THE PROLIFERATIVE AND ANAMNESTIC ANTIBODY RESPONSE OF RABBIT LYMPHOID CELLS IN VITRO

I. Immunological Memory in the Lymph Nodes Draining and Contralateral to the Site of a Primary Antigen Injection*

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Previous studies from this laboratory have suggested that a relationship exists between germinal center formation, induced by injection of an antigen into the white pulp of a spleen, and the ability of this tissue to respond upon reexposure to that antigen in vitro. Responsiveness was determined both by antibody production (1, 2) and enhanced mitotic activity (3). In studies by others (4, 5), proliferation of lymph node cells in vitro in response to challenge with antigen correlated well with delayed hypersensitivity, but not with antibody formation in vivo. The ability of the lymph node cells to give a secondary antibody response in vitro was not investigated by these authors, and was therefore not directly compared to the proliferative responsiveness. In the present experiments such studies, previously done for the spleen, have been extended to lymph nodes draining a site of antigen injection.

It is not clear in what manner long term immunological memory is mediated. In a study involving the transfer of the secondary immune response with white pulp cells injected into irradiated syngeneic recipients it was found that, in the absence of added antigen, many ^{3}H-thymidine labeled germinal center cells transform into small lymphocytes (6). It is possible that these lymphocytes belong to the population of long-lived, recirculating lymphocytes shown to be capable of transferring a secondary response (7, 8). While it seems likely that long term memory occurs as a result of nondividing small lymphocytes, which pass into the circulation and are seeded more or less equally to all lymphoid organs, there is also evidence that at least some of the memory cells remain

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fixed in the lymphoid tissue (9). It would appear, then, that in addition to the recirculating memory cells there continues to be a concentration of primed cells at the site of the initial response.

This paper describes the results of studies on the secondary response obtained with popliteal lymph node fragments, challenged in vitro at various time intervals after primary immunization via the hind footpads. If the recirculating, long-lived small lymphocyte is the major carrier of immunological memory, relatively little difference should be expected in the ability of lymph nodes throughout the body to exhibit a secondary response, even after a single local sensitizing injection. On the contrary, if there is a tendency for memory cells to remain localized in the lymph node draining the injection site, this should be readily demonstrable. For this purpose a comparison has been made, after immunization in one footpad, between the responsiveness to in vitro challenge of the draining and contralateral lymph nodes.

Materials and Methods

Animals and Immunization.—Male New Zealand rabbits, approximately 2.5 kg, were injected subcutaneously into each hind footpad with 5 mg bovine gamma globulin (BGG) or 100 Lf[1] diphtheria toxoid (DT) (Lederle Laboratories, Division of American Cyanamid Co., Pearl River, N. Y. 1050 Lf/ml, lot #60D-11). In some experiments, antigen (DT) was injected into the left footpad only. Both popliteal lymph nodes were removed aseptically at various times after immunization, ranging from 4 days to 9 months.

Secondary Response In Vitro.—Lymph node fragments were distributed over the walls of roller tubes which had previously been coated with heparinized normal rabbit plasma. Four tubes were prepared for each lymph node. 1 ml of modified Eagle's medium (2), containing 25% normal rabbit serum, was added to each tube. Antigen was added to two of the tubes, left in for 6 to 8 hr, and then rinsed off. The challenge dose of antigen was 1 Lf DT or 100 μg BGG. The tissues were then incubated at 37°C for 9 to 12 days. Medium changes were made at 3-day intervals, and the culture fluids were saved for antibody titration (10).

Proliferative Response.—Cell suspensions were obtained from the lymph nodes by gently teasing the tissue with fine forceps. The cells were filtered through sterile gauze and washed once in Hanks' balanced salt solution (BSS). One ml portions of the cell suspensions, containing 2 to 4 $\times$ 10^6 cells, were pipetted into 12 $\times$ 75 mm plastic culture tubes, and antigen was added to half of the tubes. Each experiment was set up in duplicate or triplicate and the challenge dose of antigen was the same as that used for the roller tube cultures. The tubes were incubated at 37°C in a 5% CO_2–95% air atmosphere for 24 hr. One μc of tritiated thymidine (Schwarz Bio Research, Inc., Orangeburg, N. Y., specific activity 0.36 c/mM) was added to each tube and incubation was continued for an additional 24 hr. In a few experiments additional tubes were set up which received ^{3}H-thymidine after 72 hr of incubation and were assayed for ^{3}H-DNA at the end of 4 days (11).

