PROLIFERATIVE RESPONSE OF THE SPLEEN AND LIVER TO HEMOLYSIS*

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PLATES 20 AND 21

(Received for publication, April 9, 1965)

Clearance of cellular and particulate matter from the circulation is carried out by the reticuloendothelial system (RES). This filtering function, often presumed to reflect phagocytosis, has been studied extensively from the kinetic standpoint, largely through observation of the clearance of foreign particulate matter in animals (1, 2) and of altered red cells in man (3–6). There is relatively little information as to the size of this system and its rate of cellular turnover, mainly because the RES is widely distributed and is composed of several cell lines having indistinct morphological features and derivations (7–9). The principal cells of the RES, the macrophages, sinus lining cells, and reticulum cells, appear to be responsible for the mechanism for trapping, ingesting, and dissolving particulate matter. These "true" reticuloendothelial cells exist in close structural combination with immunologically reactive cells, the lymphocytes and plasma cells, in the spleen and lymph nodes, but exist largely apart from these in the liver, bone marrow, and a number of other tissues Estimates have been made as to the relative distribution among various organs of reticuloendothelial cells based on particle uptake studies (10, 11). However, the relative uptake of cells or particles by such components of the RES as the spleen and liver varies grossly with the kind and amount of particle preparation being tested (12), rendering such estimates almost useless. Accordingly, one can only state at present that most intravascular cells or particles are cleared principally by the liver and spleen, usually to a smaller extent by the bone marrow (13), and little elsewhere.

Although the normal cytokinetics of the RES are little understood, it is known that the size and filtering activity of the RES are increased following injection of a number of foreign substances such as bacterial endotoxin (14, 15), methyl cellulose (16, 17), saccharated iron oxide (18), polyvinylpyrrolidone (19), and zymosan (20). Chronic injection of such foreign macromolecules into experimental animals has been found to cause splenomegaly and hematologic changes resembling those of "hypersplenism" in man (17, 21). Eventually, repeated injections of stable substances such as polyvinylpyrrolidone may create a sustained, "autonomous" increase in proliferative activity

* This investigation was supported in part by Grants T1-AM-5391, HE-07652, K3-HE-3943, and CA-05313 from the United States Public Health Service.

‡ United States Public Health Service Research Career Development Awardee.

that resembles neoplasia (19). Recent studies by Kelly and her coworkers (18, 22) indicate that injection of foreign organic particles stimulates division in the hepatic littoral (RE) cells (*i.e.*, the Kupffer and sinus lining cells of the liver). To what extent these proliferative responses are attributable to antigenic or toxic properties of the injected materials is speculative. Evidence that non-immunologic particles may stimulate RES proliferation may be inferred from the fact that patients with chronic, non-immunological hemolytic anemias such as hereditary spherocytosis or certain hemoglobinopathies develop splenomegaly and may show evidence of "hypersplenism" (21, 23–25). On the other hand, splenomegaly during hemolytic anemia may largely reflect red cell debris, the true reticuloendothelial mass being unknown. Nevertheless, recent studies (26, 27) have shown that spleen autotransplants grow more rapidly and function more actively as filters in splenectomized rats than in rats having intact spleen tissue; these differences are exaggerated by imposing a hemolytic process. Therefore it was proposed (27) that homeostatic regulation of the reticuloendothelial system is governed directly by the total particulate "work load."

In order to observe the "resting" and "working" RES more directly, studies have been made in rats of the cellularity and proliferative activity of the spleen and liver as judged by deoxyribonucleic acid (DNA) content and tritiated thymidine (H^3Tdr) incorporation. In addition, measurements of spleen cellularity have been made in man. A portion of these observations has been reported elsewhere in preliminary form (28).

Materials and Methods

Animal Studies.—All animal studies were made on males of a Caesarian-derived strain of Sprague-Dawley rat (Charles River C. D.).[1] Despite attempted stimulations, no red cell isoantibodies have been provoked in animals of this strain. Blood specimens for autologous or homologous survival studies were obtained by cardiac puncture and were exposed only to sterile, pyrogen-free glassware and solutions. Red cells were labeled with Cr^{51} in the form of $Na_2Cr^{51}O_4$.[2] Heat injury (5) of autologous or homologous red cells was achieved by incubating a 50 per cent saline suspension of cells, that had been washed in isotonic saline, in a water bath at 49.5°C for 1 hour. Preliminary studies revealed this to be an optimal temperature for producing non-viable rat red cells with very little hemoglobinemia; under these conditions approximately 42 per cent of injected cells are sequestered within 2 hours in the spleen, 18 per cent in the liver and 9 per cent in the bone marrow of normal rats. Phenylhydrazine[3] was injected subcutaneously as a neutralized, buffered, 1 per cent solution sterilized by passage through a membrane filter. Although the possibility of bacterial pyrogens in this preparation is difficult to ascertain, its intravenous injection in other animals did not elicit the rapid leukopenia and then leukocytosis that characteristically follow intravenous injection of pyrogens or antigens. Intravenous injections were made through tail veins. Small blood samples were obtained through small nicks near the end of the tail. Animals were sacrificed by cervical dislocation under ether anesthesia. Excised tissues were expeditiously weighed and aliquots were prepared for freezing or fixation as described below.

DNA Analysis.—Freshly thawed frozen tissue specimens were homogenized with a glass tissue homogenizer in approximately 4 volumes of distilled water at 0°C: isotonic saline was

[1] Obtained from Charles River Breeding Laboratories Inc., Wilmington, Massachusetts.

[2] Obtained from Abbott Laboratories, North Chicago, Illinois.

[3] Phenylhydrazine hydrochloride, Fisher Scientific Co., Fair Lawn, New Jersey.

found to give a much less even suspension than did water. DNA was measured on duplicate samples by the diphenylamine method of Dische as modified by others (29). Salmon sperm deoxyribonucleic acid[4] (DNA) was used as a standard, with phosphorus content as the reference. DNA extraction for subsequent measurement of radioactivity was achieved by the hot sodium chloride method (30–32) as modified by Littlefield (33).

Uptake of Tritiated Thymidine.—Tritiated thymidine[5] (H^3Tdr), specific activity 1.90 c/mole, was injected intravenously as 300 μc in 1.5 ml of sterile, pyrogen-free saline. Except in "pulse-labeling" studies, the H^3Tdr was injected 2 hours before sacrifice. Following extraction from the homogenate, the DNA was dissolved in N/100 NH_4OH in distilled water and 0.1 ml samples containing about 100 μg DNA were pipetted onto stainless steel planchets and dried in warm air. The amounts of DNA in the aliquots did not cause significant absorption of H^3 radioactivity. Radioactivity was measured in a Robinson windowless proportional flow counter perfused with 95 per cent argon/5 per cent CO_2. The counting efficiency for tritium averaged about 22 per cent. The chief source of error arose from variation in the drying patterns on planchets. Quadruplicate samples were counted for at least 10 minutes in order to reduce the over-all error to less than ± 5 per cent.

Autoradiography.—Portions of the same tissues analyzed for DNA and for H^3Tdr, were fixed in formalin and in Carnoy's fixative, both for routine histology, using hematoxylin and eosin, methyl green–pyronine, and Giemsa stains, and for autoradiography. In addition, imprints of spleen fragments were made for staining with Wright's stain. Autoradiographs were prepared with Kodak NTB emulsion (34, 35) and usually were stained in duplicate with hematoxylin and with methyl green–pyronine.

Miscellaneous.—Blood cells were enumerated or measured by standard techniques (36). The total protein content of serum was measured by the biuret reaction (37), and the various protein fractions were determined by densitometric analysis of their electrophoretic patterns in agar gel.[6]

RESULTS

Measurements of the Spleen and Liver in the Rat.—Table I presents observations and measurements made in 55 normal male rats weighing an average of 407 (range 310 to 510) gm. It can be seen that whereas the liver of male rats of this age is about 18 times the size of the spleen, its DNA content is only threefold that of the spleen. From knowledge of its DNA content it is possible to calculate the number of cells in a tissue by dividing by the fixed DNA complement of individual somatic cells of the particular species (38). This estimate may be somewhat in error with respect to granulocytes (39) and ignores the excess DNA present during the synthetic (S) phase of cell division, a relatively small error in most normal tissues, and must take into account the average number of nuclei per cell in tissues such as the liver that contain numerous binucleated cells. The cellular complement of DNA in the rat (and in man) has been found by a number of observers to be about 6×10^{-12} gm in somatic

[4] California Foundation for Biochemical Research, Los Angeles.

