RELATIONSHIP OF GERMINAL CENTERS IN LYMPHOID TISSUE TO IMMUNOLOGICAL MEMORY

I. Evidence for the Formation of Small Lymphocytes upon Transfer of Primed Splenic White Pulp to Syngeneic Mice*

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Active germinal centers are recognized as areas of rapidly proliferating blast cells in the white pulp of the spleen and in the cortex of lymph nodes. The origin and life history of these cells, their relationship to the surrounding mantle zone of small lymphocytes and, in particular, the functional significance of these two groups of cells is still unknown. Flemming (1) has proposed that germinal centers are a major site of production of small lymphocytes, but this interpretation has been disputed by others (2, 3). Hellman (4) has suggested that these structures are a reaction site against invading organisms. This is consistent with the fact that germinal centers appear specifically after exposure to antigen (5, 6). Rapid proliferation of blast cells in the germinal centers develops later than the peak of the primary response and coincides with development of primed cells after exposure to antigen (7, 8). Moreover, it can be shown by microdissection methods that the cells capable of giving a secondary response develop in the splenic white pulp at this time (9, 10). It is therefore important to determine the life history and immunological properties of the cell types arising in the white pulp after intravenous injection of antigen.

In the present paper, the incorporation of tritiated thymidine into cell suspensions derived from the white pulp during the active proliferation of blast cells is examined, and the fate of these labeled cells is followed after intravenous transfer to irradiated recipients. By these procedures, two of the problems encountered in labeling these cells in the intact animal—poor uptake of labeled precursors into germinal centers (11) and reincorporation of the radioactive label (12)—are circumvented.

This study establishes that there is a rapid development of small lymphocytes from larger dividing cells present in the white pulp during the 2nd wk after exposure to antigen. In the second paper of this series, the immunological

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properties of such primed, white-pulp cells are considered. The data suggest that development of immunological memory in recipients of the transferred cells may be linked to the formation of small lymphocytes (13). Since small lymphocytes are known to be of considerable importance for both the primary (14, 15) and the secondary immune response (16, 17), it may thus be possible to resolve the conflict between the two functions previously ascribed to germinal centers: reaction to antigen and production of lymphoid cells (18).

Materials and Methods

Animals and Immunization.—"Donor" C57Bl mice (Jackson Memorial Laboratory, Bar Harbor, Me.) were immunized intravenously at 2–3 months of age with 0.1 ml 20% sheep erythrocytes (SE) and 10 μg *Escherichia coli* endotoxin (0111 : B_4; Difco Laboratories, Detroit, Mich.). These mice were killed 7–31 days later. "Recipient" C57Bl mice received 850 rad whole body X-irradiation 24 hr prior to cell transfer. A Picker Therapeutic Unit was used at a dose rate of 40 rad/min. Inherent filtration of the tube was glass equivalent of 0.25 mm Cu and oil equivalent of 0.1 mm Al. External filtration included 0.5 mm Cu and 1.0 mm Al.

Preparation and Transfer of Cell Suspensions.—White pulp of donor mouse spleen was separated from red pulp by microdissection methods (19). The cells were released by cutting the tissue with scalpels and passing the cells through a stainless steel screen. Remaining clumps were removed by filtering the suspension through gauze. The cells were washed once and exposed to tritiated compounds in vitro. Tritiated thymidine (thymidine-^{3}H), 2 μc/ml, specific activity 0.36 c/mM (Schwarz Bioresearch, Orangeburg, N. Y.) and tritiated uridine (uridine-^{3}H), 10 μc/ml, specific activity 20 c/mM (Schwarz Bioresearch) were used. Cell suspensions were incubated with either of these compounds for 60–90 min at 37°C (with occasional agitation) in Minimum Essential Medium (Eagle) (Microbiological Associates, Bethesda, Md.) containing 20% normal mouse serum. After this period of incubation, an excess of Hanks' balanced salt solution containing 5% normal mouse serum was added to the cell suspensions before centrifugation. The cells were washed twice and smears were made of a representative sample of each donor suspension.

Syngeneic recipients received 1–3 $\times$ 10^7 cells, either intraperitoneally or by slow intravenous injection. Transfer of such relatively small numbers of labeled cells made it unlikely that reutilization of the radioactive label by the host cells had to be considered as a significant factor in the interpretation of results (12). To further preclude this possibility, cold thymidine (10 mg/g body weight) or cold uridine (10 mg/g body weight) was injected into recipients by the same route as the cells during the early hours after cell transfer.

