

IMMUNE RESPONSES IN VITRO

I. CELLULAR REQUIREMENTS FOR THE IMMUNE RESPONSE BY NONPRIMED AND PRIMED SPLEEN CELLS IN VITRO

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(Received for publication 24 March 1969)

Successful primary immunization in vitro against a variety of antigens has been reported, using cell suspension and tissue fragment cultures from the lymphoid organs of several species (1-17). Analysis of the primary type of response against heterologous erythrocytes in spleen cell suspension systems has been particularly rewarding (12-14, 17-23). This response closely parallels the in vivo primary response qualitatively and quantitatively. Essentially all the antibody-forming cells result from cell division (18); different cell populations respond to two non-cross-reacting antigens (17-20). The induction of the primary type of response in vitro requires both macrophages and lymphoid cells (21, 22). The lymphoid cell fraction is thought to contain at least two types of cells in critical numbers which interact with the macrophage during the immune response in vitro. Most antibody-forming cells in the cultures are in clusters; these cell clusters may be required for the development of this type of response (20) or may be the result of cell division (13, 23).

The experiments to be reported here used a spleen cell suspension culture system (12) and a procedure to separate macrophages from lymphoid cells (21, 22). These experiments were designed to investigate some of the cellular requirements for the plaque-forming cell (PFC) response of immunized and non-immunized spleen cells in vitro and asked: (a) Are macrophages required for the PFC response of spleen cells from immunized mice? (b) Can macrophages from the spleens of immunized mice enhance the PFC response of spleen cells from nonimmunized mice? (c) Can lymphoid cells from immunized mice enhance the PFC response of spleen cells from nonimmunized mice? The results will be discussed in terms of the function of the macrophage in the immune response and the development of immunological memory in splenic lymphoid cells.

Materials and Methods

Mice.—3-5 month old BALB/cAnN male mice, from the National Institutes of Health Rodent and Rabbit Production Section, were maintained on water and Purina laboratory chow ad libitum.

Antigens.—Erythrocytes from one sheep and one burro, maintained at the National Institutes of Health Ungulate Division, were collected under sterile conditions and stored in Alsever's solution at 4°C for up to 14 days. Erythrocytes were washed three times by centrifugation with 20–40 volumes of sterile Hanks' balanced salt solution (H-BSS) the day before use in the hemolytic plaque assay or addition to cultures. The erythrocyte dose (approximately 10^7 erythrocytes) was 0.05 ml of a 1.25% suspension in H-BSS of sheep or burro erythrocytes. In doubly immunized cultures, 0.05 ml of each kind of erythrocytes was added.

Preparation of Mice and Spleen Cell Cultures.—Mice were immunized (primed) by intravenous injection of 0.5 ml of the appropriate sterile 1.25% erythrocyte suspension in H-BSS at varying times before the preparation of the spleen cell suspension.

The procedures and reagents used were similar to those described by Mishell and Dutton (12). Spleen cell suspensions were prepared under sterile conditions by gently tearing apart the spleens in cold H-BSS lacking bicarbonate and allowing the tissue fragments to sediment briefly by gravity. The resulting cell suspension was centrifuged for 10 min at 600 *g* at 4°C. The supernatant fluid was discarded, and the sedimented cells were resuspended to $1.0\text{--}2.0 \times 10^7$ cells/ml in Eagle's minimum essential medium, monolayer type, with H-BSS, supplemented with sodium pyruvate, nonessential amino acids, and glutamine according to Eagle (24) and 10% fetal bovine serum. All tissue culture reagents were obtained from Microbiological Associates, Bethesda, Md.; fetal bovine serum was obtained from Colorado Serum Company, Denver, Col., lot 244. A portion of each cell suspension was removed for nucleated cell and plaque-forming cell determinations.

Culture Conditions.—Plastic tissue culture grade Petri dishes (35 × 10 mm) (Falcon Plastics, Los Angeles, Calif., No. 3005), six per experimental group, containing 1 ml of the cell suspension with or without erythrocyte antigen, were incubated in a water-saturated atmosphere of 7% oxygen, 10% carbon dioxide, and 83% nitrogen (Air Products and Chemicals, Inc., Washington, D.C.) at 37°C in a large Lucite box on a rocker platform (Bellco, Vineland, N.J.) oscillating at 7 cycle/min. Each culture was supplemented daily with 0.05 ml of fetal bovine serum and 0.05 ml of a nutritional mixture prepared by supplementing H-BSS with 0.01 g/ml dextrose and glutamine and amino acids in twice the concentration of that found in minimum essential medium (24).