At the end of the culture period the cells were washed and subjected to perchloroacetic acid precipitation and acid hydrolysis. Portions of the resulting hydrolysates were counted in a scintillation counter. The method used is essentially that of Reich et al. (12) and is described fully in a previous publication (3).

Antibody Determinations.—All culture fluids, obtained at 3-day intervals, were titrated for

[1] Lf, Limes flocculation dose.

antibody by two-fold serial dilution, using the passive hemagglutination technique described by Stavitsky (13).

Culture fluids obtained from DT-sensitized lymph nodes were also analyzed for precipitating antibody by means of double diffusion in agar (14) and by precipitation of ^{125}I-labeled DT (15). The ^{125}I-DT contained 50 Lf/ml and was 95% precipitable by trichloroacetic acid, and 53.5% precipitable by a hyperimmune rabbit anti-DT. When standard amounts of this ^{125}I-DT (0.05 Lf) were mixed with samples of serial dilutions of the standard antiserum, it was found that 50λ of a 1:100 dilution of this antiserum gave about 80% of maximal precipitation. Undiluted 50λ culture fluid samples were also tested to determine their ability to precipitate the ^{125}I-DT and compare them to dilutions of the standard antiserum.

TABLE I

Antibody Formation and DNA Synthesis in Lymph Node Cultures Taken at Various Times after Priming and Challenged with Antigen‡ In Vitro*

Time After primary immunization	No. of animals	Reciprocal of peak titer	Degree of stimulation§
4 days	1	No secondary response*	1.4
7 "	3	512	7.2
2 wk	1	1,024	1.1
3 "	1	1,024	0.8
1 month	2	2,048	0.9
2–3 "	2	32,000	N.T.
4–6 "	6	32,000	1.3
7–9 "	2	64,000	1.2

* Antibody formation determined by titration of medium removed at 3-day intervals. When titer of controls was equal to or greater than the titer obtained after reexposure to antigen, no secondary response was considered to have occurred.

‡ 1 Lf DT per ml of culture medium, left with tissues for the first 6–8 hr of culture.

§ Degree of stimulation $= \frac{\text{cpm in cells with antigen}}{\text{cpm in cells without antigen}}$

RESULTS

The proliferative response, measured over the 2nd day in vitro, was barely detectable with lymph node cells taken on day 4 after priming, both with DT (Table I) and BGG (Table II). On day 7, however, peak responsiveness to both antigens had already developed. The degree of stimulation obtained with BGG was much higher than with DT, and responsiveness to BGG also remained detectable over a longer period. The DT-sensitized lymph node cells did not demonstrate a significant proliferative response when taken later than 1 wk after priming, while the responsiveness of BGG-sensitized cells was still detectable up to 3 wk after priming.

When the culture period was prolonged and ^{3}H-thymidine incorporation measured over the 4th day in vitro, a small degree of stimulation could be shown even with lymph node cells taken as late as 5 to 6 months after priming (Table

III). On the contrary, the proliferative activity of lymph node cells removed 1 wk after priming was considerably less when measured over the 4th rather than over the 2nd day in vitro.

TABLE II

Antibody Formation and DNA Synthesis in Lymph Node Cultures Taken at Various Times after Priming and Challenged with Antigen‡ In Vitro*

Time After primary immunization	No. of animals	Reciprocal of peak titer	Degree of stimulation§
4 days	1	No secondary response*	1.7
7 "	2	8	18.7
2 wk	1	128	3.7
3 "	2	1024	4.3
4 "	1	2048	1.3

* Antibody formation determined by titration of medium removed at 3 day intervals. When titer of controls was equal to or greater than the titer obtained after reexposure to antigen, no secondary response was considered to have occurred.