[5] Schwarz BioResearch, Inc., Orangeburg, New York.

[6] We are grateful to Dr. F. S. Bigelow for performing the rapid microelectrophoreses in agar gel.

TABLE I

Quantitative Comparisons of Spleen and Liver in Normal Male Rats

Measurement	No. of rats	Spleen	Liver	Approximate ratio spleen/liver
Weight, *gm*	55	0.808	14.440	1/18
DNA, *mg total*	55	11.2 ± 1.0*	32.2 ± 2.4*	1/3
DNA, *mg/gm*	55	13.9	2.2	6/1
No. of cells	55	1.9×10^9	3.2×10^9	3/5
No. of RE cells‡	55	1.0×10^9	1.2×10^9	1/1
H^3Tdr uptake§	31			
cpm/mg DNA	31	63,200	10,800	6/1
cpm, total	31	641,500	336,300	2/1
Cell labeling, per cent	31	4.1	0.57	7/1

* ± 2 standard errors.

‡ This term is employed here to denote mononuclear cells aside from lymphocytes or hepatocytes.

§ 2 hours after a single intravenous injection of H^3Tdr.

TABLE II

*Relative Cellularity and Intensity of Labeling with H^3Tdr in the Various Structural Components of the Normal Rat Spleen**

Structure	Portion of total cells	Lympho-cytes	Large mononuclear cells	Granu-locytes	Cells labeled	No. of grains per labeled cell
	per cent				*per cent*	
Germinal center	4.0	11.1	88.9	0.0	42	10–30
Lymphatic mantle	27.3	88.7	11.3	0.0	1	30–60
Marginal zone	21.0	32.6	67.0	0.4	2	20–100
Red pulp	47.7	31.3	68.2	0.5	4	20–40
Total	100.0	46.4	53.3	0.3	4	—

* Based on counts of almost 50,000 cells in 7 normal rats that had been sacrificed 2 hours after a single intravenous injection of H^3Tdr.

tissues other than the liver, which has a value of 10×10^{-12} gm (38, 40). Accordingly the normal rat spleen appears to contain almost 2 billion cells (1.9×10^9) and the liver somewhat over 3 billion cells (3.2×10^9).

Differential cell counts in the autoradiographic sections of normal rat liver revealed that 74.4 per cent (range: 72.2 to 75.8 per cent) of the nuclei were those of hepatocytes and almost all of the remainder were of littoral cells. This indicates that about 43 per cent of the liver cells (25.6 per cent of nuclei $\times$ $\frac{32.2 \times 10^{-3}\text{gm DNA}}{6 \times 10^{-12}\text{gm DNA/nucleus}} \div 3.22 \times 10^9$ total cells in liver) were cells

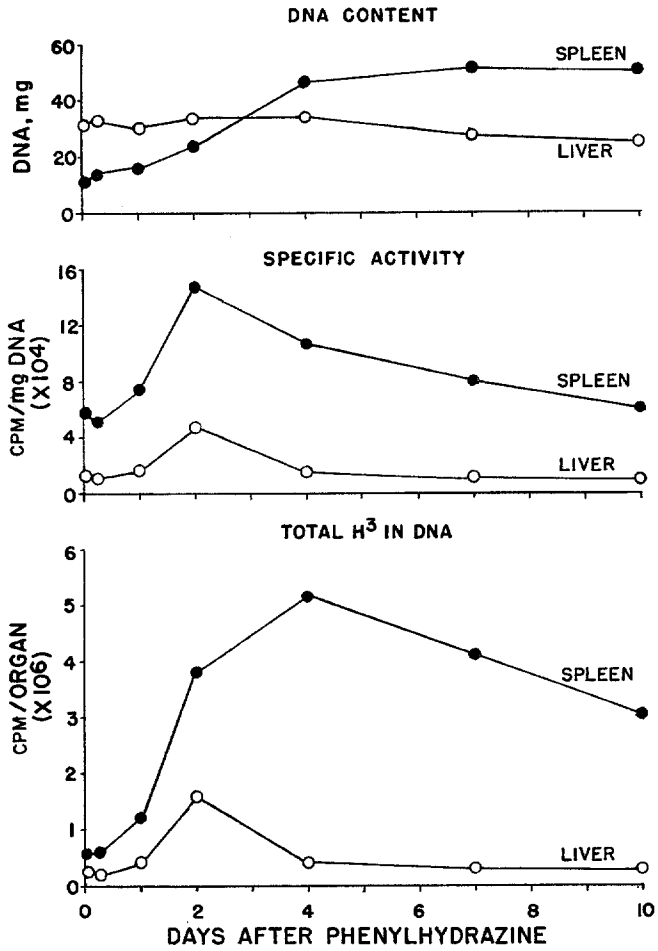
other than hepatocytes, assuming all binucleated cells to be hepatocytes. Subtracting that 6 per cent of the liver cell population made up of cells other than parenchymal or RE cells (41), it may be estimated that about 37 per cent of the liver cells in these rats were RE cells, an estimate similar to that of 38 per cent made previously by others in rats of this size (41) and in mice (18, 22). Tritium labeling was found in only 0.1 to 0.2 per cent of the hepatocyte nuclei, in accordance with previous reports (42), while that in the littoral cells averaged 1.2 per cent (range: 0.9 to 1.5). Thus, approximately 0.5 to 0.6 per cent of all liver cells in the normal rat were labeled with tritium 2 hours after the injection of H^3Tdr.

Differential cell counts in the spleen sections are much less reliable because of the difficulty in random sampling of white and red pulp and of categorizing the varieties of mononuclear cells encountered. Using a "tracking" pattern similar to that employed in lymph nodes by Litt (43) it was found that about half of spleen cells (46.4 per cent) were identifiable as lymphocytes and most of the remainder (53.3 per cent) were larger mononuclear cells (Table II). It should be noted that the histological estimate differs considerably from that obtained from imprints or smears of aspirated material, in which preparations a much higher percentage (80 to 90 per cent) of the cells are recognizable lymphocytes (44–46). Of the large mononuclear cells considerably more than half appear to be represented by reticulum cells, macrophages, and sinus lining cells. The majority of these cells are located in the red pulp, which contains about half of all the spleen cells, and in the marginal zone (Table II).

Labeling with H^3Tdr in the normal rat spleen greatly exceeds that in the liver. The average incidence of labeling (labeling index) within various structures of the normal rat spleen is presented in Table II. Labeling is heaviest in large and medium sized mononuclear cells. The cells of the germinal centers are labeled in a very high percentage (usually between 40 and 50 per cent) although the grain count per labeled cell is characteristically relatively low (average 15 grains/cell). Elsewhere in the spleen labeled cells varied in grain count by regions (Table II) but averaged 50 to 60 grains per cell. Small numbers of such heavily labeled cells are scattered among the lymphocytes of the lymphatic mantle, larger numbers are irregularly distributed in the marginal zone and still larger numbers of labeled cells, usually in clusters, are encountered in the red pulp. Labeled cells in the marginal zone and red pulp are usually faintly pyronine-positive. Most of the pyronine-positive cells in the red pulp resemble plasma cells or immature lymphocytes; some, although very few, are nucleated red cells. A representative autoradiographic view of a normal rat spleen 2 hours after an injection of H^3Tdr is presented in Fig. 1.

In the 31 normal rats that served as paired controls for the experimental animals receiving H^3Tdr, the specific activity of the isolated DNA from spleens was about 6 times that from the livers (Table I). The total uptake of H^3 into spleen DNA was about twice that into liver DNA.

