RELATIONSHIP OF GERMINAL CENTERS IN LYMPHOID TISSUE TO IMMUNOLOGICAL MEMORY

II. The Detection of Primed Cells and Their Proliferation upon Cell Transfer to Lethally Irradiated Syngeneic Mice*

By J. D. WAKEFIELD,‡ Ph.D., and G. J. THORBECKE,§ M.D.

(From the Department of Pathology, New York University School of Medicine, New York 10016)

Transfer of adoptive immunity has proved useful in detection and characterization of the cells carrying immunological memory. By introducing varying time intervals between transfer and reexposure to antigen in the recipients, the duration of responsiveness of memory cells was defined. Perkins and Makinodan (1) showed that, with donor cells taken 4–6 wk after sensitization, little change in responsiveness of the primed cells occurs over the first few days following transfer. The results of Dresser (2) and Celada (3), however, indicate that when challenge with antigen is delayed for a month or longer after transfer there is a progressive decay in the responsiveness of the transferred cells. Indeed, Celada has shown a biphasic decline of memory upon transfer of sensitized cells, which he interpreted to be due to two populations of memory cells, having half-lives of 26 and 190 days, respectively.

The importance of nondividing or slowly proliferating cells in the maintenance of long-term immunity to certain antigens has been illustrated in a number of test systems. Lack of incorporation of thymidine-^{3}H by primed cells transferred at short intervals after challenge with antigen in vivo, led Cohen and Talmage (4) to conclude that the memory cell was not part of a rapidly dividing cell population. This conclusion is compatible with the more recent findings of Gowans and Uhr (5) that long-term memory for the immune response to ϕX174 can be transferred by thoracic duct cells consisting almost entirely of small lymphocytes. X-irradiation studies have also suggested that nonreplicating cells are involved in long-term immunological memory (6–8). Moreover, long-lived small lymphocytes from peripheral blood of sensitized individuals have been identified as the cell type capable of transforming into blasts upon reexposure to antigen (9).

The precursors of these nondividing cells which must be present during the early stages of the development of immunological memory, and the stimuli needed for such precursors to differentiate into small lymphocytes, are of particular theoretical

171

interest. It is recognized that one of the major, if not the only, difference between a primary and a secondary immune response is the much greater number of cells involved in the latter. It is also clear that much of the proliferation of primed cells occurs within the first few weeks after immunization. Immunological memory develops rapidly after priming and shows its greatest increase in magnitude over the first few weeks (7, 10–12). The rapid regeneration of memory after X-irradiation (7) and after drug treatment (13, 14) is most pronounced during the first 2 wk and is probably associated with the reappearance of germinal centers in spleen and lymph node. Part of the proliferation of lymphoid cells seen in the early weeks after antigen injection is likely due to stimulation by antigen known to be located in germinal centers at that time (15, 16), but the relevance of this antigen to the process of priming has not been firmly established.

The present study uses a cell transfer system to examine this early proliferative phase of the immune response occurring in the white pulp of the spleen. If the proliferation of memory cells or their precursors is indeed dependent on the continued presence of antigen, secondary responsiveness may not continue to increase in recipient mice after transfer of dissociated cells removed from the extracellularly fixed depot of antigen (15, 16). The results in the accompanying article (17) indicate that although proliferating cells are present in the germinal centers of splenic white pulp during the 2nd wk after priming, upon transfer in the absence of added antigen, the mitotic activity of these cells is curtailed and many transform into small lymphocytes within 24 hr of cell transfer. Similar preparations of cells were transferred in the present experiments and the time of reexposure to antigen was varied. In this way, an attempt was made to analyze the relative contributions of proliferating and nonproliferating cells to the pool of responsive cells in the early weeks after priming.

Materials and Methods

Animals, Immunization Schedule.—C57Bl male mice from the Jackson Memorial Laboratory (Bar Harbor, Me.) 2–3 months of age, were used as donor and recipient mice. Donors were injected intravenously with 0.1 ml of 20% sheep erythrocytes (SE) and 10 μg endotoxin (Difco Laboratories, Detroit, Mich.). In a few control experiments, 0.5 mg bovine gamma globulin (BGG) (Pentex, Inc., Kankakee, Ill.) or 0.5 mg Keyhole Limpet Hemocyanin (KLH) was injected .

Preparation of Cell Suspension.—7–10 days after primary immunization, donor mice were killed by cervical dislocation. White pulp of donor mouse spleens was dissected from red pulp and single cell suspensions were prepared as described previously (18). The cells were washed twice in Hanks' balanced salt solution (BSS) containing 5% C57Bl serum.

Injection of Recipient Mice.—1–3 $\times$ 10^7 cells were injected intravenously into lethally irradiated mice (total dose 850 rad) at a dose rate of 40 rad/min (17). Recipient mice were challenged intravenously with 0.1 ml 20% SE immediately or at varying time intervals up to 3 days. Some mice received donor cells but were not challenged with antigen.

Assay of Adoptive Immunity.—At time periods from 1 to 9 days after cell transfer and challenge with antigen, mice were anesthetized with ether, and bled by cardiac puncture. Hemagglutinating antibody to sheep erythrocytes in the sera were titrated by serial dilution.