Cell Separation Procedure.—This technique closely followed that described by Mosier (21). The capability of macrophages to adhere to the plastic culture dishes was used to separate primed and nonprimed spleen cell suspensions into "macrophage-rich" and "lymphoid cell-rich" populations. Spleen cells were incubated for 60 min without antigen; nonadhering cells were gently aspirated and transferred to new Petri dishes. After three similar incubation periods, nonadhering cells were pooled, centrifuged, and resuspended in one-half the original volume of fresh culture medium. Cells adhering to the dishes after the first incubation period were washed gently three times with cold H-BSS to remove loosely adhering cells. Only the adhering cells (macrophage-rich population) obtained after the first culture period and the nonadhering cells (lymphoid cell-rich population) obtained after the third culture period were used. Erythrocytes were added to cultures at the completion of the cell separation procedure.

Nucleated cells in each cell population were counted; the macrophage-rich population was counted after elution of the cells from the plastic with culture medium containing 30 mM ethylenediaminetetraacetate. Direct and indirect PFC in all cell populations were also determined; PFC in the macrophage-rich population were enumerated by adding the PFC assay reagents directly to the culture dishes. Phagocytic cells were enumerated by incubating aliquots of the separated cell populations and unseparated spleen cell suspensions with titanium dioxide [Ti-Pure R-900 (0.23 μ), E. I. du Pont de Nemours and Co., Inc., Wilmington, Del.]

(0.01% w/v). After 24 hr at 37°C in the atmosphere and with agitation conditions previously described, all cells were eluted with EDTA, and smears were prepared and stained with Geimsa. The percentage of phagocytic cells in each culture was then determined.

Hemolytic Plaque Assay.—The culture dishes in each group were divided into two subgroups. The cells from dishes in each subgroup were freed from the plastic by scraping with a plastic policeman, aspirated, pooled, and centrifuged. The sedimented cells were resuspended in cold H-BSS lacking bicarbonate at dilutions expected to give 30–300 plaques/slide.

The number of plaque-forming cells was determined by the Jerne hemolytic plaque technique (25), modified for use with glass microscope slides (12, 26). Agarose (L'Industrie Biologique Francaise S.A., Gennevilliers, France), 0.5% in Tris-buffered Eagle's minimal essential medium without serum, 0.40 ml; erythrocytes, 7.5%, 0.05 ml; and the culture cell suspension, 0.1 ml, were gently but thoroughly mixed at 44°C in 10 × 75 mm tubes and poured onto microscope slides previously coated with 0.1% agarose in water.

Duplicate slides for the enumeration of indirect and direct PFC were prepared from each subgroup. Slides for assay of direct PFC (cells presumably releasing IgM antibody) were incubated for 1 hr at 37°C in a humid atmosphere; guinea pig complement (Bioquest, Baltimore, Md.), diluted 1:10 in phosphate-buffered 0.85% saline, pH 7.4, was added, and incubation was continued for 1 hr. Slides for assay of indirect PFC (27) (cells presumably releasing IgG antibody) were incubated at 37°C for 1 hr before the addition of heat-inactivated rabbit anti-BALB/cAnN 7S globulin adsorbed with sheep and burro erythrocytes and diluted to give maximum inhibition of direct plaques and maximum facilitation of indirect plaques. After 1 hr of incubation, the antiglobulin was removed by gentle washing, complement was added, and incubation was continued for 1 hr. PFC were counted at 7× magnification with indirect illumination.

The PFC per 10⁶ recovered cells in each group were determined from the average of the two subgroups. The direct PFC were calculated from slides developed only with complement, since the number of PFC developed was not significantly changed by incubation with normal rabbit serum at the same dilution as the rabbit antiglobulin. The number of indirect PFC was calculated by subtracting from the total plaques on an indirect assay slide the number of direct plaques that developed in the presence of the antiglobulin and converting the resulting number to indirect PFC per 10⁶ recovered cells.