Occasionally, unlabeled cells were transferred and recipient mice were pulse-labeled with a single injection of thymidine-^{3}H (0.5 μc/g body weight). The mice were killed 2 hr later.

Histological Examination and Radioautography.—Mice were killed at varying intervals after cell transfer. Tissue pieces of thymus, mesenteric lymph node, spleen, liver, lung, omentum, and intestine were fixed in Carnoy's solution for 30 min and stored in 60% ethyl alcohol. Other lymph nodes, bone marrow, and peripheral blood were not examined. Smears were made of cells from the spleen, mesenteric lymph node, and peritoneal washings; these were fixed in absolute methyl alcohol for 10 min. Radioautographs were prepared with NTB-3 emulsion (Eastman Kodak, Rochester, N. Y.) diluted 1:3 with distilled water. Sections and smears were stained with methyl green–pyronine.

Analysis of the percentage and intensity of labeling of the cells was made on two to four smears from each tissue; a total of at least 5000 cells was counted. Cells with three or more overlying silver grains were considered positive. For the most part, 1- and 3-wk radioautographs were used, although in some 3-wk radioautographs the density of grains in emulsions impeded classification of the underlying cells.

Classification of the Cells.—The morphologic criteria used to classify the cells were: cell and nuclear diameter, nuclear/cytoplasmic ratio, degree of condensation of nuclear chromatin, presence of nucleoli, and degree of cytoplasmic basophilia. Small lymphocytes were readily recognized because of their diameter (less than 7 μ), condensed nuclear chromatin, high nuclear/cytoplasmic ratio, and scant cytoplasms of varying basophilia. Blast cells were recognized by their large size—their nuclei having at least twice the diameter of a small lymphocyte—by their delicately stained nuclei containing two or more nucleoli, by a relatively high nuclear/cytoplasmic ratio, and by intensely basophilic cytoplasm. A small percentage of the blasts (up to 8%) could be regarded as large blasts, but the incidence varied. A third category of cells of intermediate morphology—medium-sized or large lymphocytes—was arbitrarily chosen to include all cells that could be rigorously excluded from the small lymphocyte category but which were smaller than blast cells. In particular, these cells characteristically had more cytoplasm and larger nuclei, but staining properties akin to those of small lymphocytes. The term "intermediate cells," used to designate these cells, refers only to their size and does not necessarily imply that they are intermediate with respect to cell lineage.

In a preliminary publication (20), large- and medium-sized blast cells were grouped separately, and all but the smallest lymphocytes were classified as "large." Since the small lymphocyte is the labeled cell type of major interest in the present studies, this difference in cell classification does not significantly influence the results.

RESULTS

Incorporation of Thymidine-^3H into Donor White-Pulp Cells.—Some initial studies were done in which donor mice were injected intravenously with thymidine-^3H, but it was found that exposure to the isotope of the dissociated cells in vitro gave a more reproducible pattern and a higher incidence of labeling. The pattern of labeling of white pulp, obtained 2 hr after a single injection of thymidine-^3H (0.5 μc/g body weight), was analyzed to estimate the relative numbers of labeled cells in the mantle zone and germinal centers. Approximately 85% of the labeled cells were found inside the germinal centers. As far as could be ascertained from tissue sections, the majority of the germinal center cells were labeled (75%), although much less intensely than the 4% cells labeled in the mantle zone. This pattern of labeling was also seen by others (2).

The percentage of cells in some typical donor preparations which had incorporated thymidine-^3H in vitro are given in Table I. White pulp taken at 7–12 days after antigenic stimulation showed a relatively high labeling index, approximately three- to eightfold higher than white pulp taken either 3–4 wk after immunization or from normal mice. The distribution of labeling was similar in all white pulp preparations in that labeling occurred in high proportions of the blast and intermediate cells but in a very low proportion of the small lymphocytes. In some preparations, labeled small lymphocytes were not detected at all.

There was a slightly higher incidence of labeled small lymphocytes in white pulp which, in addition to exposure to thymidine-^3H in vitro, was labeled by thymidine-^3H injection into the animals 1–4 hr prior to removal of the spleen (Table I). Results from the occasional experiments in which over 7% of the labeled cells of the donor population were small lymphocytes were excluded.