Cell suspensions of the spleens were examined for the presence of plaque-forming cells (PFC) by the technique described initially by Jerne, Nordin, and Henry (19). Briefly, known numbers of washed spleen cells in 0.1–0.02 ml volumes were mixed with 2 ml agar containing 0.1 ml 20% SE. The agar was prepared from equal volumes of melted 1.4% washed Noble agar and 2X concentrated medium 199 and kept at 42°C. It contained 50 mg/100 ml diethyl-aminoethyl (DEAE) dextran (Pharmacia, Inc., Uppsala, Sweden). This mixture was poured into 10 cm Petri dishes containing a bottom layer of agar. The agar was allowed to gel; the plates were checked for artifacts and were incubated for 1 hr at 37°C. Normal guinea pig serum, diluted 1:5 with a barbital buffer containing magnesium and calcium, was used as a source of complement, 2 ml per plate. Plates were incubated for an additional hour at 37°C. Hemolytic plaques developed around the cells synthesizing antibody of high hemolytic efficiency ("19S"). In certain experiments, plaques produced by antibody of lower hemolytic efficiency ("7S") (20, 21), were developed by exposing duplicate plates to an appropriate dilution of a rabbit antiserum to whole mouse serum, for 1 hr prior to incubation of the plates with complement. The numbers of PFC per recipient spleens were calculated per 1×10^7 donor cells injected. The numbers of PFC in recipient spleens were very low on day 1 or day 2 after challenge with antigen. Cells were therefore assayed at concentrations of 10^7 cells per ml employing pools of two to four spleens.

Treatment with 5-Bromodeoxyuridine (BUDR).—An attempt was made to evaluate the proliferative activity of the responsive cells following transfer by studying their sensitivity to treatment with a thymidine analogue. BUDR (Calbiochem Laboratories, Los Angeles, Calif.) was injected intraperitoneally to a total dose of 2–10 mg. The dose was divided into four or five injections given over a 6- to 36-hr period starting immediately after cell transfer and ending at least 6 hr before injection of the antigen. In control experiments, the same dose schedule of BUDR was used at varying time intervals after injection of antigen. In occasional experiments, the donor cells were incubated with BUDR in vitro (250 μg/ml) for 1 hr, and injected without washing into recipient mice.

Distribution of Donor Cells.—The pattern of migration of the transferred cells was followed by use of radioactively labeled cells. Donor cells were incubated with Hanks' BSS plus 20% normal mouse serum containing 10 μc/ml tritiated uridine (specific activity 2 c/mM Schwarz Bioresearch, Orangeburg, N. Y.) for 1½ hr in vitro prior to transfer. The cells were washed twice and injected intravenously. Excess cold uridine was injected intraperitoneally at frequent intervals. Recipients were sacrificed at 2–4 hr intervals within a 24 hr period after cell transfer. Radioautography of the smears and tissues were performed according to the methods described in the accompanying article (17).

RESULTS

Adoptive Secondary Immune Response of Splenic White Pulp and Whole Spleen Cells.—Lymphoid cells from primed white pulp developed a significant secondary response on transfer in the presence of antigen. Table I shows the results of assaying the spleens of the recipient mice for a period of 4 days after transfer. The response was detectable as early as day 2 after cell transfer and increased markedly over the next 2 days. Primed lymphoid cells transferred in the absence of the specific antigen produced only a very slight increase in plaque-forming cells (PFC) over background (0.025 per 10^6) in recipient spleens during this 4-day period.

The precursor cells within the white pulp developed specifically in response to priming with SE. Cells from donor mice immunized with Keyhole Limpet

Hemocyanin (KLH) (0.5 mg) or bovine gamma globulin (BGG) (0.5 mg), or from normal C57Bl mice, developed only a minor increase in PFC on days 3–4 after challenge with SE in recipient mice (Table I).

The peak of PFC detected by the direct plating technique occurred on day 5. After day 6, there was a significant reduction in the incidence of these plaques of high hemolytic efficiency. Preliminary experiments indicated that no developed plaques (7S) could be detected on day 2 and day 3. Indeed, anti-mouse γ-globulin caused some inhibition of PFC at this time. A minority of developed plaques arose by day 4 (38%) to day 5 (44%), and by day 6 these predominated (74%). These calculations are based on the assumption that all "developed" plaques are due to 7S-synthesizing cells. This is, however, not necessarily correct, since some γM-plaques appear only after developing with anti-γM.[1]

TABLE I
Secondary Response of Immune White-Pulp Cells Transferred to Syngeneic Lethally Irradiated Mice

Donor cells		PFC per 10^6 recipient spleen cells*		
Immunized with‡	Challenge with SE§	Day 2	Day 3	Day 4
SE	+	0.50	8.40	83.00
	−	0.05	0.08	0.02
KLH or BGG	+		0.06	0.11
	−		0.05	0.05

* Pooled data from 11 experiments.
‡ Donor mice immunized intravenously 7–10 days earlier.
§ 0.1 ml 20% SE injected i.v. at the time of injection of donor cells.

Significant numbers of both groups of antibody-forming cells persisted until day 9. In the following experiments, the secondary response was followed primarily over the first 4 days after exposure to antigen, and only the results of the direct plating technique were noted, unless otherwise specified.

There was no marked variation in the results obtained with lymphoid cells taken from donors 7–10 days after priming with SE. This was in contrast to white pulp taken from mice 21–30 days after priming, which had a 10- to 20-fold lower responsiveness to challenge with antigen.

White pulp from primed spleen thus contained cells that are capable of developing a secondary response on reexposure to antigen, but a major question was to identify the spleen compartment in which these cells arise. The relative incidence of responsive cells in undissected spleens and white pulp was determined by using half of each donor spleen to prepare white pulp and the re-

---

[1] Plotz, P. H., N. Talal, and R. Asofsky. 1968. Assignment of direct and facilitated hemolytic plaques in mice to specific immunoglobulin classes. J. Immunol. 100:744.

mainder for the preparation of whole spleen cells. When comparable numbers of lymphoid cells from white pulp or whole spleen were transferred, white pulp gave rise to 8- to 10-fold higher numbers of PFC than did whole spleen (Fig. 1).