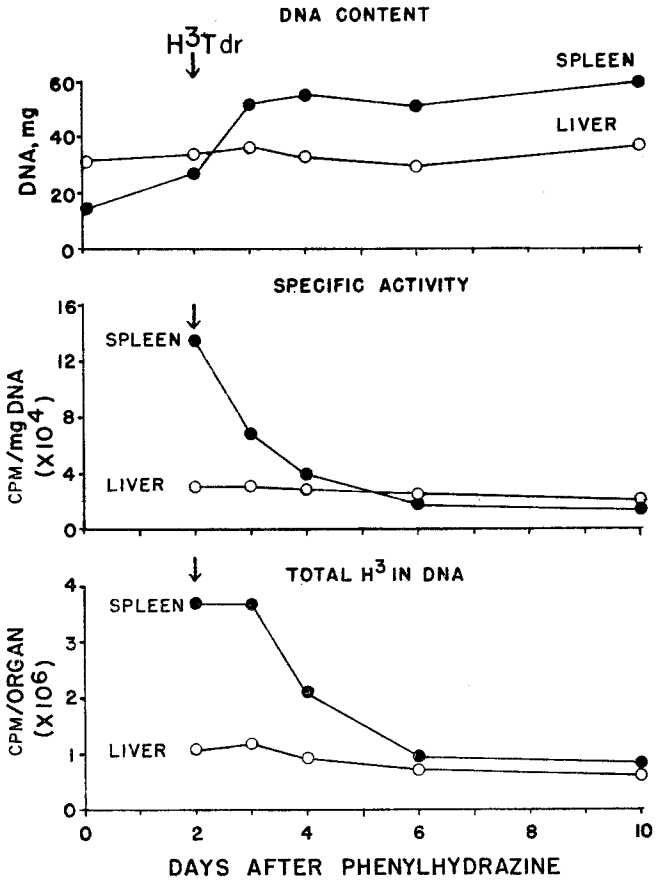
Effect of Oxidative Hemolysis on Spleen and Liver H^3 Incorporation into DNA. —A single subcutaneous injection of 20 mg of phenylhydrazine into male rats weighing approximately 450 gm caused an acute self-limited hemolytic episode.



TEXT-FIG. 1. DNA levels and H^3 activity in the spleen and liver after a single injection of phenylhydrazine: 2-hour label with H^3Tdr. The "zero" values represent the average of 3 control animals injected with saline. Each experimental point represents the average of 3 rats.

The course of this episode was as follows: (*a*) a transient fall in hematocrit from 46 to 37, followed by a rise in reticulocytes from 3 per cent to 20 per cent; (*b*) early methemoglobinemia and then sulfhemoglobinemia, Heinz bodies, and spherocytosis, and (*c*) an increase in spleen weight from 0.7 gm to a mean maximum of 2.4 gm 7 days later, with no significant change in liver weight.

These serial changes, very similar to those with a slightly larger dose of phenylhydrazine (28), resemble those observed with a single dose of acetylphenylhydrazine (47, 48). Text-fig. 1 shows the serial changes in the DNA of spleens



TEXT-FIG. 2. DNA levels and H^3 activity in the spleen and liver after a single injection of phenylhydrazine: Pulse-label with H^3Tdr. The H^3Tdr was injected (arrow) 2 days after phenylhydrazine and the first animals were sacrificed 2 hours later (day 2). The "zero" values represent the average of 8 control animals injected with saline. Each experimental point represents the average of either 2 or 3 rats.

and livers of animals during this hemolytic process. Each point represents the average of 3 rats. Although hepatic DNA levels remained fairly steady (upper portion), there was a marked and progressive rise in spleen DNA to about 5 times normal levels. The uptake of H^3 into DNA 2 hours after injecting H^3Tdr began to rise 24 hours after phenylhydrazine was given and reached a peak

specific activity 2 days after phenylhydrazine. The peak rise in total H^3 incorporation into spleen DNA was achieved on day 4, when it reached almost 10 times the normal. A sharp but more evanescent rise in the specific activity of the liver was evident on day 2 with an accompanying sixfold rise in total H^3 uptake. Hepatic DNA activity fell off sharply, approaching base line levels on day 4.

The same dose of phenylhydrazine was given another group of rats in which a single "pulse" of H^3Tdr, injected 2 days after the phenylhydrazine, was followed for serial changes. Changes in DNA levels were comparable to those in Text-fig. 1. As shown in the middle portion of Text-fig. 2, there was a rapid decline in the specific activity of spleen DNA. Total DNA H^3 radioactivity remained constant for 1 day (from the 2nd to the 3rd day after phenylhydrazine) and then dropped sharply, with half the activity leaving the spleen in about 30 hours. In the liver there was a transient rise in total DNA activity 24 hours after injection of H^3Tdr; thereafter DNA radioactivity declined slowly.

Autoradiographic Changes.—In animals sacrificed 6 hours after phenylhydrazine was injected, there was marked engorgement of the sinuses of the red pulp, particularly those adjacent to the marginal zone, with masses and clusters of red cells. In addition there were numerous red cells, many of them aggregated, within the marginal zone, which had become loosened, partially disorganized, and less pyroninophilic. Discoloration and hyalinization of red cells was evident in the sinuses and cords neighboring the marginal zone and scattered erythrophagocytosis was seen, mainly in areas of red pulp more distant from the follicles. An increase in H^3Tdr labeling was first evident at 24 hours, particularly in the nuclei of the large mononuclear cells of the marginal zone, albeit a general increase in the normal labeling pattern was evident in the red pulp and in the scattered large cells of the follicle. Tritium labeling was striking 48 hours after phenylhydrazine, particularly in the red pulp, and most of the labeled cells were to some extent pyroninophilic. Heavy labeling continued in the red pulp and marginal zone for several days, attended by a striking increase in the cellularity of the red pulp. The cells taking up H^3Tdr at 4 days were of a variety of types, including macrophages, plasma cells, lymphocytes, and erythroblasts in roughly equivalent numbers. At 7 days, red cell engorgement had subsided, the red pulp was densely populated with the various cells cited above, most of which were still taking up H^3Tdr, and the normal compact structure of the marginal zone had been restored.

In the pulse-labeling experiment in which H^3Tdr was given 48 hours after the phenylhydrazine there was a steady shift of labeled cells from the marginal zone and follicle out into the red pulp during the 1st several days, attended by a rapid decline in grain counts per cell. During the 1st 24 hours after the injection of H^3Tdr the mean grain count of smaller mononuclear cells fell from about 30 to 40 grains per cell to about 6 to 15 grains per cell, suggesting that approxi-

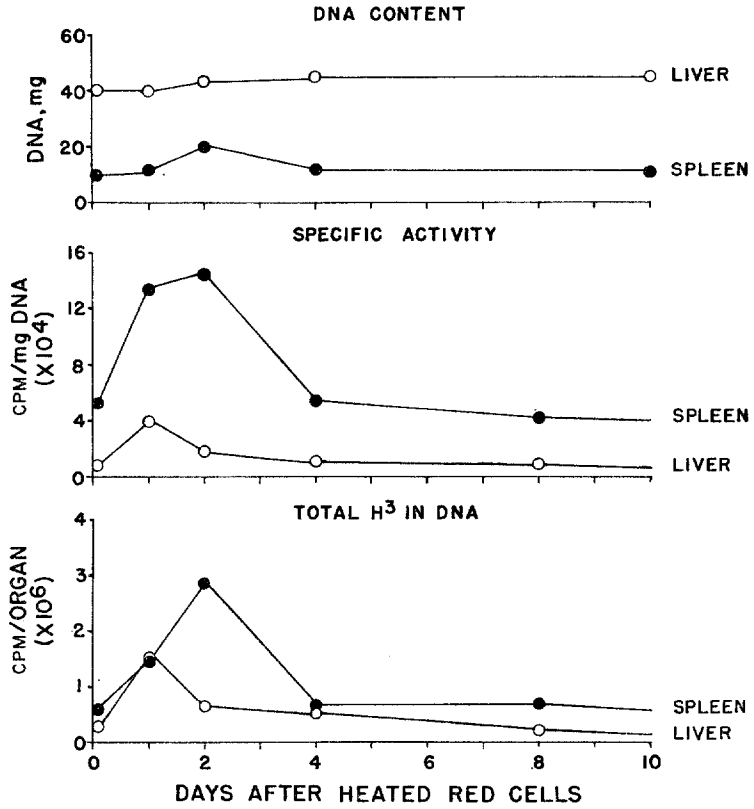
mately 1 or 2 division steps had taken place. By 4 days after H^3Tdr, most of the small cells of the highly cellular red pulp were labeled, albeit very lightly (1 to 3 grains per cell), suggesting 4 or more divisions since administration of the label. The decline in grain count in large mononuclear cells, including macrophages, was less, falling from 30 to 35 to 15 to 20 in 6 days. Mononuclear cell "ghosts" were relatively frequent in the marginal zone and red pulp 2 to 8 days after H^3Tdr (and 4 to 10 days after phenylhydrazine); these ghosts retained heavy nuclear labeling (20 to 40 grains/cell) throughout this period. The germinal cells showed their characteristic diffuse, light labeling during the first 2 days after injection of H^3Tdr. Many were still labeled at 4 days but only rare grains were encountered at 8 days. The grain counts were usually too low to allow one to estimate adequately the rate of decline.