Histological Localization of Thymidine-^3H-Labeled Cells after Intravenous Injection.—The distribution of the labeled cells after intravenous injection was followed by sampling tissues at frequent intervals. The results of two experiments are illustrated in Table II. Although there was some initial uptake of the donor cells by the lung, there was a preferential homing of these cells into lymphoid tissues within a few hours, concentrating in the spleen, mesenteric lymph node, and Peyer's patches. The numbers of labeled cells increased at a similar rate in these three lymphoid tissues, reaching a plateau at 24 hr. The

TABLE I

Labeling Pattern of White-Pulp Cells from Normal or Immunized Mice at Various Days after Primary Antigen Injection

Nature of white pulp	Exposure to thymidine-^3H*	Incidence of labeled cells, %				% labeled within each cell type		
		Total	Small Ly.‡	Inter. cells§	Blasts	Small Ly.‡	Inter. cells§	Blasts
Immune, day 7–8	In vivo and in vitro	7.5	0.9	3.3	3.3	1.0	38	75
Immune, " 7–8	In vitro	6.0	0.0	3.4	2.6	0.0	35	68
Immune, " 7–8	"	10.0	0.4	5.3	4.3	0.4	53	53
Immune, " 31	In vitro	1.9	0.4	1.1	0.4	0.3	31	80
Normal	In vitro	1.2	0.1	0.9	0.2	0.1	45	50

* In vivo labeling: injection of 0.5 μc thymidine-^3H/g body weight 1–4 hr prior to white-pulp preparation.

In vitro labeling: exposure of cells to 1 μc thymidine-^3H/ml for 1 hr.

‡ Ly., lymphocytes.

§ Inter., intermediate.

only lymphoid organ in which an accumulation of labeled cells was not observed was the thymus. Very few labeled cells were found in the liver.

Labeled cells were also absent from the mucosal lining of the small intestine. Gowans and Knight (21) found that large labeled cells from the thoracic duct preferentially home to this site. The cell type and immunological history of the cells may be important factors in this homing, since a large proportion of thoracic duct cells can be expected to have been sensitized to antigens present in the intestinal content. Moreover, in the present experiments, many of the large dividing cells rapidly changed their morphology after transfer (see below) which may have influenced their fate. It should also be noted that distinctive homing patterns have been described for other populations of lymphoid cells (22–24).

The even distribution of labeled cells over lymphatic tissues led to the con-

clusion that a significant fraction of these cells—but by no means the majority—localize in the spleen. Evidence presented in the accompanying paper (13) shows that 10–15% of uridine-^{3}H–labeled, white-pulp cells home to the spleen within 24 hr after intravenous injection. The results suggest that the spleen in these irradiated animals contains approximately 10–15% of the total lymphatic tissue.

The distribution of thymidine-^{3}H cells within the spleen was quite characteristic. By 24 hr, dense areas of lymphoid cells, containing some labeled cells, were seen in the remnants of the white pulp areas. Few blast cells were seen in these foci. Similar populations of thymidine-^{3}H– or uridine-^{3}H–labeled white-pulp cells were injected intravenously into nonirradiated recipients which

TABLE II

*Incidence of Thymidine-^{3}H–Labeled Cells per Unit Area of Tissue from Intravenously Injected Mice**

Time after cell transfer	Spleen	Mesenteric lymph node	Thymus	Lung	Liver	Small intestines	
						Mucosal lining	Peyer's patches
hr							
½	10	0	—	120	0	0	0
2–6	226	350	6	63	0	0	NE‡
8–12	175	210	0	26	1	0	NE‡
16–24	636	690	11	19	0	0	550

* 2×10^7 thymidine-^{3}H–labeled white-pulp cells injected intravenously into X-irradiated syngeneic mice.

‡ No Peyer's patches seen.

had been pretreated so as to have large germinal centers in their spleens at the time of transfer. The labeled cells localized preferentially in the mantle zone of the white pulp, and rarely entered the germinal centers.

Cytological Differentiation of Intravenously Injected White-Pulp Cells.—Smears of recipient spleens and mesenteric lymph nodes were examined to determine the cytological features of the labeled cells. The results obtained with spleen cells were analyzed in detail. A striking finding was that a marked change occurred in the type of labeled cell observed within the first 24 hr after transfer. The percentage of labeled lymphocytes increased (Table III) while the numbers of blasts and "intermediate cells" decreased. This was apparently a rapid event, since the mean percentage of labeled small lymphocytes with respect to total labeled cells increased from 7 to 33% within the first 7 hr after transfer; further increased to 65% after 24 hr; and reached a maximum of 77% by 48 hr. In spite of the dilution of the donor population with host cells, the incidence of

labeled small lymphocytes found in the spleen at 48 hr after transfer approached that observed in the initially injected population.

