cortex, bone marrow, and germinal centers. This was not the case; indeed, recovery of lymphopoiesis and lymphocyte numbers in these areas lagged considerably behind the recovery of lymph node, spleen, and blood lymphocytes. The most direct interpretation of this finding is that progenitor cells as well as mature cells were seriously damaged in these areas. It is likely that the rapid recovery of the volume of peripheral lymphatic tissue after subtotal depletion is due to the capacity of many of the remaining lymphocytes

(whether large, medium, or small) to replicate (3). Every lymphocyte which has the capacity to undergo repetitive divisions may be considered a potential stem cell for more lymphocytes. The rapid restoration of lymphatic tissue in lymph nodes and spleen in spite of the absence of germinal centers adds additional weight to the argument that established long-lived lymphocytes (immunocytes) are not produced in germinal centers and do not depend on the presence of germinal centers to proliferate.

Most lymphocytes produced in the thymus, bone marrow, and germinal centers are short-lived. The evidence to support this statement is considerable. The rapid replacement of most lymphocytes in these areas is well known (3, 4, 5, 19, 34, 35). The thymus of the young adult rat, for example, produces enough lymphocytes to replace itself in about 1 wk (3). The thymus is approximately the same weight as the spleen in the 100 g rat. The production of so many cells, all destined to survive as long-lived, recirculating, potential stem cells for more lymphocytes, would lead to explosive increase in body lymphocyte mass. Engraftment of enormous amounts of viable thymus tissue, as has been done by Matsuyama, Wiadrowski, and Metcalf (35), fails to lead to an increase in volume of lymph nodes, spleen, Peyer's patches, etc. Clearly, therefore, the vast majority of the products of thymus lymphopoiesis must survive for a short period. The indications of cell death and DNA reutilization are prominent in the lymphopoiesis in the thymus, marrow, and germinal centers. The reasons for considering germinal center lymphopoiesis as productive of cells with a limited life span have been given already.

Recently Davies et al., using marker chromosome techniques, have presented very convincing evidence that thymus-derived cells continue to proliferate in peripheral lymphatic areas (36). These workers carefully avoided the conclusion that these thymus cells which seed into peripheral lymph nodes and spleen are long-lived lymphocytes (immunocytes) which give rise to antibody-producing cells. Proliferation by these cells cloning from the thymus was induced by antigen (SRBC) injection. However, this proliferative response to foreign erythrocytes may have resulted from the type of nonspecific inflammatory proliferation demonstrated by myelocytes and monocytic precursors, as described earlier in this paper. Indeed, the early increase in mitoses which these workers noted after SRBC is more consistent with this interpretation than with the specific antigen-related lymphoproliferative response which occurs later and precedes the appearance of hemolysin-producing cells. The findings of Davies et al. indicate that thymus-derived cells become established in the periphery as replicating cells. Because of the kinetic similarities between thymus lymphopoiesis and germinal center lymphopoiesis we suggest that the germinal centers are areas of autonomous production of short-lived lymphocytes which originally emanated from the thymus. Thymectomy of neonatal mice prevents the development of germinal centers (37, 38).

Normal human thymus, in contrast to blood and lymph nodes, contains few cells which respond to PHA by proliferating in culture (39, 40). The vast majority of normal thymocytes do not transform or divide in response to PHA. Proliferating thymus cells show little detectable immunoglobulin synthesis (40) in contrast to stimulated proliferating blood lymphocytes (41, 42). Furthermore, it is clear that thymus lymphocytes have far less capacity to express various types of immune response (on a cell for cell basis) than do cells from lymph nodes or spleen (43, 44). In the present observations, rats were crippled in their immune responsiveness to SRBC by pretreatment with cortisol. They were then given thymus cells in large numbers without improvement of hemolysin production. This supports the conclusion that thymus lymphocytes are weak effector cells in this type of immune response.

The ontogeny of the thymus in fetal development and comparative studies of thymus proliferation in germfree and normal animals (45, 46) indicate that thymus lymphopoiesis is not related to exogenous antigenic experience. This is in sharp contrast to the dependency of the peripheral lymphatic system on antigenic challenge for its development. There is, therefore, ample reason on kinetic and developmental grounds to consider the thymus lymphocyte population as being distinct from that of peripheral, long-lived, recirculating immunocytes.