RESULTS

Plaque-Forming Cell Responses by Nonprimed Spleen Cells In Vitro.—The direct and indirect PFC responses were determined daily in cultures of spleen cells from nonprimed mice incubated with or without sheep or burro erythrocytes. After a 1 day lag period the number of direct PFC increased approximately exponentially from background levels (2/10⁶ spleen cells) to about 1000/10⁶ recovered cells on day 4 in cultures stimulated with sheep or burro erythrocytes. The number of direct PFC did not change markedly on day 5, but decreased thereafter (Fig. 1). The average 4 day direct PFC response to sheep erythrocytes in 34 experiments was 874; the average 4 day direct PFC response to burro erythrocytes in 15 experiments was 868. In terms of magnitude, variability, and kinetics, these in vitro primary immune responses to heterologous erythrocytes were similar to those reported by others (12, 14, 21).

Indirect PFC were not detected in significant numbers after stimulation with either sheep or burro erythrocytes, despite the use of several rabbit anti-BALB/

cAnN 7S globulin sera for facilitation. An increase in the number of direct PFC against sheep erythrocytes was always noted in unstimulated cultures, but never exceeded 10% of the response in stimulated cultures. A similar increase in direct PFC against burro erythrocytes was not detected. Indirect PFC were not detected in the unstimulated cultures.

Spleen cell suspensions from nonprimed mice were separated into macrophage-rich and lymphoid cell-rich populations and were stimulated with sheep

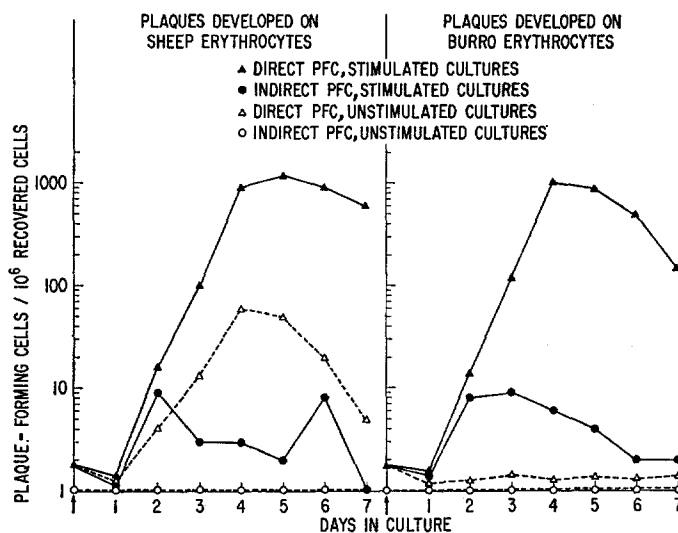


FIG. 1. Kinetics of direct and indirect plaque-forming cell responses in cultures of nonprimed mouse spleen cell suspensions incubated with and without sheep erythrocytes (left) burro erythrocytes (right). Comparable numbers of cells were recovered from stimulated and unstimulated cultures each day. Results are from a representative experiment.

or burro erythrocytes (Table I). The direct PFC response to sheep or burro erythrocytes in the recombined macrophage-rich + lymphoid cell-rich populations was about 83% of the response in unseparated, nonprimed spleen cell cultures (control response). In contrast, the responses of the separated lymphoid cell-rich or macrophage-rich populations to sheep erythrocytes never exceeded 2.8% of the control response. The average responses of these separated cell populations in unstimulated cultures (not shown in Table I) were not significantly different from their responses in stimulated cultures. The response of the lymphoid cell-rich or macrophage-rich population to burro erythrocytes was even less, never exceeding 0.3% of the control response. Daily assays of cultures of recombined cell populations showed that the kinetics of this PFC response was not significantly different from the kinetics of the response of unseparated, nonprimed spleen cells shown in Fig. 1. These data confirm the

observation that both "macrophages" and "lymphoid cells" are essential for the development of a primary type of response *in vitro* (21).