The appearance of labeled small lymphocytes was obviously not due simply to preferential homing of these cells to the spleen. A calculation of the numbers recovered from recipient spleens readily illustrated this (Table III). It seemed particularly unlikely that preferential homing could account for the presence of more than 50% of injected lymphocytes in the spleen at 48 hr after injection, in view of the even distribution of labeled cells throughout various lymphoid tissues shown in Table II. In addition, the labeled cell population sampled from mesenteric lymph nodes demonstrated a similar increase in percentage of labeled small lymphocytes.

TABLE III

*Appearance of Labeled Small Lymphocytes in Recipient Spleens after Intravenous Transfer**

		No.	Per cent total labeled cells		Absolute No. of labeled small lymphocytes × 10⁴‡	
			Mean	Range	Mean	Range
Donor white pulp		6	7	4–10	12.5	6.6–19.4
Recipient spleen at:	7 hrs	1	33	—	0.8	—
	24 "	3	65	47–87	3.6	2.4– 5.4
	48 "	4	77	52–97	7.2	4.8–11.4
	72 "	4	78.5	67–96		

* ^{3}H-thymidine–labeled white pulp cells transferred; average number of cells injected was 3.3×10^{7}.

‡ Calculation on the basis that recipient spleens at this time contain approximately 2×10^{7} cells.

Some labeled small lymphocytes, but no other labeled cells, were present in spleens of recipients killed as late as 7 days after cell transfer. The mean grain count over these small lymphocytes was 10, as compared with 12 on days 2 and 3.

Use of the Intraperitoneal Route for the Study of the Fate of White-Pulp Cells.— One of the major disadvantages of studying the fate of intravenously injected lymphoid cells is their rapid distribution over the various lymphatic tissues which limits the numbers of cells that can be sampled from any particular organ. Moreover, recirculating small lymphocytes preferentially home to lymphatic tissues, so that it becomes difficult to assess the proportion of injected labeled cells that become small lymphocytes by studying any given lymphoid organ. Although formation of small lymphocytes undoubtedly occurred, as studied by intravenous transfer, a more convincing demonstration of this phenomenon might be feasible by transferring the cells into a site where rapid dissemination of cells did not occur.

The intraperitoneal route was chosen because preliminary experiments with uridine-^{3}H–labeled, white-pulp cells indicated that there was a much greater recovery of transferred cells from the peritoneal cavity. The majority of cells remained locally for 24 hr. Examination of spleen and other lymphatic tissues after intraperitoneal injection of uridine-^{3}H–labeled cells demonstrated that very few donor white pulp cells migrated to these organs during the first 24-hr interval. After injection of 1–2 $\times$ 10^7 cells, contamination with host cells in this particular site was only 10–20% during this time period. On the average, a 40% recovery of donor cells was obtained at 24 hr.

In a typical experiment, the initial thymidine-^{3}H–labeling index of the donor population was 13%. At 24 hr after transfer, 11.0–13.6% of the cells recovered from the peritoneal cavity were labeled in spite of dilution with host cells. This suggested that some replication of the labeled cells had occurred. At later time intervals, a fairly high labeling index was still seen: 7.0–7.6% at 48 hr and 5% at 72 hr after cell transfer. The decrease in labeling index between 24 and 72 hr was partially due to an increased dilution with host cells and to an actual loss of labeling because of mitotic activity. Undoubtedly, some labeled cells migrated out of the peritoneal cavity since, at 48–72 hr, a few were seen in mesenteric lymph node and spleen.

Cytological Differentiation of Intraperitoneally Injected White-Pulp Cells.— Differential counts of labeled, white-pulp cells recovered from the peritoneal cavity indicated that a series of changes took place in the transferred cells similar to that seen after intravenous transfer (Fig. 1). The initial percentage of labeled small lymphocytes per total labeled cells obtained by incubation with thymidine-^{3}H in vitro was 0–5%, whereas 27.0–33.5% were present at 24 hr after transfer. This appearance of labeled small lymphocytes was concomitant with a rapid drop in the percentage of blast cells; the percentage of "intermediate cells," however, remained relatively constant. From 24–48 hr, there was a continued, linear increase in the percentage of labeled small lymphocytes; a somewhat smaller increase was still observed over the next 24 hr. This latter increase was accompanied by a drop in all other labeled cell types. The changes in the labeled cell cytology observed over the first 24 hr were extremely reproducible since similar differential counts were obtained in all of five experiments (Fig. 1). Comparable results were obtained in an additional experiment on the fate of the transferred cells in nonirradiated hosts.