Lymphoid cells derived from both white pulp and whole spleen showed an exponential rise in the numbers of PFC after challenge. The rate of increase in activity was the same for both, suggesting that the populations of cells in the white pulp and whole spleen responding to antigen were similar, but that there
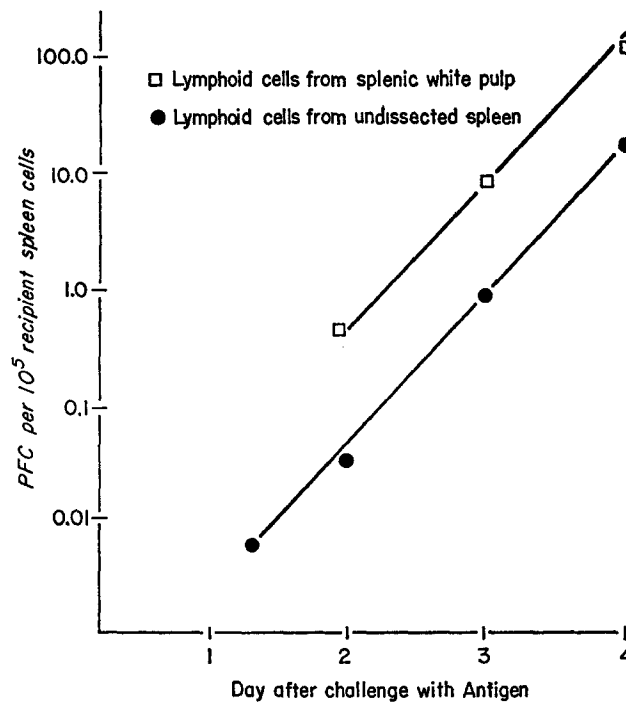

FIG. 1. Relative plaque-forming activities of spleen cells from recipients at various days after receiving comparable numbers of cells derived from white pulp and whole spleen. Donors were injected with SE 7 days prior to transfer.

was a significantly higher initial concentration of precursor cells in the white pulp at the time of second exposure to antigen. Assuming that all these PFC were the result of cell division and not of cell recruitment, the doubling time for the responding cells could be calculated to be 6.5 hr. There was no detectable increase over background in the numbers of PFC on day 1 after challenge. Thus, in this cell transfer system, the duration of the latent period before the appearance of PFC differed from that found in actively immune mice, where an increase in PFC at 24 hr after challenge could be detected (22).

The preponderance of primed cells in the white pulp was a consistent finding

in a series of experiments in which the activities of white pulp and whole spleen taken day 7–10 after the primary injection of antigen were directly compared. There was variation in the ratio of activity obtained with white pulp to that obtained with whole spleen on day 4 after transfer. In two of three additional experiments ratios as high as 20.5 and 52 were found, while in the third the ratio was only 3. Table II represents a typical experiment in which other parameters of the immune response evoked by these populations of cells were compared.

The Adoptive Immune Response Observed after Delayed Exposure to Antigen.— The cell transfer system also allows a study of the state of replication of the

TABLE II

A Comparison of the Secondary Response in Transferred Immune* White Pulp and Whole Spleen and the Effect of Delay in Reexposure to Antigen

Interval between cell transfer and reexposure to SE‖	Antibody formation‡ in recipients of cells§ from:					
	White pulp			Whole spleen		
	PFC per 10⁵ spleen cells	Total PFC per spleen	Reciprocal HA serum titer	PFC per 10⁵ spleen cells	Total PFC per spleen	Reciprocal HA serum titer
0 days	103¶	8,974	32	8¶	427	16
2 days	283	57,353	2048	12	2313	128

* Donor mice injected intravenously with 0.1 ml 20% SE 7 days previously; recipients received similar dose.

‡ Assayed 4 days after reexposure to antigen.

§ Half of each donor spleen was used for whole spleen suspension; white pulp was prepared from the remainder.

‖ 1 × 10⁷ cells injected per recipient.

¶ Represent averages of results obtained with three recipients.

responsive population prior to reexposure to antigen. By varying the times of reexposure to antigen after cell transfer, a characteristic pattern of responsiveness in the recipients was observed. Table II indicates that, in a typical experiment, there was a marked increase in responsiveness when injection of antigen was delayed 2 days. This increased activity is illustrated by the relative numbers of PFC in the spleens and by the serum titer of these mice. Cells derived from both white pulp and whole spleen early after sensitization showed this increased responsiveness after a delay in exposure to antigen, but this "lag effect" was not seen with spleen cells taken from mice 30 days after primary immunization.