Factors controlling production of thymocytes may be quite different from those influencing immunocyte lymphopoiesis. There is, for example, strong evidence that the thymus epithelium secretes a hormone which promotes thymic lymphopoiesis (47, 48). It may well be that such thymus hormonal influence is necessary for lymphopoiesis in the peripheral lymphatic tissue as well; however, there is very little evidence as to whether this influence affects germinal center production of short-lived lymphocytes or mantle zone production of long-lived immunocytes.

Both kinetic varieties of lymphocytes are, we suggest, importantly involved in the immune response, but at different points. The long-lived immunocyte system is clearly concerned with the expression of the specific immune response to antigen. The short-lived lymphocyte appears to be more concerned with the steps which lead to this expression. The present experiments suggest a relationship between the anti-inflammatory action of cortisol and depletion of short-lived lymphocytes. Suppression of the inflammatory phase of the reaction to SRBC by cortisol leads to impairment of phagocytosis and antigen processing. Granulocytic and monocytic phagocytic cells are not obviously damaged by cortisol nor is their production curtailed. The major cytological effect of cortisol is destruction of short-lived lymphocytes and their progenitors. Partial replacement of these short-lived cells by administration of thymus cells to cortisonized animals may partially repair the defect in antigen processing but does not provide the animal with antibody-producing cells. Short-lived lympho-

cytes appear to be an essential component of the interaction between antigenic material and phagocytic cells which leads to germinal center formation in the rat spleen after SRBC.

The present work fails to define sharply a discrete role for the vast numbers of short-lived lymphocytes produced in the thymus, marrow, and lymphatic tissues of normal adult animals. Nevertheless, the correlation of kinetic, cytological, and immunological features of the response of normal and cortisol-treated animals to SRBC indicates, we believe, that these cells are involved in the earliest phases of particle trapping, phagocytosis, and antigen processing. This does not exclude other suggested roles for lymphocytes produced in the thymus or migrating through the thymus from the marrow. Some of these cells may evolve into immunocytes in the periphery (47, 49). The vast majority, however, have kinetic and functional features which resemble more closely those of other short-lived cells participating in the inflammatory response than those of long-lived immunologically responsive lymphocytes.

SUMMARY

The sequence of morphological changes in the rat spleen following SRBC injection associated with hemolysin production has been correlated with estimates of proliferative activity by splenic lymphatic tissue. Formation of new, reactive germinal centers containing macrophages which engulf nuclear debris is a prominent feature of the response. This is prevented by pretreatment of the animal with cortisol. Indirect evidence is provided that short-lived lymphocytes produced in germinal centers may be a necessary component in the induction of other cells to proliferate and differentiate into hemolysin-producing cells. The reasons are discussed for considering short-lived lymphocytes, such as those produced in the thymus, bone marrow, and germinal centers, as differing from long-lived lymphocytes capable of antibody synthesis.