An indication of the rate of transformation to small lymphocytes can be seen in one experiment in which the donor population contained no labeled small lymphocytes whereas, as early as 2 hr after transfer, small lymphocytes comprised 2.3% of the labeled cells (Fig. 1). The appearance of labeled small lymphocytes was less rapid, however, than had been observed in the spleen in experiments using intravenous transfer. At 7–8 hr, labeled small lymphocytes made up 15% of the total, labeled cell population after intraperitoneal transfer

versus 33% after transfer intravenously. Similarly, at 24 hr, these values were 30 and 65% after intraperitoneal and intravenous transfer, respectively.

Some further insight into these changes can be gained by studying the incidence of labeled cells within each class of cells and in the total cell population. In a typical experiment, pairs of mice were sampled over a period of 3 days after cell transfer. The labeled donor population contained 5% labeled cells—including seven labeled small lymphocytes per 1000 cells. In spite of some dilution with host cells, labeled small lymphocytes constituted 30‰ of

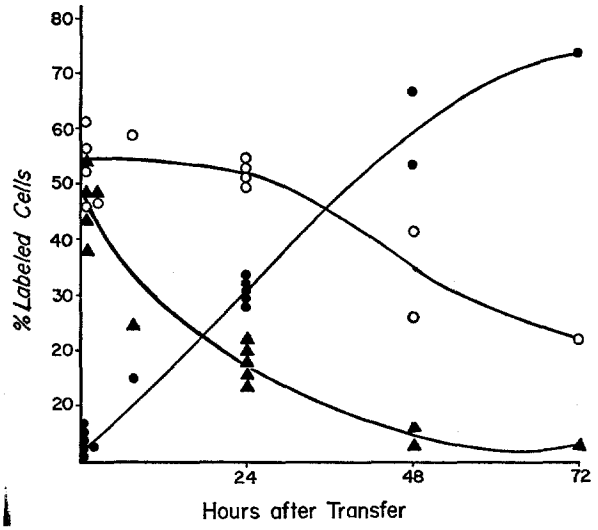

FIG. 1. Fate of thymidine-^{3}H-labeled white-pulp cells after intraperitoneal injection. Results obtained in five different experiments are given and expressed as per cent of total labeled cells recognized as blast cells (▲——▲), as intermediate cells (○——○), or as small lymphocytes (●——●) among the peritoneal cells sampled at varying time intervals after transfer.

the total population at 24 hr after transfer. This increase in labeled small lymphocytes, also noted with respect to the total population of small lymphocytes, was accompanied by a sharp reduction in the total number of labeled blasts, whereas the incidence of labeled intermediate cells did not change (Fig. 2). Over the next 24 hr, the rise in total labeled small lymphocytes continued in spite of a small decrease in their labeling index.

Beyond 24 hr, blast cells were no longer an important part of the population. The incidence of labeled blasts fell to 5 per 10^3 cells by 48 hr (Fig. 2) and approximately 27% of the blasts still present at this time were labeled.

The mean grain counts of the transferred cells in this experiment are given in Table IV (Experiment I). It can be seen that the intensity of labeling of the

small lymphocytes increased over the first 24 hr after which time it remained constant. The other two cell groups had a significantly higher level of labeling initially, and showed a progressive decrease in their mean grain counts. The

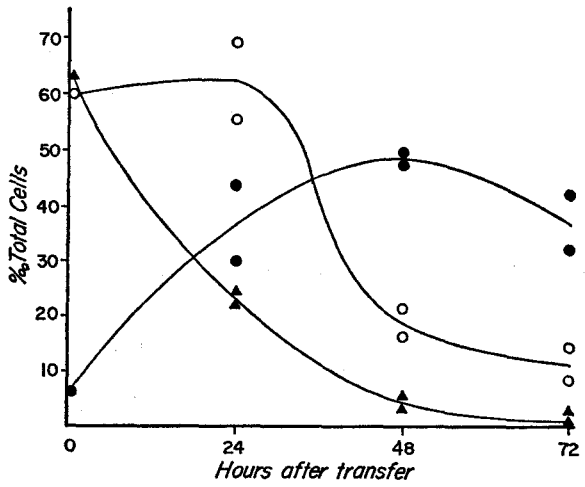

FIG. 2. Incidence of labeled cells in the peritoneal cavity at varying times after intraperitoneal transfer of thymidine-^{3}H-labeled white-pulp cells. Results of one typical experiment are given as individual values in two simultaneously sampled recipients. The data are expressed as numbers (per 1000 cells) of labeled cells recognized as blast cells (▲——▲), as intermediate cells (○——○), or as small lymphocytes (●——●).