Some insight into the nature of this lag effect can be gained by following the evolution of the secondary response over the first few days after reexposing the

cells to antigen at two different time periods. Fig. 2 illustrates the results of an experiment in which the response in recipients challenged immediately was compared with the response obtained after a lag period of 24 hr before challenge. The actual rate of increase of PFC was very similar to that produced by
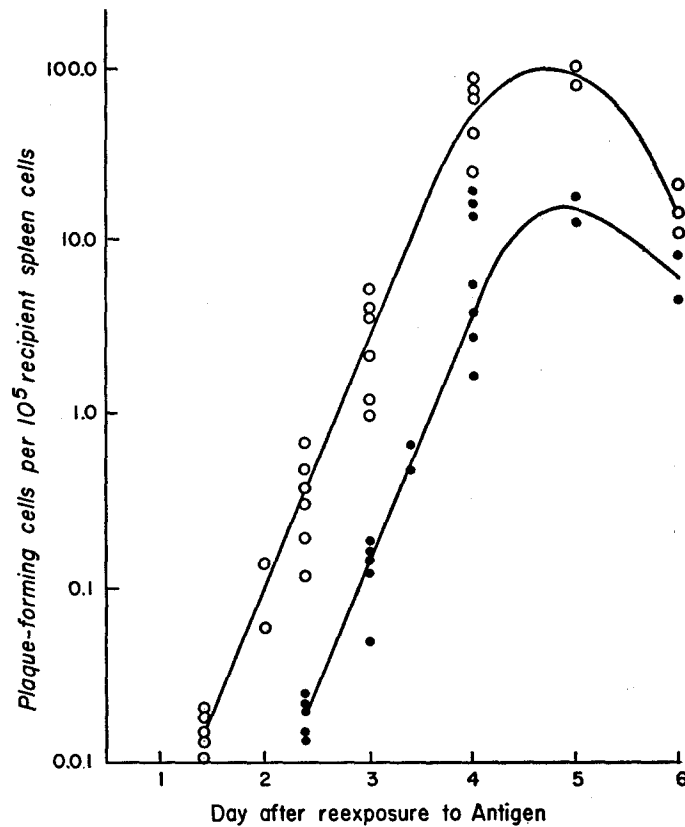

FIG. 2. Numbers of plaque-forming cells in recipient spleens at various days after transfer of spleen cells from donor mice sensitized with SE 7 days previously. Comparison of the response obtained upon immediate reexposure to antigen (●——●) with the response seen upon reexposure to antigen with a 24 hr delay (O——O).

cells transferred and challenged with antigen immediately. However, there was a 10-fold increase in the numbers of PFC observed throughout the period of assay in spleens from recipients challenged at 24 hr and, in addition, PFC above background (0.025 PFC per 10^5) were detectable in this group 24 hr earlier after challenge than in the controls. Not only was the response earlier, but the peak level of the response increased 10-fold. The day of peak response with

respect to the time of reexposure to antigen and the relative incidence of 7S and 19S plaques, on days 4–6 after challenge, were similar in the two groups.

The length of the lag period resulting in optimal responsiveness of the transferred cells was determined by exposing the cells to antigen either immediately or on day 1, 2, or 3 after cell transfer. The most impressive increase in responsiveness was observed with a lag period of 24 hr, but in some experiments there was a slight additional increase if challenge was delayed for 48 or 72 hr. When the apparently crucial period of the first 24 hr was studied in detail, it was noted that a delay of even a few hours in reexposure to antigen resulted in enhanced responsiveness. Table III indicates that most of the increased responsiveness was attained within the first 12 hr after transfer as evidenced by larger numbers of plaques and higher serum titers in the recipients.

TABLE III

*Increase in Responsiveness to Sheep Erythrocytes during the First 24 Hr after Transfer of Primed Whole Spleen Cells**

Interval between cell transfer and reexposure to SE	PFC per 10^5 recipient spleen cells		Reciprocal of HA serum titer	
	Day 3 (Exp. 1)‡	Day 4 (Exp. 2)‡	Day 3 (Exp. 1)‡	Day 4 (Exp. 2)‡
hr				
0	0.7§	13.4	4§	8
2–6	—	56.5	12	—
8–12	4.1	63.9	32	48
20–24	3.1	96.8	64	128

* Donor mice injected intravenously with 0.1 ml 20% SE 7 days previously; recipients received similar dose.

‡ Indicate days of assay for two different experiments.

§ Represent averages of results obtained with three to six recipients; days 3 and 4.

A Study of the Proliferative Activity of the Transferred Cells: The Influence of 5-Bromodeoxyuridine (BUDR).—The extent of cellular proliferation of the responsive cells during the lag phase was estimated by exposing the cells to a thymidine analogue known to be incorporated into DNA of dividing cells (23). BUDR was chosen since this drug can completely suppress the secondary response in vitro if given during the proliferative phase after exposure to antigen. However, it is not toxic to the cells at other stages of the immune response (24). Doses were chosen in excess of those used in vitro (250 μg/ml) but very much less than those causing toxicity in nonirradiated mice (1000 mg/kg/day) (25).

In the first experiment depicted in Table IV, a total of 2 mg BUDR was injected at 4-hr intervals during the first 18 hr after cell transfer. 6 hr after the last injection of the drug, antigen was injected intravenously. Under these conditions, a secondary response to SE was evoked, but this response was lower than that of untreated control mice challenged with antigen at the same time

(24 hr). The incidence of PFC in the spleens of drug-treated mice was similar to that found in recipients challenged with antigen at the time of cell transfer. A comparable pattern of response was noted in the following two experiments, even though the BUDR dose was greatly increased and the drug was administered at 2-hr intervals. When the recipient mouse spleens were assayed on day 3 rather than on day 4 after antigen, as in Experiment 3, the effect of the BUDR was even more clearly demonstrated, but it was not possible to completely suppress the secondary response. These findings indicate that the lag effect can be abolished by BUDR, and that the cells responsible for the lag effect were synthesizing DNA in the recipients prior to exposure to antigen. The fact that the BUDR could only partially inhibit the secondary response suggests that a proportion of the population of primed cells were most likely not dividing at the

TABLE IV

Influence of Bromodeoxyuridine, Administered Prior to Challenge with Antigen, on the Responsiveness of Transferred Spleen Cells*

Interval between cell transfer and reexposure to SE	BUDR	PFC per 10^5 recipient spleen cells		
		Exp. 1	Exp. 2	Exp. 3‡
1 hr	—	19.6§	7.9	1.0
1–2 days	—	44.0	14.9	7.5
1–2 "	+‖	14.8	8.0	0.5

* Donor mice injected with 0.1 ml 20% sheep erythrocytes (SE) 7 days previously.