‡ 100 μg BGG per ml of culture medium, left with tissues for the first 6–8 hr of culture.

§ Degree of stimulation = $\frac{\text{cpm in cells with antigen}}{\text{cpm in cells without antigen}}$

TABLE III

The Effect of Time Interval after Priming on the Proliferative Response of Lymph Node Cells Measured 2 and 4 Days after Reexposure to Antigen In Vitro

Time After primary immunization*	Degree of stimulation	
	Day 2§	Day 4
1 wk	9.6	2.1
5–6 Months	1.5	3.5
	1.5	2.4
	0.9	1.3

* Priming was by injection of 100 Lf Diphtheria toxoid into hind footpads. Popliteal lymph node suspensions reexposed to 1 Lf/ml DT upon initiation of cultures.

‡ Degree of stimulation = $\frac{\text{cpm in cells with antigen}}{\text{cpm in cells without antigen}}$.

§ Refers to day of culture on which ^{3}H-thymidine (1μc) incorporation was measured.

Despite the fact that the proliferative responsiveness decreased with the time after immunization at which the lymph nodes had been removed, peak antibody titers obtained during the secondary response with fragments from the same nodes increased steadily. The ability of the nodes to give a secondary response to DT appeared to reach a peak by 2–3 months (Table I). Nodes from the BGG-sensitized rabbits were not studied later than 4 wk after priming, but it was clear that their ability to give an anamnestic response was increasing up to this time.

The antibody formed during the secondary response in vitro was resistant to 0.1 M mercaptoethanol in all cases and was therefore considered to belong to the 7S class (16). The analysis of the culture fluids by means of double diffusion in agar, showed a good correlation between hemagglutinating antibody titers and the ability of the fluids to precipitate DT. Culture fluids exhibiting passive hemagglutinin titers of 1:16,000 and higher gave precipitin lines, while those with titers of 1:8000 or lower gave no detectable lines (Table IV). The results

TABLE IV

Relationship between Passive Hemagglutinin Titers and Precipitating Antibody to DT in Culture Fluids

Time after primary	No. of experiments*	Reciprocal of peak PHA titer	Precipitation in agar‡	^{125}I-DT % ppt§
2 wk	1	1,024	−	1.9
3–4 "	3	2,048	−	11.4
3 months	1	64,000	++	92
4 "	1	32,000	+	80
	1‖	8,000	−	7.5
5 "	2	16,000	+	75
7–9 "	2	64,000	+	73

* Culture fluids showing peak hemagglutinin titers were pooled from 1 to 3 experiments.

‡ Precipitin line formed with 2.5 or 5 Lf DT per ml.

§ Per cent of maximal precipitation of 0.05 Lf ^{125}I-DT caused by 50μ of culture fluid.

‖ Contralateral lymph node; all the other cultures in this table were from draining lymph nodes.

obtained by precipitation of a standard amount of ^{125}I-DT also agreed well with the hemagglutinin titers (Table IV). Very little precipitation was obtained with culture fluids from lymph nodes removed 2 to 4 wk after priming, while draining lymph nodes removed 2½ to 9 months after the primary injection formed good precipitating antibody.

Secondary Response in Left and Right Popliteal Lymph Nodes after Priming in the Left Footpad.—The left popliteal lymph node was able to give a moderate secondary antibody response as early as 7 days after antigen injection (Fig. 1; Table I). No secondary response was detectable with lymph nodes removed on day 4. The increase in immunological memory over the following weeks is more clearly shown in Fig. 1 than in Table I, because titers due to the remaining primary response in the tissue were subtracted before plotting of the data. The curves representing the response in nodes removed after 2 or more wk were similar to those seen in a typical secondary response in vitro in that titers were

negligible on day 3, and peak titers were obtained on day 9. There was still a marked increase in responsiveness between 1 and 2 months after sensitization (Table I).