TABLE IV

Changes in Mean Grain Counts of Transferred White Pulp Cells

		Experiment I			Experiment II		
		Small lympho-cytes	Inter-mediate cells	Blasts	Small lympho-cytes	Inter-mediate cells	Blasts
Donor cells		8	19	41	—*	19	22
Recipient	2 hr				9	15	20
Peritoneal	24 "	14	18	21	16	16	15
cells at:	48 "	14	14	12			
	72 "	14	12	—*			

* No labeled cells of this type present.

rate of disappearance of the label was considerably higher for the blasts than for the intermediate cells. The 50% reduction in the mean grain count of blast cells from 41 to 21 over the first 24 hr agrees well with the findings of Fliedner et al. (2). The mean grain counts on one other experiment are also included in

Table IV. Though labeled small lymphocytes were absent from the donor population in Experiment II, there was an increase in the labeling intensity of the small lymphocytes between 2 and 24 hr.

Interpretation of grain count data obtained with emulsions exposed for 1 wk may pose problems in that lightly labeled cells can appear negative. Longer exposure times, however, result in higher grain counts which is not an advantage in all respects, since grain counts above 15–20 over cells of the size of small lymphocytes already become spuriously low. The observed increase in labeling of small lymphocytes, therefore, may have been higher than the grain counts indicate.

TABLE V

*Incorporation of Thymidine-^3H In Vitro by Cells Sampled from Spleen or Peritoneal Cavity of Recipient Mice**

Route of injection of donor cells	Time after cell transfer	No. of labeled cells/1000 in:	
		Recipient mice*	Control mice‡
	hr		
i.p.	5	11.5§	0.7
	8	6.0	NE
	24	3.0	1.0
i.v.	5	2.5‖	2.5
	8	3.0	NE
	24	2.5	NE

NE, not examined.

* Recipient mice received unlabeled white-pulp cells 24 hr after X-irradiation.

‡ Control mice were irradiated but were not injected with cells.

§ Cells sampled from the peritoneal cavity.

‖ Cells sampled from spleen.

Incorporation of Thymidine-^3H by Donor Cells after Transfer.—The DNA-synthesizing activity of the transferred cells may be followed either by pulse-labeling recipients in vivo or by sampling cells from sites where donor cells are known to be present and exposing them to thymidine-^3H in vitro. When unlabeled white pulp cells were transferred by the intraperitoneal route, there was a significant incorporation of the precursor by cells gained from the peritoneal cavity of recipient mice 5 hr after transfer (Table V). The label was, almost exclusively, in blast and intermediate cells. This activity declined steadily and by 24 hr the incidence of thymidine-^3H incorporation by cells present in the peritoneal cavity of recipients was not much different from that observed in peritoneal cells of X-irradiated control mice. On the other hand, it was not possible after intravenous transfer to detect a difference in the DNA-synthesizing

activity of recipient and control spleen cells thus indicating an absence of actively dividing donor cells (Table V). This is in keeping with the relatively low yield of labeled blast cells in spleens of mice injected intravenously with labeled, white-pulp cells (20). Pulse labeling of recipients in vivo and sampling 2 hr later further demonstrated that little mitotic activity took place in the donor population in the absence of antigen over the period from 24 to 48 hr after transfer.

DISCUSSION

The appearance of labeled small lymphocytes after intravenous or intraperitoneal transfer of pulse-labeled white-pulp cells is most readily explained by production of small lymphocytes from precursor cells in the donor population. An absolute increase in numbers of labeled small lymphocytes in the organs examined was observed in many experiments. Labeled small lymphocytes were absent from the donor cell population in some experiments yet appeared, nonetheless, in significant numbers in the recipients. The increase in mean grain counts over labeled small lymphocytes also provides clear evidence that active production of these cells from more highly labeled precursor cells occurred in the white pulp suspensions after transfer.