As compared to the cytological upheaval taking place in the spleen, the reaction of the liver to phenylhydrazine-induced hemolysis was restrained. Six hours after the injection of phenylhydrazine, erythrophagocytosis was seen in the Kupffer cells and there was some increase in the number of red cells in the sinuses. A twofold increase in H^3Tdr labeling was seen at 24 hours and at 48 hours labeling was 4 to 6 times that of controls. Most of the increased labeling was in the littoral cells, although parenchymal cell labeling was also increased slightly. From 2 to 10 days after the onset of hemolysis clusters of small round cells were observed wedged within the venous sinuses. These clusters contained mixtures of lymphocytes, plasma cells and erythroblasts and almost all were labeled 2 hours after H^3Tdr. On pulse labeling the decline in grain count in littoral cells was much slower than was observed in small mononuclear cells of the spleen and resembled that of spleen littoral cells: 2 days after H^3Tdr (and 4 days after phenylhydrazine), the grain count had declined from an average of between 25 and 30 per cell to about 20 per cell and the littoral cell grain count was not halved until 7 or 8 days. Many cells containing hemosiderin were unlabeled. The clumps of small mononuclear cells encountered in the hepatic sinuses showed a rapid decline in grain count in parallel with similar cells in the spleen.

Effect of Injecting Heat-Injured Red Cells on Spleen and Liver.—

H^3 incorporation into DNA: In order to induce an abrupt episode of red cell destruction without anemia, each of a group of rats was injected with 10 ml of a 60 per cent suspension of heat-injured isologous red cells suspended in pyrogen-free saline. An equal volume of the saline was injected into controls. H^3Tdr was given intravenously to one group of animals 2 hours before sacrifice and to another at 24 hours after injection of red cells for a "pulse label" study. There were no consistent changes induced in controls, and the data for all 6 controls were averaged to provide the "zero" values for Text-fig. 3. As depicted in Text-fig. 3, there was a transient (60 per cent) increment in spleen DNA

levels 2 days after the hemolytic event. At both 24 and 48 hours a striking, threefold increase in specific labeling of spleen DNA, accompanied by a five-fold transient increase in total labeling of DNA. The specific activity and total activity of liver DNA also increased sharply (to 4 times control levels) at day

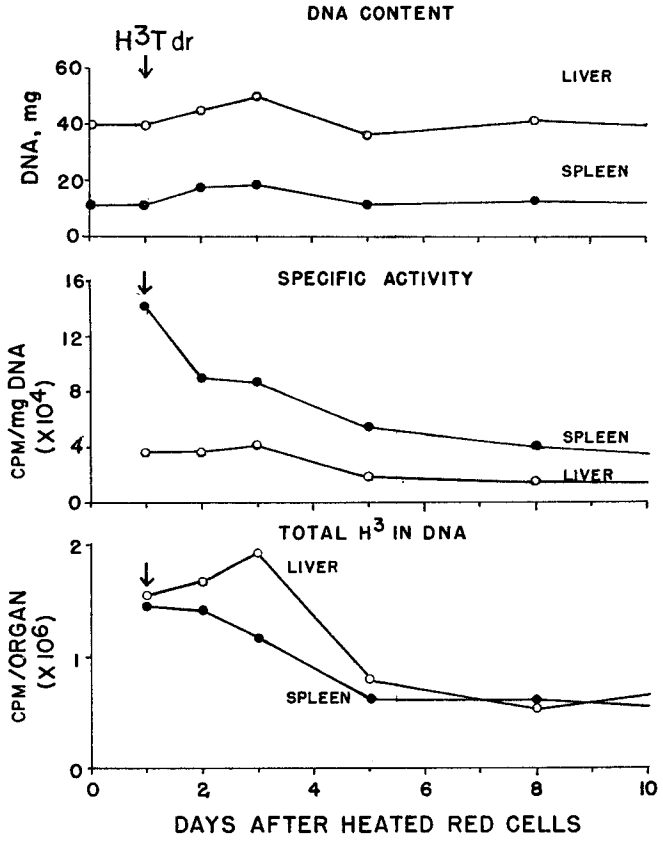


TEXT-FIG. 3. DNA levels and H^3 activity in the spleen and liver after a single injection of heated red cells: 2-hour label with H^3Tdr. The "zero" values are the means from 6 control animals injected with saline. Each experimental point represents the average of either 2 or 3 rats.

1, and fell thereafter even more abruptly than did the spleen uptake of H^3Tdr. Thus in both organs there was an intense but brief spurt of DNA synthesis.

The autoradiographic changes in the spleen at various times following injection of heated cells and 2 hours after H^3Tdr are portrayed in Figs. 2 *a* to 2 *f*. The changes resemble those reported above for phenylhydrazine albeit they are less extreme, more short-lived and do not involve a proliferation of erythropoietic elements. At 24 hours the perifollicular red pulp is seen to be engorged with red cells and there are a number of red cells trapped among the cells of

the marginal zone. Little erythrophagocytosis is evident except among macrophages at a distance from the follicles. A considerable increase in cell labeling is evident in the marginal zone, and also in the red pulp, but not necessarily in those cells adjacent to the sequestered red cells (Figs. 2 *c* and 2 *d*). At 48 hours



TEXT-FIG. 4. DNA levels and H^3 activity in the spleen and liver after a single injection of heated red cells: Pulse-label with H^3Tdr. The "zero" level of DNA is the average of 6 control rats injected with saline. Each experimental point is the average of 2 animals. The H^3Tdr was injected (arrow) 24 hours after the heated cells and the first animals were sacrificed 2 hours later (day 1).

an increase in pulp cellularity is evident, and nuclear labeling with H^3Tdr is intense, although uneven (Figs. 2 *e* and 2 *f*).

The uptake of H^3 into DNA after pulse-labeling with H^3Tdr is portrayed in Text-fig. 4. As in the study depicted in Figs. 2 *a* to 2 *j*, there was a modest, transient increase in spleen DNA at 2 to 4 days after the heated cells were injected, possibly accompanied by a rise in hepatic DNA. The specific activity

of spleen DNA declined rapidly in the spleen to half its original level in about 3 days, whereas the liver showed no change or possibly a slight rise in the first 2 days after H^3Tdr and then gradually declined thereafter. The total H^3 in spleen DNA changed little for a day or 2 and then fell to half the 2 hour value at between 3 and 4 days after the injection of H^3Tdr, declining rather slowly thereafter. Total H^3 in hepatic DNA actually rose for 2 days and then rather abruptly fell between 3 and 5 days after H^3Tdr. These findings indicate a transient input of label, apparently of labeled cells (see below), into the liver at a time when splenic activity was dropping off.

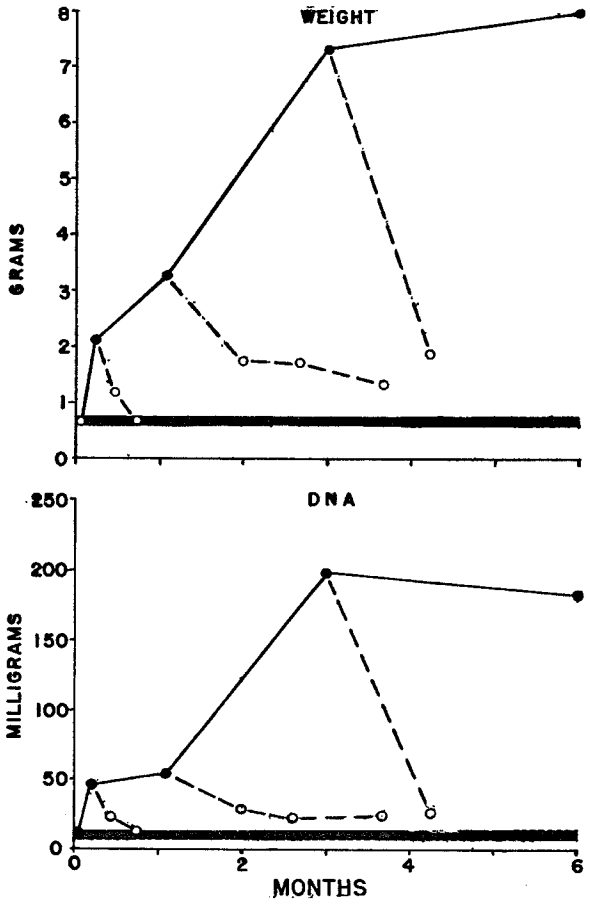
On autoradiography after pulse-labeling with H^3Tdr 24 hours after injection of heated cells, the changes observed were very similar to those described above after phenylhydrazine. As before, the labeled cells were at first encountered in the marginal zone, red pulp, and the larger cells of the follicle, but within 1 or 2 days the labeled cells moved out into the red pulp. Again, the decline in grain count was more rapid in small mononuclear cells than in the larger, littoral cells. In contrast to the phenylhydrazine experiments, only rare erythroblasts were present. In the liver there was the slow decline of grain count in the labeled littoral cells noted previously. On the 1st, 2nd, and 4th day after H^3Tdr clumps of labeled small mononuclear cells (lymphocytes and plasma cells) were seen in the hepatic sinusoids, presumably accounting for the transient increment in total labeled DNA in the liver shown in Text-fig. 4.