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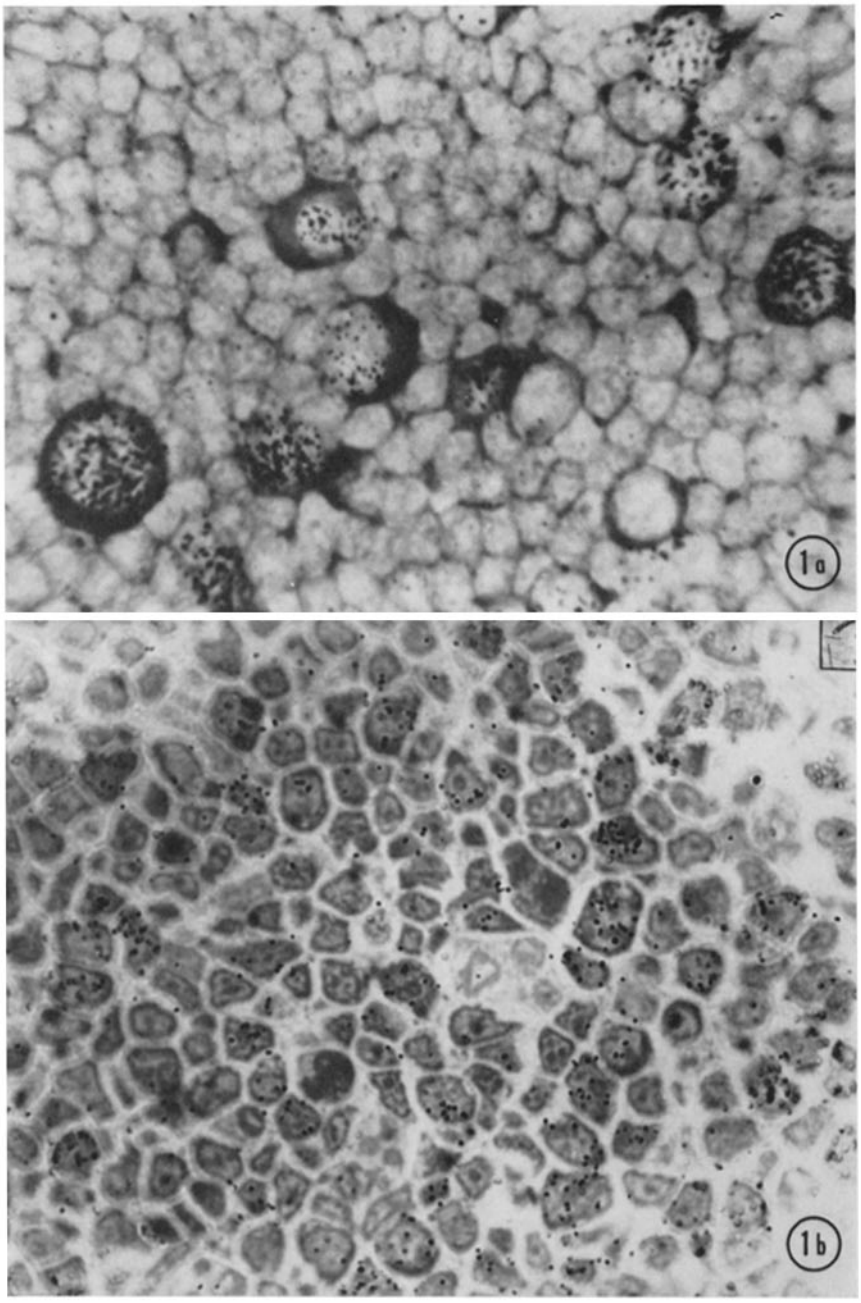
EXPLANATION OF PLATES

PLATE 115

FIG. 1 *a*. Radioautograph of section through the densely packed lymphocytes in the cuff surrounding a germinal center in the lymph node of a normal rat sacrificed 1 hr after 0.5 μc/g of ^{3}HT. The majority of cells are small lymphocytes. The scattered immature cells show a high grain count per labeled cell. × 1250.

FIG. 1 *b*. Radioautograph of thin section through the germinal center of a lymphoid follicle in a normal lymph node. A darkly tingible body macrophage is near the center. A high percentage of lymphoid cells are in DNA synthesis, and hence labeled with ^{3}HT, or in mitosis. The average number of grains per labeled cell is lower than in the labeled cells in the surrounding cuff of lymphocytes. × 1000.