Other factors that could contribute to a relative increase in labeled small lymphocytes might be the known preferential homing of small lymphocytes to the sites examined in the host animal (21–24), or preferential loss of other labeled cell types. However, the number of labeled small lymphocytes present among the donor cells cannot account for the actual incidence of such cells observed both after intravenous and intraperitoneal transfer. Undoubtedly, some of the highly labeled blasts undergo irradiation damage (25) and others lose the label through cell proliferation. Although a loss of label through dilution did occur in the early hours after transfer, this did not seem to be a major problem since by 24 hr a very low level of mitotic activity remained. The relatively slow decrease in mean grain count over these cells between 24 and 72 hr after transfer also reflected a low rate of cell division.

The rapid appearance of labeled small lymphocytes after transfer suggests that a significant number of the labeled donor cells were immediate precursors of small lymphocytes. There was a continuous increase in numbers of small lymphocytes over the first 48 hr after transfer. Thereafter, the percentage of labeled small lymphocytes among the remaining labeled cell population continued to rise, but no further increase in absolute numbers was noted. Although the nature of the immediate precursor cells of the small lymphocytes cannot be established with certainty, the results suggest that they belong to the cell group which has been classified as "intermediate cells" in these experiments. These intermediate cell types may indeed represent a stage in the differentiation of blast cell to small lymphocyte (26). The changes in relative incidence of blasts

and intermediate cells, together with their relative rates of loss of label, are consistent with this interpretation. Assuming that there is a constant production of small lymphocytes from intermediate cells, a transformation of blasts into intermediate cells involving one cell division would account for the relatively constant incidence and labeling index of the intermediate cells seen during the first 24 hr. The decrease in these cells observed during the next 24 hr is most likely caused by relative exhaustion of their precursor blasts and by their transformation into lymphocytes. No further increase in labeled small lymphocytes would occur, as was indeed observed, when the number of labeled blasts and intermediate cells decreased to insignificant levels.

The data do not rule out the possibility that blast cells may transform into small lymphocytes independent of the intermediate cells, nor that such transformation may involve only a minor fraction of this cell group. The characteristic large lymphoid blasts, arising throughout lymphoid tissue during the early part of the immune response, are known to be capable of transformation into small lymphocytes, under various experimental conditions (27, 28). This cell type is invariably present in lymphoid tissues and particularly in germinal centers during their early developmental stage, but the relationship between these large blasts and the typical medium-sized blasts of germinal centers has not been determined. The donor cell populations used in the present experiments were taken at the peak of germinal center development and contained few labeled blasts of this larger variety. It is unlikely, therefore, that they were directly responsible for the rapid appearance of the relatively large numbers of labeled small lymphocytes in the recipients.

A more likely interpretation is that the medium-sized blasts which constitute the majority of labeled cells in the donor populations, gave rise either directly or indirectly to the labeled small lymphocytes. By far, the majority of these blasts and intermediate cells were found within the germinal centers, although a number of similar cells was also seen in the surrounding mantle zones in the intact white pulp. The relative importance of these larger cells in the mantle zone has not been evaluated. At least some of these cells may themselves have originated in the germinal centers (29).

The question arises whether the white pulp containing germinal centers also produced small lymphocytes before transfer. One major difference between structurally intact white pulp and the suspensions transferred in these experiments is that the reticular framework, which is known to fix antigen (30) and antigen-antibody complexes (31) onto its surface, is dissociated and probably removed from the lymphoid cells in the preparation of the donor suspension. Since these immune complexes may constitute the major stimulus for blast cell division within the germinal centers, a diminished proliferative tendency, as was observed after transfer, might have been expected. Indeed, a rapid redevelopment of germinal centers can be induced in the recipients by transferring the

cells with antigen (20). This is not seen in the absence of antigen and occurs much more slowly after transfer of unimmunized lymphoid cells. The kinetics of the lymphocyte production observed in the present experiments may therefore not be comparable with the situation in the actively immune animal, where the amount or nature of the antigen present in these centers possibly regulates the rate of proliferation of blast cells and the tendency of these cells to differentiate into small lymphocytes or other cell types, such as plasma cells.