In a study comparable to the one reported above, 14 rats were bled of 2 ml of blood. Ten were reinjected with the autologous red cells after these had been heated for 1 hour and 4 received an equivalent volume of saline and the anticoagulant (citrate). One day after injection, H^3 uptake into spleen DNA 2 hours after H^3Tdr was increased to 179 per cent that of the controls. Hepatic incorporation of H^3 into DNA was increased at the same time to 196 per cent of controls. Both values declined to the control range by the 2nd day.

In still another comparable study 10 rats were injected with 3 ml of autologous blood, the cells of which had been rendered non-viable by sterile incubation for 18 hours. Seven control rats that had been similarly bled received incubated saline. Experimental animals were sacrificed in pairs at various intervals after injection of the cells and 2 hours after H^3Tdr. As after heated cells, these non-viable cells caused an increased labeling of spleen DNA to 211 per cent of control values in 24 hours. Hepatic DNA labeling was even more strikingly increased at 24 hours, reaching 331 per cent of control levels. This relatively greater effect on the liver is of interest, for most cells treated in this way are sequestered in the liver (49), whereas with the heated cell preparations employed the spleen is the more active site of sequestration.

The Effect of Chronic Hemolysis on the Rat Spleen.—A large group of weanling rats was given twice weekly injections either of neutral phenylhydrazine (6 mg/100 gm body weight) or buffered saline. At various intervals of time there-

after, animals were sacrificed and examined. At predetermined times phenylhydrazine was stopped in some rats and the animals were sacrificed at intervals thereafter. During these injections the animals maintained a chronic, com-



TEXT-FIG. 5. Effect of chronic hemolysis on the rat spleen. Each point represents the average of 3 rats. The shaded area denotes the limits of the values in 17 saline-injected control animals. The black circles and the solid lines connecting them represent results in animals that had received phenylhydrazine twice weekly until 3 days before sacrifice. White circles represent animals in whom phenylhydrazine was previously discontinued at a time indicated by the departure of the interrupted line from the continuous line.

pensated, hemolytic anemia with elevated reticulocyte counts (60 to 70 per cent) and normal hematocrit levels (range 44.5 to 49.4, as compared to the controls with a range of 45.6 to 52.3). Body weight was slightly below normal in hemo-

lytic animals, averaging 95 per cent of controls after one month, and 89 per cent of controls after 6 months, of hemolysis.

As shown in Text-fig. 5, spleen weight rose dramatically, to reach 10 times the normal in 3 to 4 months. DNA levels increased even more strikingly, reaching 18 to 20 times the normal in the same period. On cessation of the hemolytic process, splenomegaly regressed, as denoted by interrupted lines. However, spleens were over twice the weight and 3 times the DNA content of normal 33 days after the last dose of phenylhydrazine and both parameters were still increased by two- to threefold 74 days after cessation.

Spleen imprints of animals after 6 months of hemolysis revealed that between 5 and 20 per cent of the imprinted cells were nucleated red cells, whereas in control (mature) rats such cells were rare. Histologically, germinal centers were prominent and contained increased tingible bodies, the mantles were narrowed, the marginal zones were poorly defined, and all sinuses were markedly engorged with red cells and red cell pigment. The pulp was very cellular and contained conglomerates of all cellular types native to spleen, at least 10 per cent of the cells being blood cell precursors. The livers were somewhat enlarged to 27 per cent above the mean control weight by 6 months, but in all instances regressed to normal weight within a month of cessation of hemolysis. Liver histology was normal except for pigment in the Kupffer cells and scattered dense clusters of erythroblasts, lymphocytes, and plasma cells in the sinuses. There was also moderate portal infiltration with lymphocytes and some apparent proliferation of bile ducts.

In 24 hemolytic and 23 control animals serum proteins were analyzed at the time of sacrifice, after from 1 to 6 months of injections. Total protein levels were almost identical (mean for controls 6.31 gm per cent; mean in hemolytic anemia 6.34 gm per cent). On agar gel electrophoresis it was evident that the phenylhydrazine-injected animals developed hypergammaglobulinemia within 1 month, and this became more striking with time. After 3 months of hemolysis the γ-globulin of treated rats averaged 1.52 gm per cent as compared to 0.64 gm per cent in controls; at 6 months these values were 1.58 and 0.72 gm per cent, respectively.

Four rats that had received phenylhydrazine for 3 months and 6 saline-injected controls were given Cr^{51}-labeled normal, isologous red cells (1.2 ml of a 50 per cent suspension) intravenously 5 days after the last injection. Blood Cr^{51} was measured on tail samples for 4 weeks thereafter. In normal rats the red cell survival curve was almost rectilinear on arithmetic graph paper, half-survival being 21 days. In rats that had received phenylhydrazine (and were still splenomegalic) the survival curve for normal red cells was complex: the initial component involved about half of the cells and gave a half-survival value of about 7 days. Thereafter a slower component was manifest, having about half of the initial slope. On sacrifice, at 28 days, at which point about 60 per

cent of the injected activity was recoverable in the carcass, the distribution of Cr^{51} between spleen and liver was about the same in both groups, namely in a ratio of about 3 to 1.

DNA Content of Normal Human Spleen.—Spleen tissue was obtained at autopsy from 31 adult patients dead of various causes. Preliminary studies revealed that the measured levels of DNA in samples of human and rat spleen do not change over a period of incubation *in vitro* at 37°C of over 5 hours (al-

TABLE III

DNA Content and Calculated Cell Numbers in Human Spleens

Patients	Age	Weight	DNA concentration	DNA content	Total cell content
	yrs	*gm*	*mg/gm*	*mg*	
"Normal" spleens (20)	66 (42–83)	111 (60–190)	7.7 (4.5–10.8)	851 (402–1380)	1.4×10^{11}
Spleens enlarged for diverse reasons (11)	64 (40–84)	271 (200–540)	7.8 (6.6–9.6)	2106 (1180–5184)	3.5×10^{11}
Hereditary spherocytosis					
Adult L	34	700	10.2	7140	11.9×10^{11}
Adult P	42	745	16.3	12144	20.2×10^{11}
Adult D	38	444	9.8	4329	7.2×10^{11}
Adult S	24	420	14.4	6048	10.1×10^{11}
Adult Da	40	466	14.1	6571	11.0×10^{11}
Adult Di	61	542	11.7	6320	10.5×10^{11}
Adult E	33	430	10.8	4631	7.7×10^{11}
Child H	2	84	17.4	1462	2.4×10^{11}
Child K	4	128	9.4	1206	2.0×10^{11}
Idiopathic thrombocytopenic purpura					
Adult H	18	155	18.7	2899	4.8×10^{11}
Adult S	17	160	14.2	2269	3.8×10^{11}

though diphenylamine-reactive DNA is gradually lost after cellular disruption by homogenization). Samples were obtained within a few hours of death and were frozen with methanol-dry ice and stored for later analysis. The results are tabulated in Table III, in which spleens were designated as "normal" if their weights were below 200 gm. In patients with such normal-sized spleens, the mean spleen weight was 111 gm, as compared with an expected 139 gm in unselected autopsied patients of comparable age (50). The total DNA content of these spleens averaged 851 mg (range 402 to 1380). Assuming a mean nuclear content of 6×10^{-12} gm of DNA (40), this indicates that human spleens contain about 140 billion (1.4×10^{11}) cells. In patients with mild splenomegaly of

diverse causes, principally involving infection, the total DNA content averaged 2106 mg, indicating about 350 billion cells.

By comparison, the average liver weight in these patients was 1827 gm, and the average DNA content was 3332 mg (range 2040 to 4950). Employing an average figure of 10×10^{-12} gm of DNA per human liver cell (51) this indicates a cell population of about 330 billion, or about 2.4 times that of the "normal" spleen.