‡ Experiment 3 assayed on day 3 after challenge; the remaining experiments were assayed on day 4.

§ Mean of three mice.

‖ Total dose BUDR: Experiment 1, 2 mg; Experiment 2 and 3, 10 mg.

time of exposure to the drug (Table IV). It is possible, of course, that some cells escaped the effect of the drug, but greater effectiveness was not achieved when the level of drug treatment was greatly increased.

The effect of two further schedules of BUDR administration was examined. The susceptibility of the responsive donor population to BUDR at the time of transfer was of interest, since such a population contains both replicating and nonreplicating cells (17). Moreover, the time of susceptibility of the transferred cells to BUDR in recipient mice was determined more precisely. In Experiment 1 (Table V), the cells were exposed to the drug either in vitro or in vivo during the first 6 hr after cell transfer. Both these regimens of drug treatment resulted in a reduction but not an elimination of the secondary response. The BUDR caused an almost complete abolition of the lag effect seen in control mice challenged at 8 hr, as illustrated by the concentration and total numbers of PFC in the spleen, as well as by the serum titers. The effectiveness of a

similar dose schedule of BUDR on the adoptive immune response by these cells 2 days after challenge with antigen was clearly demonstrated in Experiment 3 (Table V). Other experiments not represented in the tables, have shown that BUDR given immediately after antigen in these transfers, did not affect the response. In this respect the sensitivity of these primed cells to BUDR in vivo was similar to that found for the secondary response in vitro (24).

The Effect of Passive Antibody on the Responsiveness of the Transferred Cells.— A low rate of antibody formation occurs in the cells at the time of transfer. It

TABLE V

The Effect of Varying the Time of Administration of Bromodeoxyuridine on the Responsiveness of Transferred Spleen Cells*

Exp. No.	Exposure to BUDR	Interval between cell transfer and reexposure to SE	PFC per 10^5 recipient spleen cells	Total PFC recipient spleen	Reciprocal HA titer
		hr			
1	Before SE in vitro§	0	7.9	121	8
	None	0	15.0	389	24
	Before SE in vitro‡ and in vivo§	8	19.3	579	24
	None	8	60.6	2171	48
2	None	0	23.4	234	8
	Before SE in vivo§	24	57.0	1140	64
	None	24	152.0	7625	128
3	None	0	8.7	590	48
	After SE in vivo‖	0	1.2	96	4

* Assayed day 4 after reexposure to sheep erythrocytes (SE).

‡ Cells incubated with 250 μg/ml BUDR for 1 hr prior to injection.

§ 4 × 2 mg BUDR with 2-hr intervals, given intraperitoneally within the first 6 hr after transfer.

‖ 4 × 2 mg BUDR with 2-hr intervals, given intraperitoneally between 48 and 54 hr after antigen.

might be argued that this antibody, after accumulating in the recipient for 8–24 hr, induces a change in the fate of injected antigen which results in a magnified responsiveness of the transferred cells. The responsiveness of cells given at the same time as 0.05 ml of serum from donor mice (agglutinin titer 1:1024) was therefore studied. It was found that a 24-hr delay in the reexposure to antigen resulted in a fivefold increase of the response whether or not the cells had been given with antibody. Neither was the response upon immediate challenge affected.

Fate of the Transferred Cells: Localization in the Spleen.—In the preceding publication, a specific homing of thymidine-³H–labeled white-pulp cells to

lymphoid tissues of recipient mice was noted (17). The pronounced increase in responsiveness of the transferred cells over the first 24 hr could possibly be due to a delay in the homing of the cells to specific lymphoid areas. The rate of accumulation of heavily uridine-^{3}H–labeled white-pulp cells in lymphoid organs of the recipient was therefore examined.

Smears of spleens, injected with 2×10^7 uridine-^{3}H–labeled spleen cells, indicated that at 1–8 hr 13–15% of the cells present in recipient spleens were labeled. At 24 hr, this percentage had dropped slightly (10%). Since at this time recipient spleens contain approximately 2×10^7 cells, this indicates that 10–15% donor cells localize in the spleen during the first 24-hr period.

Sections of spleens taken within 10–15 min after transfer showed the presence of a few labeled cells in the red pulp areas. By 1 hr, there was an increase in the total number of labeled cells in the spleens, most of which were in the red pulp and concentrated at the border of white and red pulp (Fig. 3). The numbers of labeled cells in the red pulp then decreased, while those in the white pulp increased over the first 4–6 hr after transfer (Fig. 4). In view of the intense labeling of the white pulp areas between 6 and 24 hr, it could not be determined with certainty at which time the maximal incidence of labeled cells was reached. The localization of the labeled cells within the white pulp appeared to remain constant from 4–24 hr after transfer. The cells were concentrated in peripheral follicular areas, away from the major central arterioles (Fig. 4).

When white pulp cells were transferred together with the specific antigen, large blast cells began to appear in the white pulp of the recipient animals at 48 hr. These cells progressively filled and surrounded the white pulp areas of recipient spleens on days 3 and 4. Densely packed foci of dividing blast cells representing reformation of germinal centers developed at this time (Fig. 5). Very few blast cells were seen during the first 4 days after transfer when the cells were injected without antigen or with an unrelated antigen (18).