Some evidence for production of small lymphocytes by tonsillar germinal centers in rabbits has been obtained from pulse-labeling experiments by Koburg (32). This author found accumulations of labeled small lymphocytes in the lymphocyte caps of active germinal centers in the tonsil. Within 5 min after injection of thymidine-^{3}H, labeled cells could be detected in the posterior regions of the germinal centers and, after 72 hr, nearly 100% of the small lymphocytes in the caps were labeled. Control experiments were performed which strongly indicated that this formation of labeled small lymphocytes occurred locally in the tonsil.

The density of small lymphocytes in the mantle zones does not necessarily reflect the activity of the follicle centers but is directly dependent on the level of small lymphocytes in the circulating blood. In fact, bare germinal centers can be seen in cases of peripheral blood lymphopenia after extracorporeal blood irradiation (33), thymectomy at birth (34), or during recovery after whole body X-irradiation (8). It is possible that under these circumstances lymphocytes are still produced in the germinal centers but have immediate access to the recirculating pool and do not accumulate in mantle zones.

Obviously, there are alternative sources of lymphocyte production besides germinal centers. Large lymphoid blasts independent of the presence or absence of blast cell accumulations in germinal centers may transform into lymphocytes (27, 28); and lymphocytes are also produced in the thymus (26, 35), bone marrow (36), and bursa of Fabricius (37). In fact, in some species relatively normal lymphocyte levels in tissues and blood are present at birth in spite of the total absence of germinal centers in the lymphoid tissues during embryological development (38).

The fact that, after pulse-labeling in vivo, the intensity of labeling of cells in the mantle zones is much higher than the labeling index of the germinal center cells has also been used as an argument against the production of lymphocytes by germinal centers (2, 39). Craddock, et al. (40) observed a biphasic grain count distribution over the large lymphocytes of lymph nodes following in vivo pulse-labeling. Such a high incidence of lightly labeled cells was not found with the donor cell suspension in the present experiments probably because the tissue was dissociated prior to incubation with thymidine-^{3}H. This suggests that there was no intrinsic difference in the ability of the larger cells in germinal centers and mantle zones to incorporate thymidine into DNA. It is

more likely that the low labeling of germinal center cells in vivo is due to a relative lack of isotope within the centers (11).

In the present experiments, the grain counts of the small lymphocytes remained relatively constant over the first few days after transfer, despite the fact that a small fraction of the small lymphocytes became rapidly labeled after a pulse of thymidine-^{3}H. Some small lymphocytes of similar grain count could still be detected on day 7 after transfer—the last day of observation. This suggests that although their precursors divide rapidly, many of the small lymphocytes do not.

A variety of functions have been ascribed to small lymphocytes. Some lymphocytes, originating in the marrow, appear to transform into macrophages (41), and lymphocytes from the thoracic duct and peripheral blood may transform into blast cells on exposure to antigen (42, 43) or phytohemagglutinin (44). This latter type of lymphocyte is long lived (45), continuously recirculates through the lymphoid tissue (21), and appears of major importance in immunological memory (16, 17). It is therefore relevant to establish the functional properties of the small lymphocytes shown to be produced in the present study. The accompanying article indicates that cells endowed with immunological memory develop in the white pulp of the mouse spleen during germinal center proliferation and that both dividing and nondividing cells contribute to the responsive population (13). Experiments are in progress to determine whether the small lymphocytes produced in the transferred white pulp revert to blasts on reexposure to antigen.

SUMMARY

The fate, proliferation, and developmental potentialities of cell suspensions made from white pulp containing large germinal centers have been studied in the mouse by transfer of cells labeled with thymidine-^{3}H to lethally irradiated, syngeneic recipients. Radioautographic analyses were made using both smears and sections of a variety of tissues. Thymidine-^{3}H–labeling patterns of white pulp showed that, initially, labeling occurred in a majority of blast and "intermediate cells" but in very few or no small lymphocytes. After intravenous transfer, most of the labeled cells localized in the lymphoid tissues of spleen, lymph nodes, and Peyer's patches. Few cells migrated to the thymus, lung, liver, and intestinal mucosa.

Both after intravenous and after intraperitoneal transfer there was a rapid increase in the incidence of labeled small lymphocytes and a decrease of labeled blasts and intermediate cells. This was accompanied by an increase in the grain count of the small lymphocytes and a progressive decrease in the grain counts of the blast cells. Exposure of nonlabeled donor cells to thymidine-^{3}H at various time intervals after transfer indicated that dividing cells were present early after transfer but that their incidence progressively decreased. Between 24 and 48 hr, very little cell division was detectable.

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