DNA Content of Hereditary Spherocytosis Spleens.—The spleens of 9 patients with mild to moderate hereditary spherocytosis were obtained at surgery and samples thereof were analyzed for DNA. None of the adult patients had received transfusions and none of the spleens was particularly large for this disorder. As shown in Table III, values for total DNA content ranged up to 12,144 mg and despite their characteristically increased content of sequestered red cells, all showed relatively high concentrations of DNA. Estimated total cellularity ranged from 5 to 15 times that of spleens from autopsied adults and exceeded that of their livers.

DNA Content of Idiopathic Thrombocytopenic Purpura Spleens.—The spleens of two patients with idiopathic thrombocytopenic purpura (ITP) were of normal size, as is usual in that disorder. The DNA concentrations were over twice normal, however, and the spleens contained from 2 to 3 times as many cells as in the normal.

DISCUSSION

The cellular content and proliferative activity of "resting" and "working" spleens were quantitated by combining biochemical and autoradiographic analyses. Cell numbers were estimated in spleens and livers by making use of the DNA constancy of the nuclei of somatic cells, an approach previously utilized for lymphatic tissues by Monden (46, 52).

The Normal Rat Spleen and Liver Cellularity.—Assuming a mean nuclear DNA content of 6×10^{-12} gm (38, 40) it is evident that the normal rat spleen contains more than half as many cells as does the much larger liver. Differential cell counts made on histological sections indicate the surprising fact that the normal spleen contains approximately as many non-lymphocytic RE cells (about 1 billion) as does the liver. Earlier estimates by others (44–46) reporting much higher lymphocyte concentrations were based upon imprints or aspirations, techniques which readily detach lymphocytes and other blood-borne cells but which characteristically do not dislodge RE cells that are structurally enmeshed in reticulin (53). It is noteworthy that the third major organ of the reticuloendothelial system, the bone marrow, has been found in the rat to contain a very similar number of RE cells: approximately 1 billion (13). Thus the markedly different abilities of these 3 RE organs to clear material from the blood stream appears to be dependent upon such factors as blood flow and specific affinities rather than upon cellular numbers.

Proliferative Activity of the Normal Rat Spleen. Special Features of Germinal Center Cells.—Although possessing only one-third as much DNA as the liver, the spleen incorporated about twice as much H^3 into its DNA following a single ("flash") injection of H^3Tdr, giving spleen DNA a specific activity about 6 times that of the liver. Commensurate with this, autoradiography revealed the over-all spleen labeling index to be about 7 times that of the liver, confirming the relatively high proliferative activity of the "resting" spleen. In the normal spleen 2 distinctive labeling patterns are seen on autoradiography: (*a*) most DNA synthesis is being carried out by medium-to-large mononuclear cells in the red pulp and in the marginal zones bordering the lymphatic follicles (Table II). Labeled cells constitute from 2 to 4 per cent of the population in these areas and show rather heavy nuclear labeling with tritiated thymidine. (*b*) The cells of the germinal centers were labeled in an extremely high percentage (40 to 50 per cent), but labeling was characteristically light, with grain counts only one-half to one-third those noted elsewhere in the spleen. This light-labeling of a high proportion of cells was noted in an earlier report of this work (28) and has been reported in greater detail and discussed recently by Fliedner *et al.* (54) and by Cottier and his associates (55). Previously, Craddock and Nakai (56) observed light-grained labeling to be a feature of thymic cells. Whether this light labeling pattern reflects a prolonged DNA synthetic period, differences in geometry or in the penetration of labeled thymidine, or a larger intracellular pool of thymidine, is uncertain; the fact that heavily labeled cells were frequently seen adjacent to the germinal centers is somewhat against the latter 2 explanations. However, Fliedner *et al.* (54) have evidence for a relatively short synthetic period. Furthermore, the fact that many germinal center cells also have a remarkable propensity for phagocytizing nuclear material (as the "tingible bodies" of Fleming) implies the availability of a large pool of nucleic acid precursors with a resulting dilution of label. Although these cells have been found to contain cytoplasmic debris and γ-globulin (57) in addition to nuclear material from various cell lines, the finding that most of the nuclear debris in tingible bodies is derived from plasma cells and lymphocytes (58) suggests that immunological information is being supplied or recovered. The recent observation by Fliedner and his associates (54) that the tingible bodies label rapidly with H^3Tdr has supported the view that many germinal center cells may die *in situ*, possibly with reutilization of their own nuclear material. The observations above and the conclusion that germinal centers are characteristic of secondary immune responses (59) suggest the possibility that this structure may provide a mechanism for immunological memory.

Histological Site of Red Cell Sequestration in the Spleen.—In order to impose a particulate "workload" on the RES, acute hemolytic episodes were induced in several ways: by injection of phenylhydrazine; by injection of heated homologous or autologous red cells; and by injection of stored autologous red cells. In each instance red cell sequestration in the spleen was most prominent in the

marginal zones and the adjacent areas of the red pulp, manifested by masses of trapped (but not phagocytized) red cells. Phagocytosis became striking only after several hours, as noted previously (64), and was chiefly evident in the deeper areas of the red pulp. This perifollicular pattern of sequestration, noted in an earlier report (28), was evident with all forms of red cell injury studied and is analogous to a number of previous observations of others who have observed that particulate matter of many sorts is first trapped in the perifollicular marginal tissue (60–63). Recently, Snook (63) observed that such particles as carbon and saccharated iron oxide appear first in the marginal zone where the particles are seen to be trapped between (but not within) the numerous non-reticular marginal cells residing just beyond the marginal sinus. During the subsequent several hours, the trapped particles are seen to move outward into the red pulp where they are found to be within macrophages. Similarly, the present and earlier studies (28, 64) with red cells indicate that altered red cells are initially trapped among the cells of the marginal zone and then are lysed *in situ* or are devoured by macrophages moving in from the more peripheral red pulp. It may be noted that the physical barrier for cells that is provided by the marginal zone in rat spleens may be supplemented variously in other species by other devices for braking arterial blood flow, including the high cuboidal arteriolar endothelium seen in rabbits, and specialized arterial sheaths, as in dogs (62). In its histological structure as well as gross relative size, human spleen resembles that of the rat.

Proliferative Response to Hemolysis.—Within 24 hours of the onset of hemolysis in all experiments, there followed a sharp increase in the over-all rate of DNA synthesis, with a resulting increment in DNA content. As indicated by DNA radioactivity after pulse-labeling, a large portion of the cells containing newly-synthesized DNA emigrated from the spleen between 1 and 6 days after incorporation of the label. Although there was a generalized increase in tritium labeling the most striking site of labeling and new cell formation was in the perifollicular regions of the marginal zones and the neighboring red pulp, the same regions that were most active in sequestering red cells. Subsequently, labeled cells from the follicle and marginal zones migrated into the red pulp and underwent serial divisions: as judged by grain counts at 7 days after a pulse label, the littoral cells and macrophages had undergone 1 or 2 divisions, while smaller cells, of the lymphocyte and plasma cell series, had undergone 4 to 6 divisions. Within several days this proliferative reaction led to a considerable increase in the cellularity of the red pulp, this hyperplasia involving an increase in all cell lines native to the spleen. In summary, splenic sequestration of red cells stimulated a marked proliferative reaction in the perifollicular region, which formed macrophages, lymphocytes, plasma cells and, in anemic states, erythroblasts. Many of these cells emigrated from the spleen, and clumps of them were observed transiently in the hepatic sinuses.

In the liver, red cell sequestration was less pronounced than in the spleen, but erythrophagocytosis in littoral cells was more prominent and occurred earlier. As in the spleen, there followed in all instances an abrupt 4- to 6-fold increase in DNA synthesis, as indicated by the incorporation of H^3Tdr into DNA, this reaction principally involving the RE components of the liver. Generally, the newly-labeled RE cells underwent only 1 or 2 divisions within the week after hemolysis and the net increment in liver size was inappreciable. Except for the appearance of clumps of small round cells found transiently within the hepatic sinuses and presumed to arise from the spleen, no cells of the lymphocyte, plasma cell, or erythroid series appeared to be generated by this hepatic reaction. The reaction of the hepatic RE cells to hemolysis resembled that described by Kelly and her associates (18, 22) following the injection of foreign organic particles.