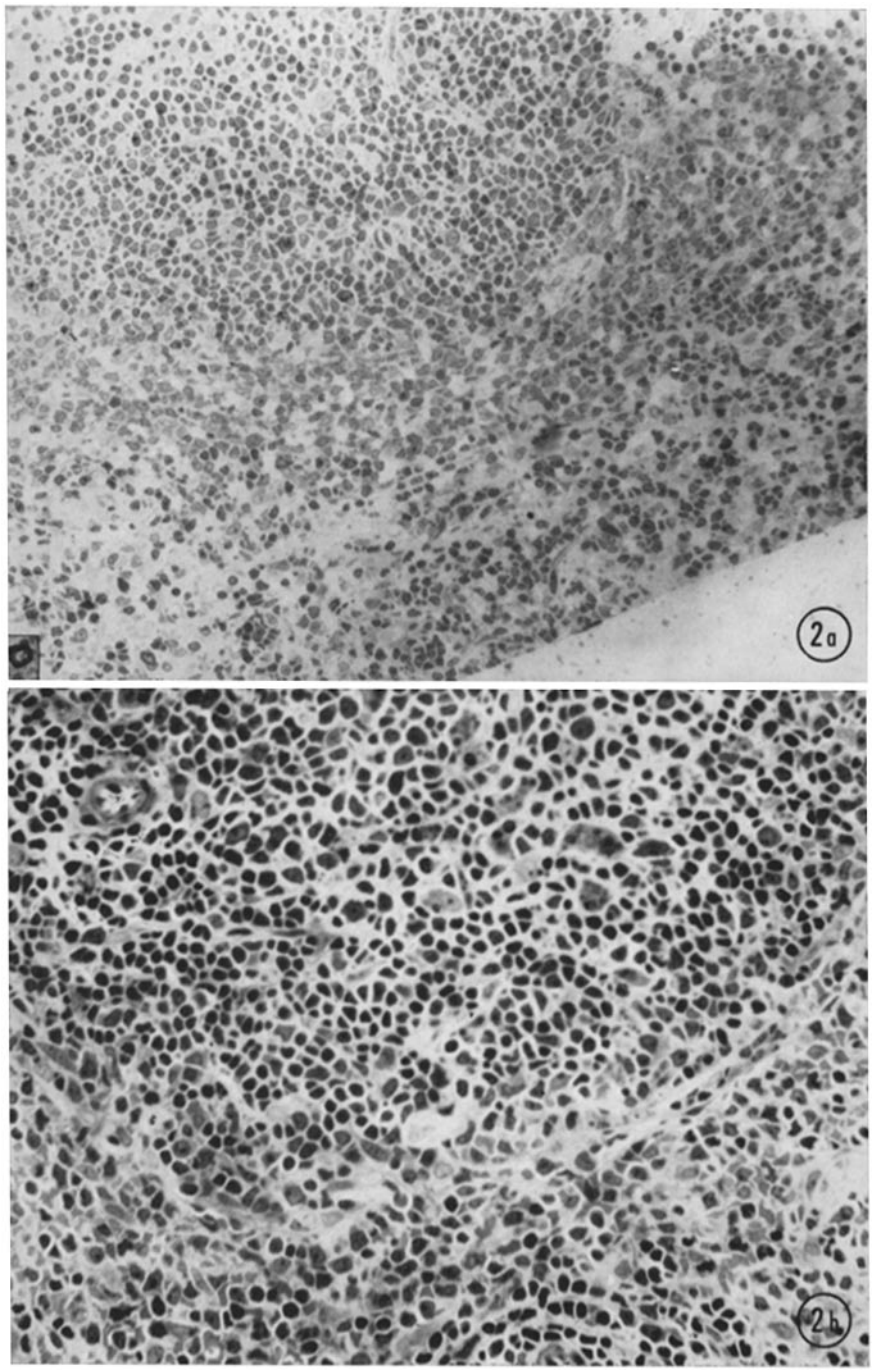


(Craddock et al.: Foreign red blood cells and short-lived lymphocytes)

PLATE 116

FIG. 2 *a*. Thin section of normal rat spleen showing portion of a lymphoid nodule, marginal vascular zone containing paler staining endothelial and phagocytic cells and red pulp.

FIG. 2 *b*. Thin section of a reactive splenic lymphoid follicle in a normal rat 6 days after SRBC. Note the large macrophages containing phagocytized material scattered through the nodule. × 250.