Equivalent PFC responses were obtained in cultures of the recombined cell populations when the erythrocyte antigen was present for 4 days or when only the macrophage-rich population had been exposed to the erythrocyte antigen for 30 min, after which the free erythrocytes were removed and the lymphoid cell-rich population was added. This suggests that the macrophages, after brief

TABLE I
Plaque-Forming Cell Response of Unseparated Spleen Cells and Separated Cell Populations from Nonprimed Mice to Sheep and Burro Erythrocytes

| Cell population | PFC/10 ⁶ recovered cells to SRBC*, † | % of unseparated spleen cell response to SRBC§ | PFC/10 ⁶ recovered cells to BRBC*, † | % of unseparated spleen cell responses to BRBC§ |
|---|--|--|--|---|
| Unseparated spleen cells + SRBC or BRBC | 856 | 100 (Control response) | 913 | 100 (Control response) |
| Macrophage-rich + lymphoid cell-rich + SRBC or BRBC | 704 | 83.6 (77.3-89.3) | 763 | 83.1 (80.2-84.3) |
| Lymphoid cell-rich + SRBC or BRBC | 11 | 1.22 (0.42-2.67) | 2 | 0.13 (0.03-0.25) |
| Macrophage-rich + SRBC or BRBC | 13 | 1.37 (0.67-2.80) | 2 | 0.13 (0.05-0.29) |

* SRBC = sheep erythrocytes; BRBC = burro erythrocytes.

† Plaque-forming cell response to the immunizing erythrocyte from a representative experiment.

§ The response of the cell population is expressed as a percentage of the unseparated spleen cell response (control response). The average of five experiments is shown; the range of the percentage of the control response is in parentheses.

exposure to antigen, obtained the ability to stimulate the lymphoid cell-rich population to develop a primary PFC response *in vitro*.

Plaque-Forming Cell Responses by Primed Spleen Cells In Vitro.—The direct and indirect PFC responses were determined daily for 7 days in cultures of spleen cells prepared from mice primed with sheep erythrocytes 1, 3, 4, 5, 7, 10, 20, and 28 days before and incubated with or without sheep erythrocytes.

In stimulated and unstimulated cultures, the number of PFC did not change markedly on the first day of culture. The direct and indirect PFC then increased almost exponentially to a maximum on the fourth or fifth day of culture, regardless of how long after *in vivo* priming the culture was initiated (Fig. 2). The maximum direct and indirect PFC responses in unstimulated cultures never exceeded 15% of the corresponding PFC response in stimulated cultures initiated at the same interval after priming. In experiments using separated cell

populations from primed animals, which are described elsewhere in this paper, PFC responses in unstimulated cultures never exceeded 15% of the response in stimulated cultures.

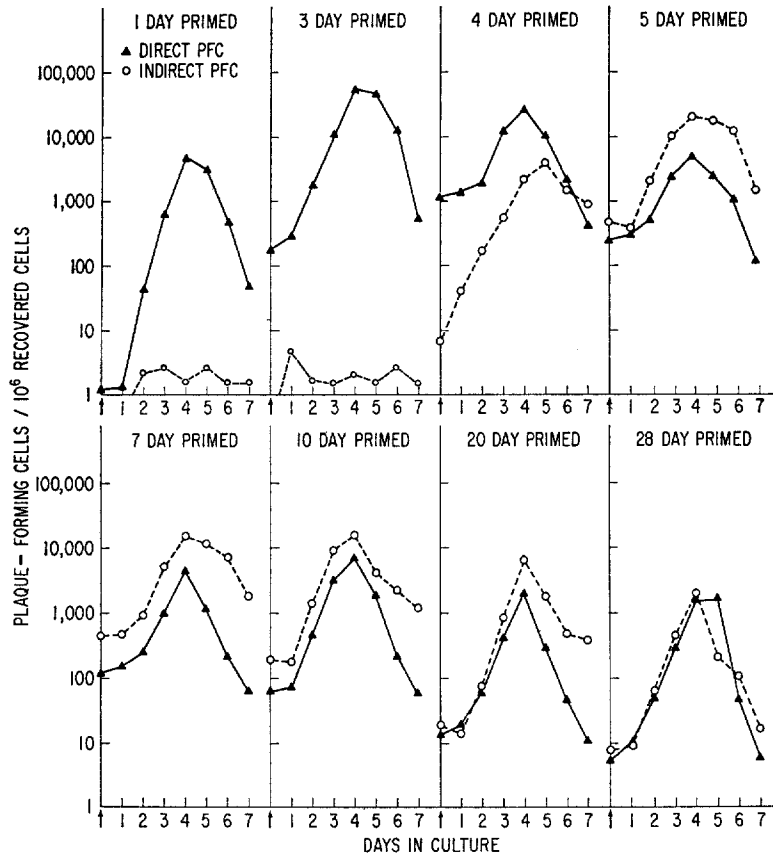


FIG. 2. Kinetics of direct and indirect plaque-forming cell responses in cultures of mouse spleen cell suspensions from mice primed 1, 3, 4, 5, 7, 10, 20, and 28 days before the initiation of cultures. Results are from a representative experiment; only data from stimulated cultures are shown.