The present studies were designed to demonstrate whether or not the sequestration of particulate matter *per se*, even autogenous cells, stimulated reticuloendothelial hyperplasia. The reactions in general resembled those described by others (65–69) after the injection of antigens, usually particulate antigens. The effects of phenylhydrazine might conceivably be attributed in part to a hapten action, although its prompt invariable effects and the failure to demonstrate a serum antibody active against phenylhydrazine-treated cells argue against such an immunological event. Furthermore, the similar reactions to autogenous cells that had been injured by heating or storage can not reasonably be attributed to immunological phenomena. The marked proliferative reaction to phenylhydrazine involved an erythropoietic response as well, and, indeed, uptake by the spleen of H^3Tdr has been employed by Kurtides and his associates (70) as a measure of erythropoietin action. On the other hand, only a minority of the splenic hyperplasia, and none of the hepatic response, involved erythropoietic cells. The sharp proliferative response to heated autologous cells involved no erythropoietic response in either tissue. It is reasonable to conclude from the present studies that the sequestration ("phagocytosis") of particulate matter does stimulate cell division in the RE system by non-specific (non-immunological) mechanisms. Further, it is possible that the cytoproliferative reactions ascribed by many to specific antigenic stimulation arise in fact from such a non-specific mechanism. The interpretation that the cytoproliferative response to antigens is largely if not entirely the result of this type of non-specific stimulation is supported by the numerous examples of particulate or colloidal materials which markedly enhance the production of antibody against antigen simultaneously administered (71–75). The source of energy for the proliferative response to hemolysis is unclear, but there is ample evidence that phagocytosis and pinocytosis (76, 77) act to stimulate the metabolism of the phagocytic cells, particularly through acceleration of the hexose monophosphate shunt. Thus, the proliferative reaction of the RES to hemolysis may be the

result of metabolic stimulation by particulate matter and may be akin to the reparative type of proliferative reaction (78).

Effect of Chronic Hemolysis in the Rat.—The imposition of a chronic "compensated" hemolytic state by repeated injections of phenylhydrazine induced a marked splenomegaly and moderate hepatomegaly and a 15-fold increase in spleen cellularity, as determined by DNA analyses. Apart from the hematopoietic elements, which constituted up to 20 per cent of the cells, there was hyperplasia of all reticuloendothelial elements: reticulum cells, macrophages, lymphocytes, and plasma cells. As the functional counterpart of the plasma cell proliferation, γ-globulin levels were elevated to almost 3-fold normal. It is difficult to exclude fully the possibility that phenylhydrazine acted as a hapten, but the failure to detect serological evidence for this, the fact that the γ-globulin elevation was diffuse rather than "monoclonal," and the exceptional magnitude of the elevation for this species argue against the possibility of occult immunization. It appears more likely that a generalized hyperplasia of spleen elements non-specifically increases γ-globulin synthesis. Presumably the γ-globulin produced represents antibody directed against the various ambient antigens, in which case the hemolysis acts as an adjuvant; it is also possible that some of the γ-globulin is non-functioning. In either event, it appears that splenic hyperplasia will with time engender hyper-γ-globulinemia. Attesting to this clinically is the frequent association of splenomegaly and diffuse hyper-γ-globulinemia in such disorders as alcoholic cirrhosis in which a primary immune mechanism is not believed to exist. Although there is conflicting evidence as to whether these patients produce excessive amounts of antibody (79, 80) it may be necessary to employ an appropriate intravenous particulate antigen to elicit differences, as was true in comparisons between normal and splenectomized individuals (81, 82).

Spleen Cellularity in Normal Man and in Hereditary Spherocytosis.—As judged by DNA analyses in autopsied adults who had died with normal-sized spleens, the adult human spleen contains about 140 billion cells (range 70 to 230 billion), almost half (42 per cent) the number found in the liver. In miscellaneous disorders involving mild or moderate splenomegaly the number was increased to about 360 billion. In many instances involving infection, sequestered granulocytes may have contributed to this figure; however, the largest spleen (540 gm) was from an uninfected patient with cirrhosis and congestive splenomegaly whose spleen contained an estimated 864 billion cells.

The cellularity of the enlarged spleens invariably found in patients with hereditary spherocytosis was of particular interest, for this hemolytic disorder arises from a defect primary to the red cell. In 7 adults, none of whom had been transfused, and in whom there was a relatively moderate rate of hemolysis and moderate splenomegaly, the calculated cell content of the spleen ranged from 720 to 2020 billion, and averaged about 8 times the "normal." Histologically,

the distribution of cell types was essentially normal, but with relatively more cellular marginal zones and red pulp. These findings bear out remarkably well the estimates of Von Hamm and Awny (83), who, on the basis of histological scanning, concluded that the spleens of 3 patients with this disorder showed a marked increase in RE cells, varying from 5.9 times normal in a 320 gm spleen to 24.2 times normal in a 2200 gm spleen. In this hemolytic process, which involves the liver little if at all, spleen cellularity may become many times that of the normal liver. If one extrapolates from the values for DNA synthesis in the rat spleen as compared to liver, the total rate of DNA synthesis in the spleens of patients with hereditary spherocytosis on the average may exceed that of the liver by 25- or 30-fold. These studies confirm the observations in rats that chronic hemolysis does stimulate reticuloendothelial hyperplasia in the spleen, without apparent "toxic," "foreign," or immunological mechanisms. It would be anticipated that this high rate of proliferation may constitute a metabolic burden. There is good evidence that the marrow proliferation of chronic hemolytic disorders increases the need for one of the factors required in DNA synthesis, folic acid (84, 85). The same reasoning may be applied to spleen hyperplasia and in many hemolytic disorders the two burdens may be additive.

It has been noted (21) that hemolytic disorders that are largely the result of splenic sequestration of red cells usually remain well-compensated for by increased marrow activity. Ultimately, the basis of this equilibrium must depend upon the comparative rates of cell formation in the 2 tissues. The present and previous (13, 28) observations in rats indicate that the RE populations of the 2 tissues are similar and it must be inferred that the potential rate of red cell formation exceeds the rate at which a maximally proliferating splenic RE cell population can destroy red cells. The normal human marrow produces about 200 billion red cells daily and this may readily be increased to 6- or 8-fold this in compensated hereditary spherocytosis (86). In such a patient with a spleen containing 400 billion cells, fewer than one-half of which are capable of red cell destruction, equilibrium would involve the destruction by each spleen RE cell of at least 6 or 8 red cells daily.

Splenomegaly, Hyperglobulinemia, and "Autoimmunity."—As noted above, spleen hyperplasia appears non-specifically to augment γ-globulin synthesis. For this reason and because of the proximity in the spleen of antigen-trapping and antibody-forming cells it would be predicted that particles trapped in the spleen will have an enhanced antigenicity, as compared to particles trapped in the liver or bone marrow. It is of interest in this regard that the authors have seen several patients with "Coombs-positive" acquired hemolytic anemia, who had had negative Coombs test early in the course of hemolysis. The frequent association of splenomegaly, hypergammaglobulinemia, and various immunological phenomena may in some instances be initiated by non-immunological disorders.

SUMMARY

Combined chemical and autoradiographic studies in rats injected with tritiated thymidine indicate that acute red cell sequestration stimulates reticuloendothelial (RE) proliferation. In the spleen DNA synthesis is most markedly stimulated in the marginal zone which is also the initial site of red cell sequestration. This proliferative response involves several division steps and eventuates in a colonization of the red pulp with increased numbers of all cell lines native to the spleen. In both spleen and liver there occurs also a generalized stimulation of division in the macrophages and littoral cells which involves only 1 or 2 division steps.

Chronic compensated hemolytic anemia achieved in rats by injections of phenylhydrazine caused functional overactivity of the RE system, including increased sequestering function and hypergammaglobulinemia. This splenic hyperplasia did not entirely regress after cessation of the injections. In man the splenomegaly of a chronic non-immunological hemolytic anemia, hereditary spherocytosis, was found to involve a marked (average: 8-fold) hyperplasia of all spleen cellular elements.

Neither the acute nor chronic proliferative reaction appears to arise from immunological or "toxic" stimuli and the findings support the view that the size of the RE system is a function of its particulate "work load." It is suggested that the cytoproliferative aspects of immune responses may depend upon non-specific, usually particulate stimulation. After prolonged stimulation, hyperplasia of the RES may become partly irreversible.

The authors wish to acknowledge gratefully the editorial assistance of Miss Winifred M. Sheldon and the technical assistance of Mrs. Diana Curren Bennett.