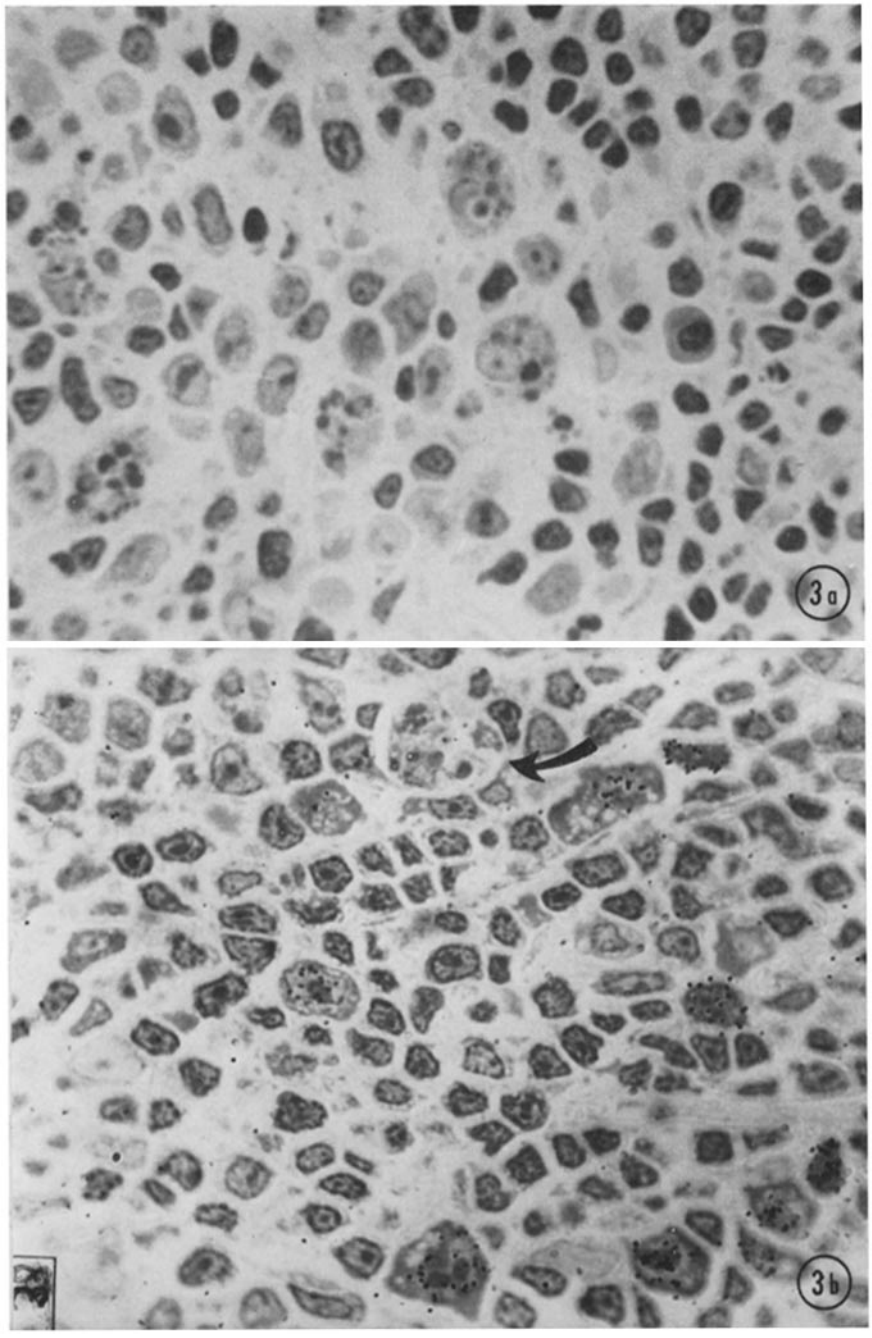


(Craddock et al.: Foreign red blood cells and short-lived lymphocytes)

PLATE 117

FIG. 3 *a*. Thin section of spleen of normal rat 5 days after SRBC. There are many tingible body macrophages in this germinal center. × 1000.

FIG. 3 *b*. Radioautograph of thin section of same normal rat spleen showing a large tingible body cell (arrow) and large pyroninophilic lymphoblasts which have incorporated ^{3}HT. This field is at the periphery of an expanding lymphoid follicle. A labeled Russell body cell is below the arrow. × 1000.