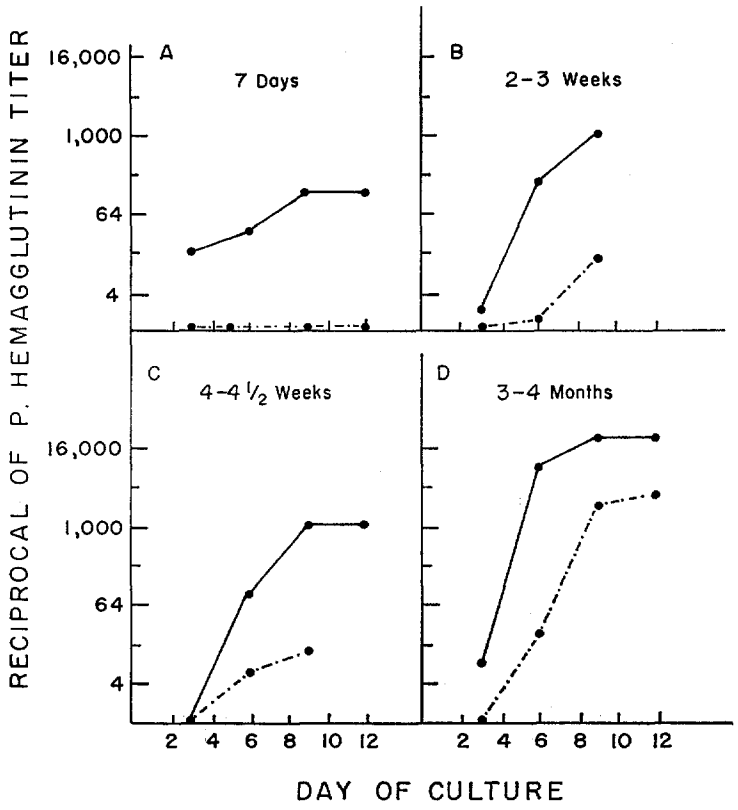

FIG. 1. Secondary antibody response of draining (●——●) and contralateral (●·-·-·-●) popliteal lymph nodes, challenged with 1 Lf DT in vitro at various times after primary immunization into a single hind footpad. Passive hemagglutinin titers of culture fluids removed at 3-day intervals are plotted against time after initiation of culture. In order to clearly show the secondary response, the titers plotted represent the differences between titers obtained with and without reexposure to antigen. The time intervals between primary in vivo and challenge in vitro are: *A*, 1 wk; *B*, 2–3 wk; *C*, 4–4½ wk; *D*, 3–4 months.

The right popliteal lymph node did not give any response on day 7, and formed little or no antibody when challenged in vitro 2 to 4 wk after the primary injection. The appearance of antibody production in these cultures was slower than in cultures of lymph nodes draining the injection site, since, in some cultures the medium changes did not show detectable antibody until day 9. At 3 to 4 months after sensitization there was a moderate response in these lymph

nodes, but even by this time their level of production of hemagglutininating and precipitating antibody remained significantly below that of the draining lymph nodes (Fig. 1, Table IV). Moreover, at no time did the cells from the contralateral node show a detectable proliferative response.

DISCUSSION

The higher initial responsiveness of the left than of the right lymph node must be due to the fact that it is draining the site of antigen injection. The maintenance of the difference between left and right lymph nodes over a period of 3 months is most likely the result of persisting antigen, which is known to remain localized in the lymph node cortex for a similar time period after injection (17). Evidence from the literature indicates that the antigen localized in the lymphoid follicles is usually present as antigen-antibody complexes and remains fixed (18, 19). It is, therefore, unlikely that the slow, extended increase in the ability of the lymph node on the right side to give a secondary response in vitro is due to small amounts of antigen entering this node, particularly since circulating antibody is present in the animals during this period. Probably this acquisition of immunological memory is due to circulating memory cells such as were clearly shown to exist by Gowans and coworkers (7, 8). These cells are most likely produced in the lymph nodes containing antigen and are slowly distributed to lymphoid tissue throughout the body. The continued increase in ability to give a secondary response, as well as the maintenance of a difference between left and right nodes, suggests that, for a long time after immunization with a single dose of soluble antigen, immunologic memory has a component consisting of slowly proliferating (20), sessile (9) cells.