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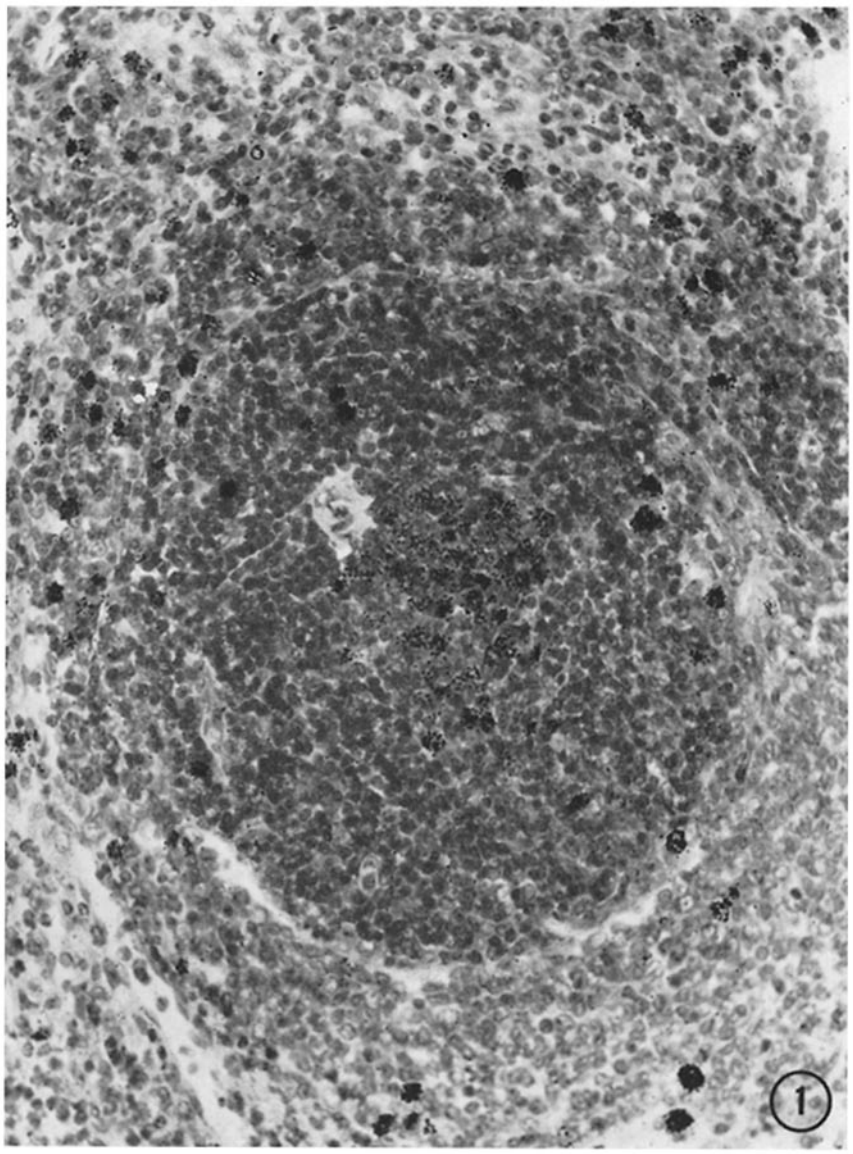
EXPLANATION OF PLATES

PLATE 20

FIG. 1. Normal rat spleen: autoradiograph 2 hours after H^3Tdr. The lymphatic follicle is clearly outlined by the encircling marginal sinus, which in turn is surrounded by an eccentric mass of relatively pale cells that comprise the marginal zone. The high labeling index of the germinal center cells, in the middle of the follicle and their distinctive low grain counts, contrasts with the comparatively low incidence of labeling of cells elsewhere with relatively high grain counts. Methyl green–pyronine. × 400.

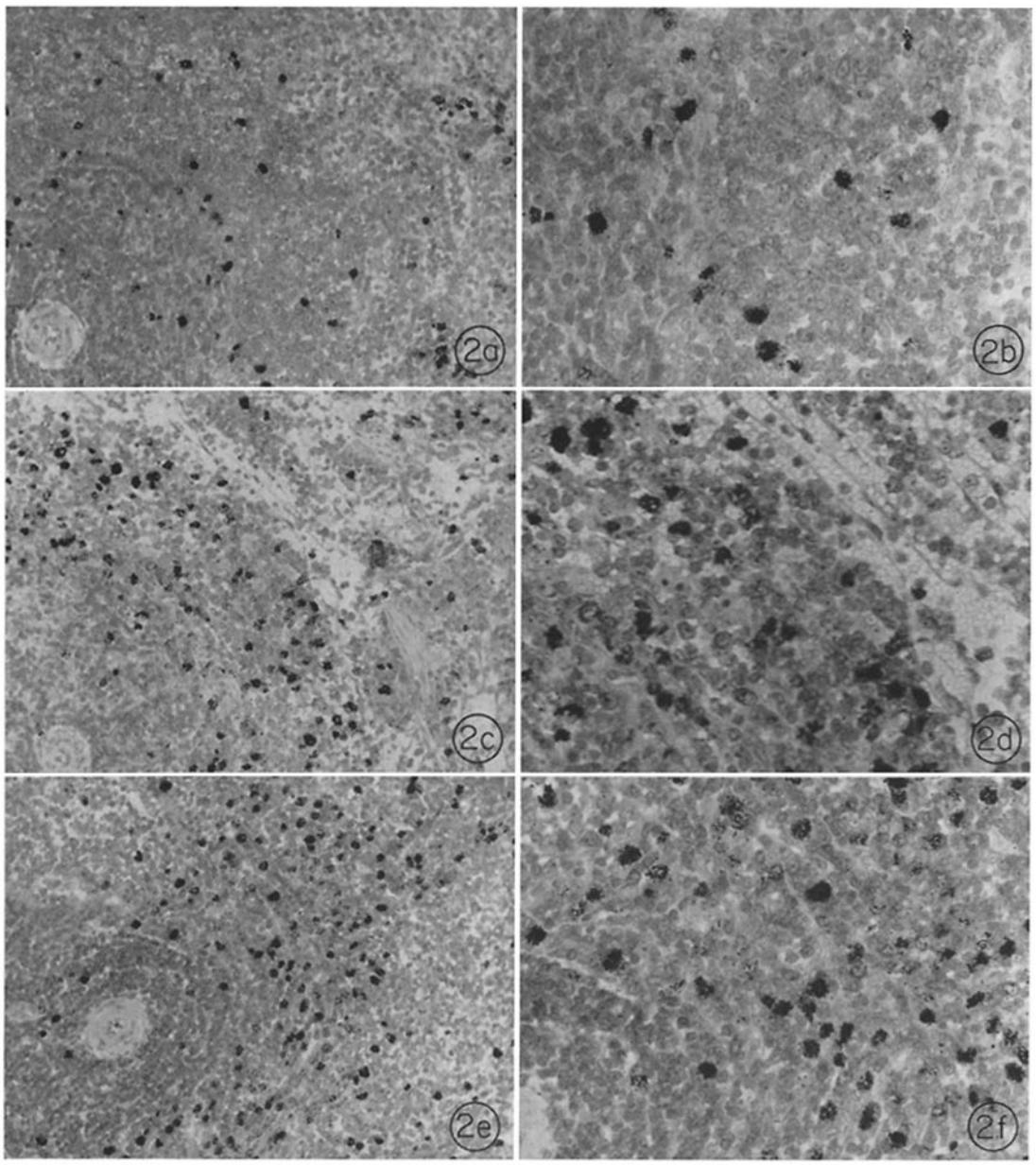


(Jandl *et al.*: Response of spleen and liver to hemolysis)

PLATE 21

FIGS. 2 *a* to 2*f*. Autoradiographic changes in the rat spleen after an injection of heat-injured homologous red cells: 2-hour label with H^3Tdr. In each photograph on the left, the view, from left to right, is of the central arteriole, lymphatic mantle, marginal zone, and red pulp. In each photograph on the right the view is centered on the marginal zone and shows lymphatic mantle on the left and red pulp on the right. (Methyl green–pyronine):

Fig. 2 *a*. Control, × 90. Fig. 2 *b*. Control, × 180. Fig. 2 *c*. 1 day after heated red cells, × 90. Fig. 2 *d*. 1 day after heated red cells, × 180. Fig. 2 *e*. 4 days after heated red cells, × 90. Fig. 2 *f*. 4 days after heated red cells, × 180.



(Jandl *et al.*: Response of spleen and liver to hemolysis)