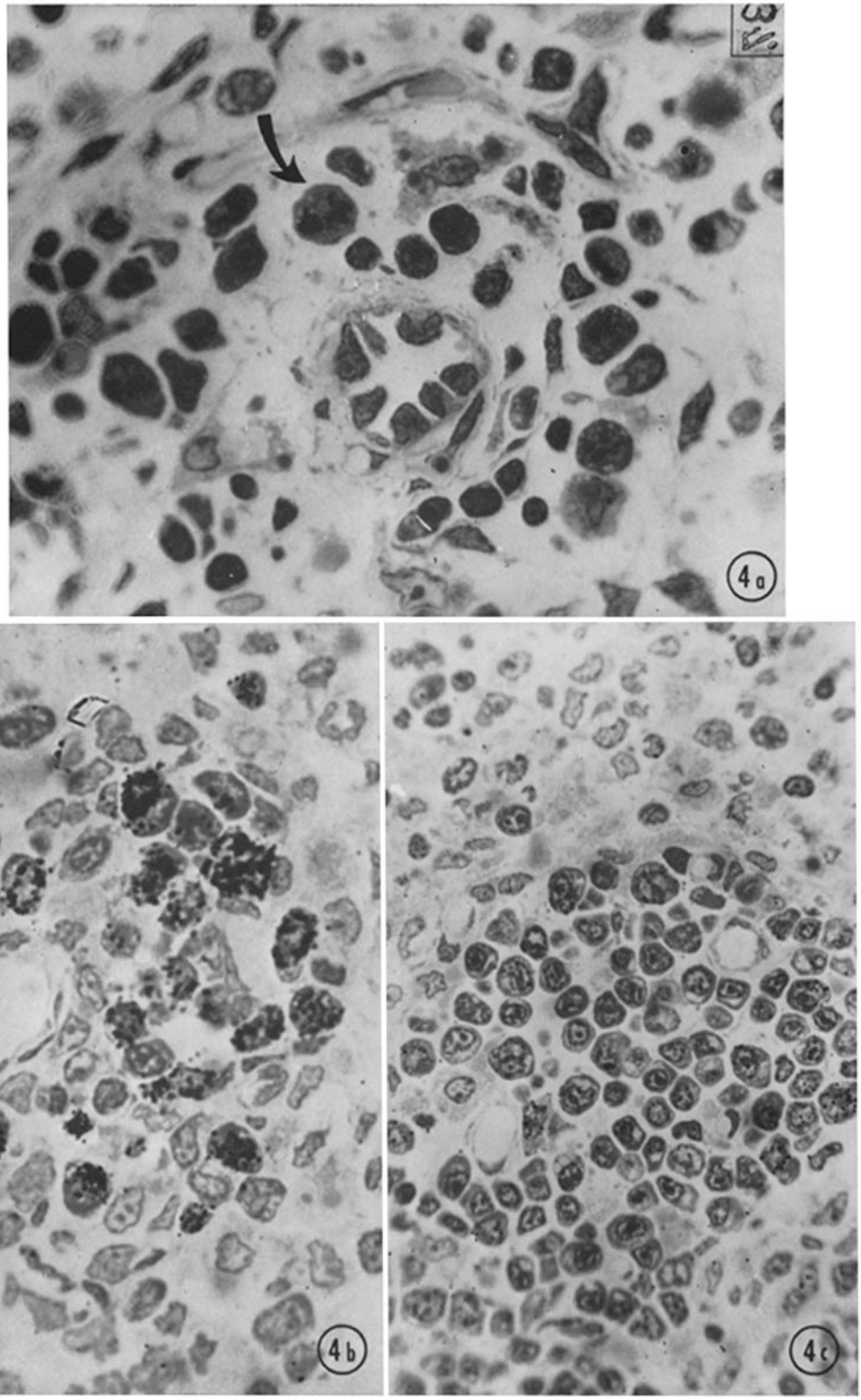
(Craddock et al.: Foreign red blood cells and short-lived lymphocytes)

PLATE 118

FIG. 4 *a*. Residual lymphocytes and plasmacytoid cells surrounding a vascular sinus in the lymph node of a rat given four daily doses of cortisol, 5 mg per 100 g body weight. The last cortisol injection was 8 hr before sacrifice. Note the mitotic cell (arrow). Epon embedded, section at 1 μ, toluidine blue–pyronine stain. × 2000.

FIG. 4 *b*. Radioautograph of a lymph node 3 days after four daily doses of cortisol. There is a focus of very active lymphopoiesis and many highly labeled cells without the presence of macrophages or tingible body cells. × 1200.

FIG. 4 *c*. Lymph node 10 days after four doses of cortisol. × 900.