In cultures of unseparated spleen cells from mice 1 and 3 days after priming, significant numbers of indirect PFC were not detected at the time the cultures were initiated, or at any time thereafter. However, if indirect PFC were detected at the time the culture was initiated (4 or more days after priming), these PFC increased exponentially to a maximum on the fourth or fifth day of culture. In subsequent experiments, in which responses of separated cell populations and unseparated spleen cells were compared, a single assay after 4 days of culture was used.

At several intervals after priming, spleen cell suspensions were prepared and diluted to contain from 5×10^7 to 1×10^6 cells/culture and were incubated with 10^7 sheep erythrocytes for 4 days. Maximum PFC responses were found in cultures containing $1-2.5 \times 10^7$ spleen cells. At higher or lower cell concentrations, the response was markedly decreased.

Plaque-Forming Cell Responses of Separated Cell Populations from Primed Mice In Vitro.—The foregoing experiments clearly demonstrated the requirement for cells from the macrophage-rich population of nonprimed spleens to develop a primary type of PFC response to sheep erythrocytes in vitro. The

TABLE II
*Cell Populations Used to Study Cellular Requirements for an In Vitro Response of Primed Spleen Cells**

| | | |
|--|---|--------------------|
| 1. Unseparated primed spleen cells | ± | Sheep erythrocytes |
| 2. Macrophage-rich population | ± | Sheep erythrocytes |
| 3. Lymphoid cell-rich population | ± | Sheep erythrocytes |
| 4. Macrophage-rich + lymphoid cell-rich populations (recombined) | ± | Sheep erythrocytes |
| 5. Macrophage-rich population + nonprimed spleen cells | ± | Sheep erythrocytes |
| 6. Lymphoid cell-rich population + nonprimed spleen cells | ± | Sheep erythrocytes |
| 7. Nonprimed spleen cells | ± | Sheep erythrocytes |

* Macrophage-rich and lymphoid cell-rich populations were derived from primed spleen cell suspensions by procedures described in the text.

following series of experiments were designed to investigate some of the cellular requirements for the in vitro PFC responses of primed spleen cells.

Mice were primed with sheep erythrocytes 1, 3, 5, 7, 10, 20, and 28 days before the preparation of spleen cell suspensions and separated cell populations. The experimental cell populations tested are shown in Table II. Assays for direct and indirect PFC were done after 4 days of culture.

Approximately 10% of the spleen cells in suspension from primed mice adhered to the plastic dishes during the first hour of culture; about 98% of these cells engulfed titanium dioxide, and only 2% of the PFC per 10^6 primed spleen cells adhered to the dishes (Table III). In contrast, only 2% of the nonadhering cells were capable of phagocytosis, and 95% of the PFC per 10^6 primed spleen cells were recovered in this fraction. About 45% of the cells in the original suspension were recovered in the lymphoid cell-rich population; hence, it was resuspended in one-half the original volume of culture medium to restore the cell concentration to $1.0-2.0 \times 10^7$ cells/ml.

The number of direct and indirect PFC detected in cultures of the experimental cell populations was expressed as a percentage of the direct and indirect PFC responses in the primed, unseparated spleen cell cultures restimulated in vitro (direct and indirect PFC control responses).

The direct PFC response in the recombined macrophage-rich + lymphoid cell-rich populations restimulated *in vitro* decreased gradually from 86% of the direct PFC control response in cultures initiated 1 day after priming to 70% of the direct PFC control response in cultures initiated 28 days after priming (Fig. 3). An indirect PFC response was not detected in cultures of these recombined cell populations until 5 days had elapsed between priming and the initiation of the culture. In cultures established 5 days after priming, the indirect PFC response in the recombined cell populations was 80% of the indirect PFC control response. A decrease in the indirect PFC response with

TABLE III
*Some Properties of Cell Populations from Primed Mouse Spleens**

| Population | Average per cent of PFC/10 ⁶ cells | Average per cent phagocytic cells | Average per cent of total cells |
|---------------------|---|-----------------------------------|---------------------------------|
| Primed spleen cells | 100 | 12.6 (9.32-15.3) | 100 |
| Macrophage-rich | 1.6 (0.0-3.0) | 98.3 (97.8-99.2) | 9.7 (8.35-12.7) |
| Lymphoid cell-rich | 95.3 (90.0-115.2) | 1.91 (1.06-2.37) | 45.3 (43.8-46.6) |

* Data from 34 spleen cell suspensions prepared 1, 3, 5, 7, 10, 20, and 28 days after priming. Numbers in parentheses indicate the range of each of the determinations. Preparation of cell populations and determination of plaque-forming cells and phagocytic cells are described in the text.

time similar to that in the direct PFC response was observed in cultures of the recombined cell populations.