In some cases, very low antibody production was also seen in the absence of added antigen, but this occurred only in cultures of lymph node tissue from the injected side. As was also seen previously (21), this response is similar to the secondary response in vitro in that peak titers occur on days 6 to 9 of the culture period. It has been suggested that this is due to antigen remaining in the node at the time of culture, which is in agreement with the observation that cultures from the contralateral lymph nodes fail to show this background response.

The fact that a much higher proliferative response is seen on day 7 than at any other time after sensitization suggests strongly that peak numbers of responsive cells are present at this time. However, the steady increase in the secondary immune response in vitro with time after priming would support the reverse. Even in nodes removed late after priming, at least some cells must be able to proliferate in response to antigen, since the ability of such lymph node fragments to form antibody during the secondary immune response in vitro has been shown previously to depend on proliferation of the cells during the first few days of culture (22). Moreover, a slight but definite proliferative response in

cell suspensions could be demonstrated when ^{3}H-thymidine addition was delayed until day 4 of the culture period.

Two possible explanations for this apparent discrepancy come to mind. The first is that two entirely different cell populations are involved. The early proliferative response could be due to delayed type sensitive cells, and thus represent the "thymus-dependent" cell population which has been suggested to proliferate in response to antigen without giving rise to antibody (23). However, the time of appearance of this proliferative response is a few days later than the peak of thymus-dependent cell proliferation noted by others (24). The time agrees much better with the peak of proliferation in germinal centers, as was also noted in previous studies on the spleen (3). This does not suggest a relationship to delayed type sensitivity, since germinal centers are thymus-independent (25, 26), and are much more clearly related to the humoral antibody response (27, 28) particularly of the 7S anamnestic type (1, 29, 30). The other possible explanation is that the early proliferative response indeed occurs in cells carrying immunological memory for antibody production, but that the majority of these cells are still immature. These cells might produce antibody of such low affinity upon challenge that either their antibody response remains below detectable levels, or else they are unable to retain antigen sufficiently long to allow their progeny to continue the process of differentiation into antibody-producing cells. The marked decrease in proliferation seen over the first few days in vitro with cells reexposed early after priming as opposed to cells taken later, would be in agreement with such a possibility. Recent studies in this laboratory have confirmed this finding with a different antigen.[2] An additional factor could be that the proliferative response in cells which are already actively dividing follows a course different from the one induced in resting cells.

The immature memory cells formed in the draining lymph node are probably dispersed throughout the rest of the lymphoid tissue, resulting in a decrease in the number of responsive cells which remain locally. Late after priming they may give a slower proliferative response because the majority of the cells are no longer in division at the time of explantation. These cells, however, have now differentiated into stable memory cells, capable of producing antibody of increased affinity (31). Thus, the ability to give a high, sustained secondary antibody response upon challenge in vitro may be limited to those cells able to bind antigen firmly, and capable of capturing antigen over a relatively extended time period. The progeny of these cells give rise to colonies of antibody producing cells during the 1 to 2 wk period of the secondary response in vitro.

SUMMARY

Popliteal lymph nodes were obtained from rabbits 4 days to 9 months after a primary injection of diphtheria toxoid or bovine γ-globulin into the footpad.

[2] Siskind, G. W., and G. J. Thorbecke. 1968. Unpublished observations.

The ability of cells from these nodes to proliferate upon reexposure to antigen in vitro was compared to the height of the secondary response produced by tissue fragments. In addition, a comparison was made between the responsiveness of draining and contralateral lymph nodes.

While the secondary antibody response in vitro increased markedly with the time after immunization at which the lymph nodes were taken from the animals, the degree of proliferation induced by antigen was highest with cells from lymph nodes taken early after priming (peak day 7) and was very much lower with lymph node cells taken longer than 3 wk after priming. This striking difference between these two responses has been discussed.

Contralateral lymph nodes were much inferior to draining nodes in their ability to give a secondary antibody response in vitro, and never gave a detectable proliferative response. This difference became less marked with time after priming, but could still be demonstrated after 4 months. These results suggest a concentration of primed cells in the lymphoid tissue draining the site of injection, and a slow release of these cells into the circulation, to be distributed to the remaining lymphoid tissue.

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