DISCUSSION

The major finding presented concerns the increase in responsiveness of primed cells when challenge with specific antigen is delayed for 8–24 hr after transfer. The fact that a similar increase with primed or unprimed cells has not been observed by others (1–3) is most likely due to the nature of the cells transferred. In the present experiments, the lag effect could be shown with cell populations taken 7–9 days after the primary injection of antigen, but not with cells taken at 1 month. The increase in responsiveness after transfer (before challenge) is of finite duration and a plateau of responsiveness is reached which lasts for at least 2 more days.

It appears that the increased response is largely due to proliferating cells present in the transferred population. When the responses of cells challenged on day 0 and on days 1 or 2 after transfer are compared, the kinetics and

magnitude of the response show clearly that the lag effect is not due to an increased rate of production of PFC, but to an actual increase in the numbers of responding cells present at the time of challenge. The observation that BUDR abolishes the lag effect strongly suggests an important role of cell division in providing the increased numbers of responding cells.

It is possible that the increased numbers of responding cells after a lag period are partly due to the homing of sensitized cells to regions of the lymphoid system favorable for the early stages of antibody formation. Cells accumulate in the white pulp over a period of at least 4 hr. However, it appears unlikely that homing is an important factor since cells taken at longer periods after priming, which presumably have a similar homing tendency, do not develop a lag effect. A comparable increase in responsiveness develops in vitro when white pulp from primed animals is used for the induction of a secondary response. Delay in re-exposure to antigen in vitro resulted in a higher response only when tissue was sampled during the proliferative phase of priming (12). Increased numbers of responding cells may also develop through a maturation of precursor cells present in the donor population. Proliferation and maturation frequently accompany each other in developmental processes but the relative contribution of maturation to the formation of sensitized cells cannot readily be evaluated.

Determination of some of the cellular events accompanying the lag effect might elucidate the development of the memory cell. Proliferating cells were indeed present among the donor cells at the time of transfer and continued to proliferate for at least 8 hr after transfer; thereafter the mitotic activity decreased progressively (17). This agrees with the finite time period over which the lag effect was observed and coincides with the BUDR-sensitive phase. The majority of the donor cells were small lymphocytes and many of the proliferating cells transformed into small lymphocytes during this 24-hr period. These data, as well as the fact that a proportion of the responsive cells present in the donor inoculum were insensitive to the action of BUDR suggest that nondividing or relatively slow-dividing cells formed part of the responsive population. The observations are therefore in agreement with the hypothesis that small lymphocytes constitute the responsive cells resistant to BUDR and that the lag effect is due to the proliferation of cells on their way to becoming small lymphocytes. Differentiation of small lymphocytes from dividing precursors during the first 2 wk after sensitization may therefore be a natural step in the development and maturation of the memory cell.

White pulp is particularly responsive to reexposure to antigen. When the response of a given number of transferred white-pulp cells is compared with that obtained with whole spleen cells, much higher numbers of PFC are seen in the recipient spleens. The kinetics of the two responses suggest that there is a higher concentration of responding cells in the white pulp at the time of challenge. This confirms previous observations on transferred antibody responses in

rabbits (26). The lag effect can also be clearly shown with transferred white-pulp cells. The majority of proliferating cells present in the white pulp on days 7–9 after priming are in the germinal center and by day 30, when a lag effect cannot be demonstrated, germinal center proliferation has subsided. It seems likely, therefore, that the high concentration of memory cells early after the primary injection, as well as the cells responsible for the lag effect are related to the presence of germinal centers in the spleen at this time. The lower activity of spleen cells taken on day 30 after priming is most likely due to a dissemination of memory cells.

SUMMARY

White-pulp cells and whole spleen from donor mice immunized with sheep erythrocytes were transferred intravenously to heavily irradiated mice. The numbers of plaque-forming cells and the amount of hemagglutinating antibody produced after reexposure to antigen were measured.

When reexposure to sheep erythrocytes was delayed, a much greater response occurred in the transferred cells. Peak responsiveness was reached at 24 hr after transfer. This "lag effect" was greatly reduced by repeated injections of 5-bromodeoxyuridine into the recipient mice prior to challenge with antigen. It was therefore concluded that much of the increase in responsiveness was due to a proliferation of "primed" cells after cell transfer. The fact that a significant response was given by the transferred cells in spite of 5-bromodeoxyuridine treatment suggested that some of the primed cells were nondividing.

White pulp was a much richer source of responsive cells than was whole spleen.

BIBLIOGRAPHY

1. Perkins, E. H., and T. Makinodan. 1964. Relative pool size of potentially competent antibody-forming cells of primed and nonprimed spleen cells grown in in vivo culture. J. Immunol. 92:192.

2. Dresser, D. W., 1961. A study of the adoptive secondary response to a protein antigen in mice. Proc. Roy Soc. London, Ser. B. 154:398.

3. Celada, F. 1967. Quantitative studies of the adoptive immunological memory in mice. II. Linear transmission of cellular memory. J. Exptl. Med. 125:199.

4. Cohen, E. P., and D. W. Talmage. 1965. Onset and duration of DNA synthesis in antibody-forming cells after antigen. J. Exptl. Med. 121:125.

5. Gowans, J. L., and J. W. Uhr. 1966. The carriage of immunological memory by small lymphocytes in the rat. J. Exptl. Med. 124:1017.