The direct PFC response in cultures of the separated lymphoid cell-rich population from spleens 1 day after priming was 17% of the direct PFC control response. The ability of this population to respond *in vitro* steadily increased with time after priming, and the response was not significantly different from the response in cultures of the recombined cell populations 20 and 28 days after priming (Fig. 3).

1 year after *in vivo* priming, the direct PFC response in cultures of the separated lymphoid cell-rich population was 27% of the direct PFC control response.

An indirect PFC response first developed in cultures of the separated lymphoid cell-rich population established 5 days after priming, when it was 36% of the indirect PFC control response. The indirect PFC response in cultures of the separated lymphoid cell-rich population established 7 or more days after priming was not significantly different from the response in cultures of the recombined cell populations.

1 year after *in vivo* priming, the indirect PFC response in cultures of the

separated lymphoid cell-rich population was 59% of the indirect PFC control response. The direct and indirect PFC responses in cultures of the macrophage-rich population never exceeded 4% of the control response (Fig. 3).

The macrophage-rich population from spleens 1 day after priming, when added to cultures of unseparated, nonprimed spleen cells, enhanced the direct PFC response 3-fold over the response of unseparated, nonprimed spleen cells

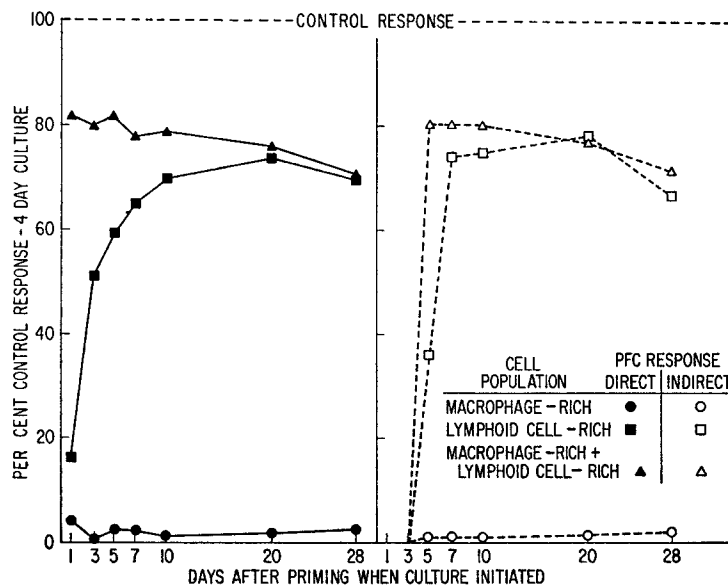


FIG. 3. Plaque-forming cell responses of separated spleen cell populations from primed mice, expressed as a percentage of the response in unseparated spleen cell cultures (control response). The symbols represent the average of three to five experiments at each interval after priming; only data from stimulated cultures are shown.

alone. However, the macrophage-rich population from spleens 3 or more days after priming had no enhancing effect on the response of nonprimed spleen cells.

The macrophage-rich population from spleens of primed mice did not stimulate the development of an indirect PFC response when added to cultures of nonprimed spleen cells.

The pattern of direct and indirect PFC responses in cultures of the primed, separated lymphoid cell-rich population mixed with unseparated, nonprimed spleen cells was similar to the pattern of responses in cultures of recombined macrophage-rich + lymphoid cell-rich populations from primed mice. The response of the separated, primed lymphoid cell-rich population was enhanced by unseparated, nonprimed spleen cell suspensions in the first few days after priming.

PFC Responses of Separated Cell Populations from Primed Mice to a Second,

Non-cross-reacting Erythrocyte.—The preceding experiments indicated that primed, separated lymphoid cells can respond to the priming erythrocyte in vitro in a macrophage-poor environment. The following experiments were performed to determine whether the 2% phagocytic cells in the primed, separated lymphoid cell-rich population could support a primary type of response to a non-cross-reacting erythrocyte.