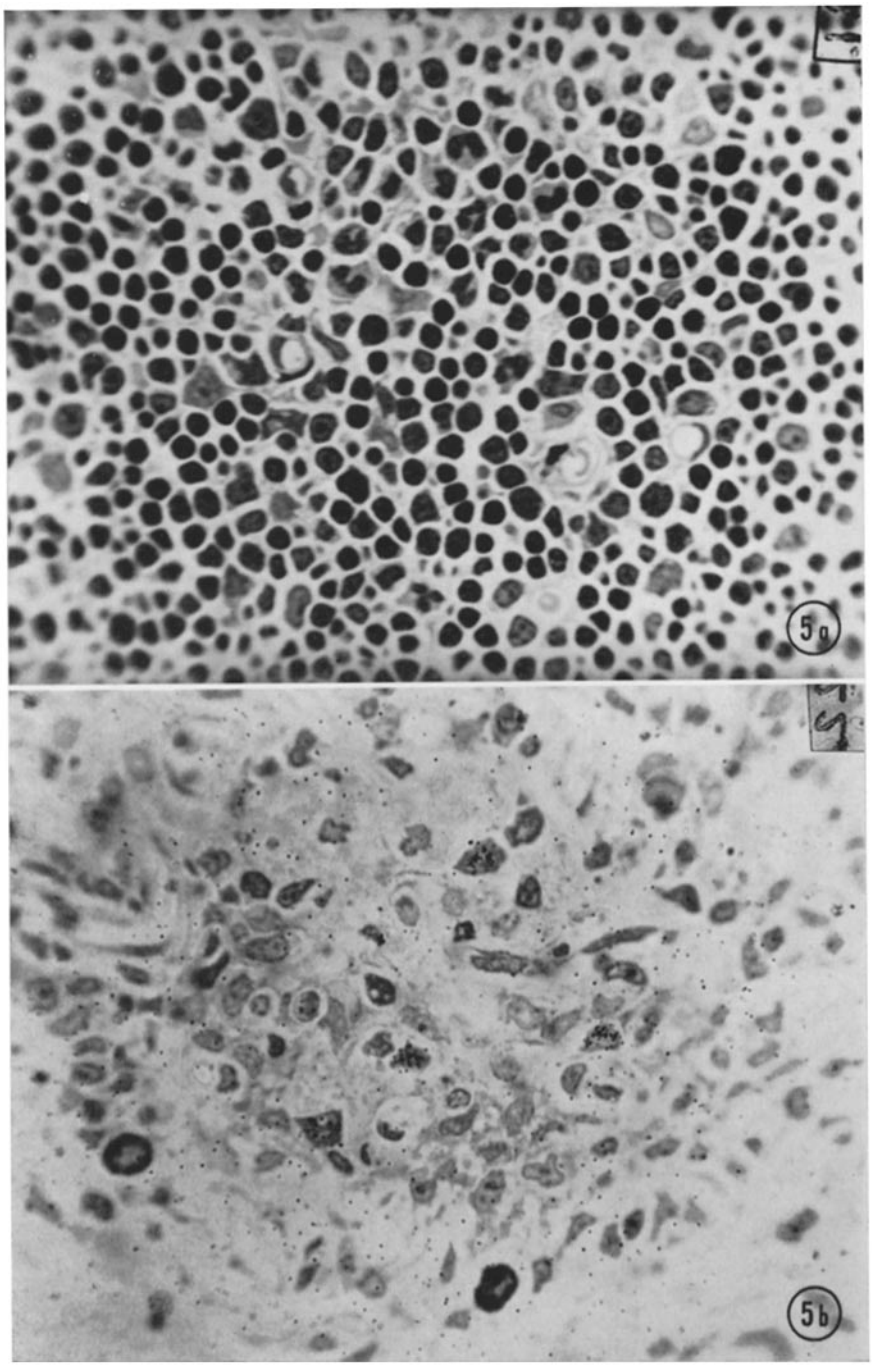


(Craddock et al.: Foreign red blood cells and short-lived lymphocytes)

PLATE 119

FIG. 5 *a*. Normal rat thymus cortex near junction with medulla showing lymphoid cells and scattered focal myelopoiesis. × 600.

FIG. 5 *b*. Thymus of rat shown in Fig. 4 *c*. There is still, at 10 days after cortisol, marked collapse of the tissue with prominent stromal cells and mast cells (the large cells with dark cytoplasm). The few highly labeled cells are myelocytes. There is no evident lymphopoiesis. There is considerable background scatter of radioactivity. × 600.



(Craddock et al.: Foreign red blood cells and short-lived lymphocytes)