6. Porter, R. J. 1960. Studies on antibody formation. Effects of X-irradiation on adaptation for the secondary response of rabbits to bovine gamma globulin. J. Immunol. 84:485.

7. Thorbecke, G. J., E. B. Jacobson, and R. Asofsky. 1964. Gamma globulin and antibody formation *in vitro*. IV. The effect on the secondary response of X-irradiation given at varying intervals after a primary injection of bovine gamma globulin. *J. Immunol.* **92:**734.

8. Nettesheim, P., T. Makinodan, and M. L. Williams. 1967. Regenerative potential of immunocompetent cells. I. Lack of recovery of secondary antibody forming potential after X-irradiation. *J. Immunol.* **99:**150.

9. Nowell, P. C. 1965. Unstable chromosome changes in tuberculin stimulated cultures from irradiated patients. Evidence for immunologically committed long-lived lymphocytes in human blood. *Blood* **26:**798.

10. Thorbecke, G. J., R. Asofsky, G. M. Hochwald, and G. W. Siskind. 1962. Gamma globulin and antibody formation *in vitro*. III. Induction of secondary response at different intervals after the primary: the role of secondary nodules in the preparation for the secondary response. *J. Exptl. Med* **116:**295.

11. Fecsik, A. I., W. T. Butler, and A. H. Coons. 1964. Studies in antibody formation. XI. Variation in the secondary response as a function of the length of the interval between two antigenic stimuli. *J. Exptl. Med.* **120:**1041.

12. Cohen, M. W., E. B. Jacobson, and G. J. Thorbecke. 1966. Gamma globulin and antibody formation *in vitro*. V. The secondary response made by splenic white and red pulp with reference to the role of secondary nodules. *J. Immunol.* **96:**944.

13. Hurliman, J., J. D. Wakefield, and G. J. Thorbecke. 1966. The effects of immuno-suppressant drugs administered during germinal center proliferation on preparation of a secondary antibody response. *In* Germinal Centers and the Immune Response. H. Cottier, N. Odartchenko, R. Schindler, and C. C. Congdon, editors. Springer-Verlag, New York. 225.

14. Sterzl, J. 1967. The effect of immunosuppressive drugs at various stages of differentiation of immunologically competent cells. *In* Immunity, Cancer and Chemotherapy. E. Mihich, editor. Academic Press, New York.

15. Miller, J. J., and G. J. V. Nossal. 1964. Antigens in immunity. VI. The phagocytic reticulum of lymph node follicles. *J. Exptl. Med.* **120:**1075.

16. Cohen, S., P. Vassalli, B. Benacerraf, and J. T. McCluskey. 1966. The distribution of antigenic and nonantigenic compounds within draining lymph nodes. *Lab. Invest.* **15:**1143.

17. Wakefield, J. D., and G. J. Thorbecke. 1968. 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. *J. Exptl. Med.* **128:**153.

18. Wakefield, J. D., M. W. Cohen, J. McCluskey, and G. J. Thorbecke. 1966. The fate of lymphoid cells from the white pulp at the peak of germinal center formation. *In* Germinal Centers and the Immune Response. H. Cottier, N. Odartchenko, R. Schindler, and C. C. Congdon, editors. Springer-Verlag, New York. 183.

19. Jerne, N. K., A. A. Nordin, and C. Henry. 1963. The agar plate technique for recognizing antibody-producing cells. *In* Cell Bound Antibodies. D. B. Amos and H. Koprowski, editors. Wistar Institute Press, Philadelphia. 109.

20. Sterzl, J., and I. Riha. 1965. Detection of cells producing 7S antibodies by the plaque technique. *Nature.* **208:**858.

21. Dresser, D. W., and H. H. Wortis. 1965. Use of an antiglobulin serum to detect cells producing antibody with low haemolytic efficiency. *Nature.* **208:**859.

22. Hege, J. S., and L. J. Cole. 1966. Antibody plaque-forming cells: kinetics of primary and secondary responses. *J. Immunol.* **96:**559.

23. Dutton, R. W., A. H. Dutton, and J. H. Vaughan. 1960. The effect of 5-bromo-uracil deoxyriboside on the synthesis of antibody *in vitro. Biochem. J.* **75:**230.

24. O'Brien, T. F., and A. H. Coons. 1963. Studies on antibody production. VII. The effect of 5-bromodeoxyuridine on the *in vitro* anamnestic antibody response. *J. Exptl. Med.* **117:**1063.

25. Butler, W. T., and A. H. Coons. 1964. Studies on antibody production. XII. Inhibition of priming by drugs. *J. Exptl. Med.* **120:**1051.

26. Thorbecke, G. J., E. B. Jacobson, and G. M. Hochwald. 1965. Radiation effects and studies *in vitro* in the evaluation of the possible role of secondary nodules in the preparation for the secondary response. *In* Molecular and Cellular Basis of Antibody Formation. J. Sterzl, editor. Czechoslovak Academy of Sciences, Prague. 587.
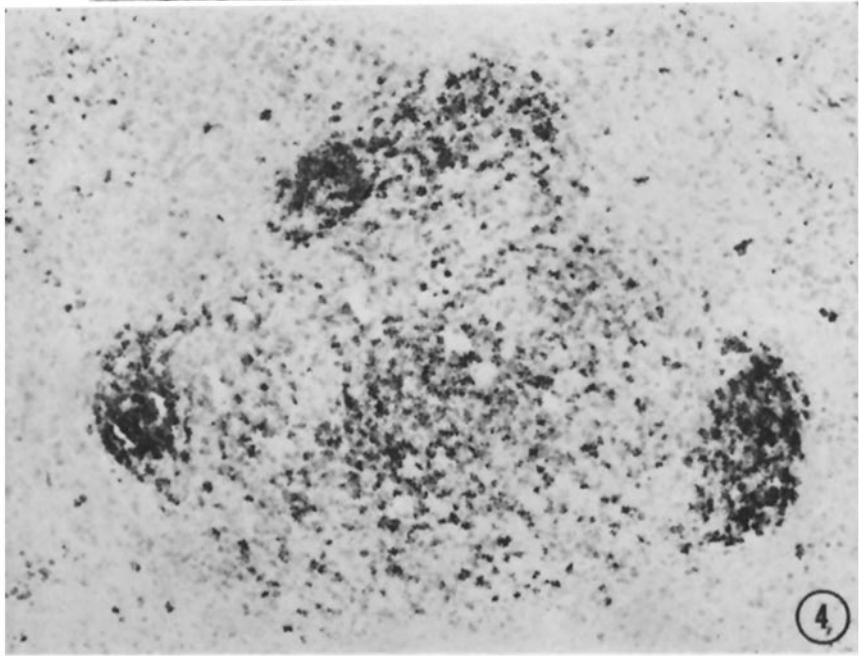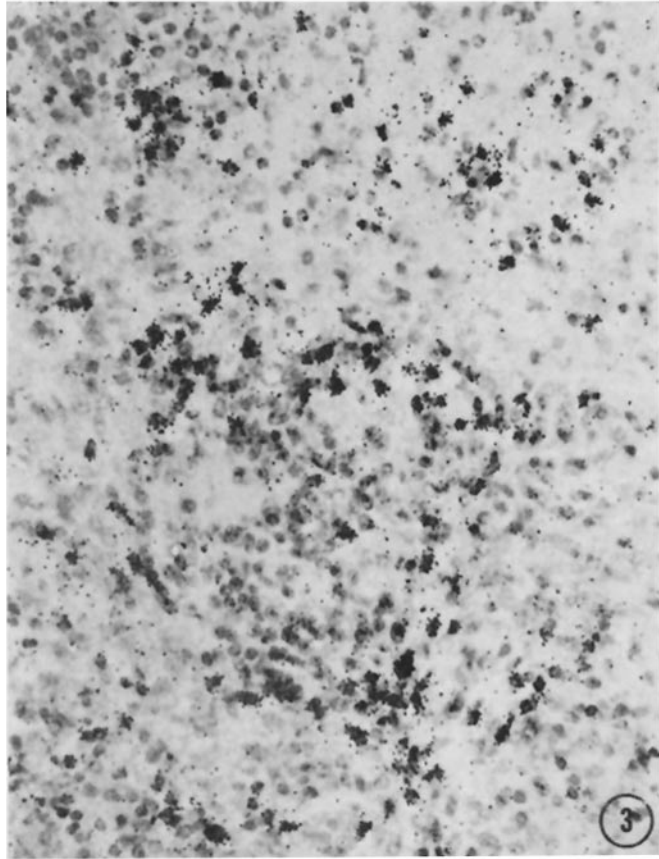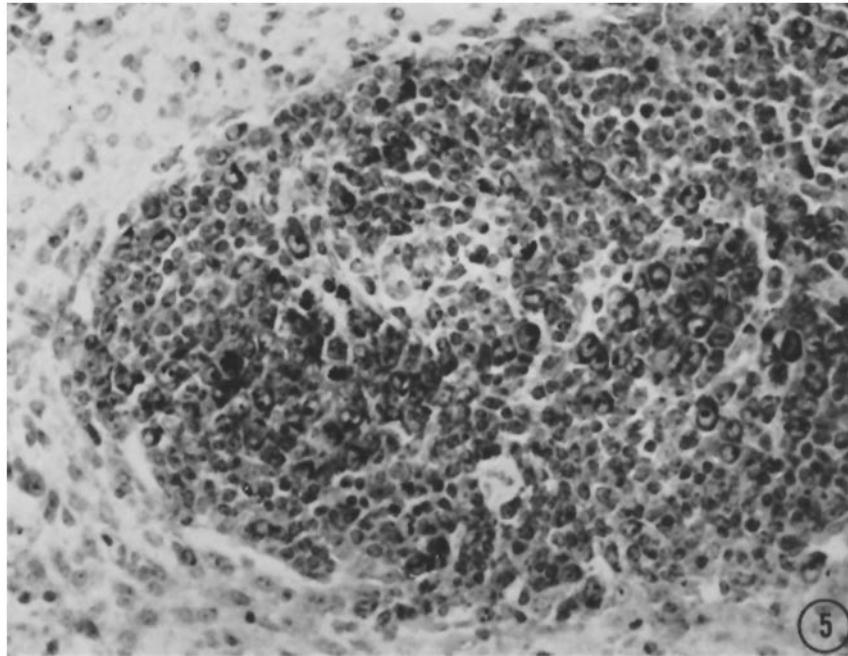
FIG. 5. Blast cell proliferation in white pulp of recipient spleen 3 days after transfer of sensitized white-pulp cells with antigen. Note formation of germinal centers. Methyl green-pyronine. ×100.

FIG. 3. Distribution of uridine-³H–labeled white-pulp cells over white and red pulp of recipient spleen at 1 hr after intravenous injection. Methyl green-pyronine. ×100.

FIG. 4. Typical localization of uridine-³H–labeled, white-pulp cells in peripheral areas of the white pulp of recipient spleen at 8 hr after intravenous injection. Methyl green-pyronine. ×50.