Mice were primed with burro or sheep erythrocytes 1, 3, 5, 7, 10, 20, and 28 days before the preparation of spleen cell suspensions and separated cell populations. The first four experimental groups in Table II were included in this protocol. Spleen cell suspensions from mice primed with sheep or burro erythrocytes were prepared on the same day; 16 culture dishes in each group were divided into four subgroups. Sheep erythrocytes were added to the dishes in one subgroup; burro erythrocytes, to the dishes in the second subgroup; both erythrocytes, to the dishes in the third subgroup; and no erythrocytes were added to the dishes in the fourth subgroup. For each separated cell population and unseparated spleen cell suspension, therefore, the response of primed cells to restimulation in vitro and the primary response in vitro to a second erythrocyte antigen were determined. Assays for direct and indirect PFC responses to sheep and burro erythrocytes were done after 4 days of culture.

The patterns of the direct and indirect PFC responses in cultures of the separated cell populations stimulated in vitro with the priming erythrocyte did not differ significantly from the responses shown in Fig. 3.

The cultures of primed, unseparated spleen cells stimulated in vitro with the erythrocyte not used for priming had only direct PFC responses in the range of the primary response to that erythrocyte in cultures of nonprimed spleen cells, as shown in Table I and Fig. 1. These responses are referred to as the control primary response. Cultures of primed, recombined macrophage-rich + lymphoid cell-rich populations stimulated in vitro with the erythrocyte not used for priming had only direct PFC responses of about 80% of the control primary response (Fig. 4). In contrast, cultures of primed, separated lymphoid cell-rich populations stimulated with the erythrocyte not used for priming had responses not exceeding 4.5% of the control primary response. Cultures of separated macrophage-rich populations stimulated with the erythrocyte not used for priming had similar low responses.

Thus, when primed spleen cell suspensions are separated into macrophage-rich and lymphoid cell-rich populations and stimulated in vitro with a second erythrocyte, the response observed is a typical primary type of response, consisting only of direct PFC and requiring both macrophages and lymphoid cells for its development. Cultures stimulated with both erythrocytes had a typical "primed" response to the priming erythrocyte, depending on the time after priming and on the cell population. Simultaneous responses to the erythrocyte not used for priming were similar to the primary type of responses shown in

Table I and in Fig. 4. The foregoing experiments demonstrated that the 2% phagocytic cells contaminating separated lymphoid cells from primed mice were unable to support a primary type of response to a second, non-cross-reacting erythrocyte.

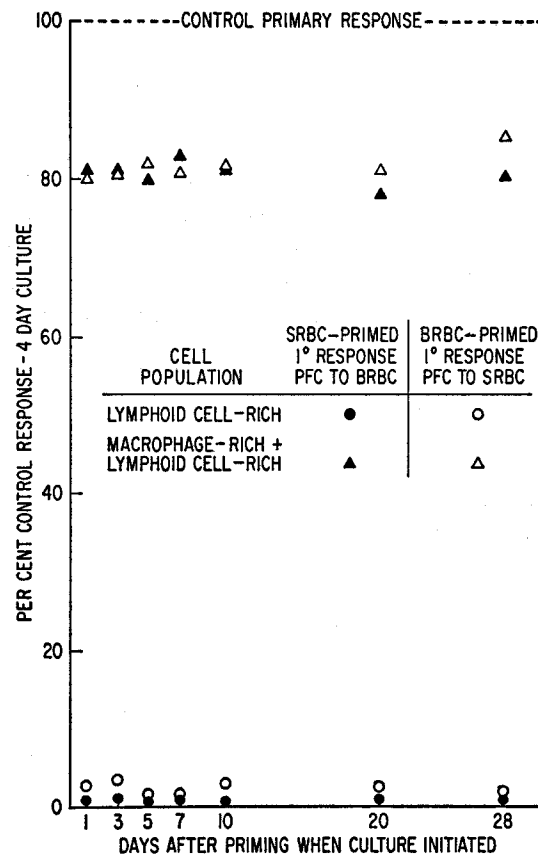


FIG. 4. Plaque-forming cell response of separated spleen cell populations from primed mice to a second, non-cross-reacting erythrocyte, expressed as a percentage of the response in un-separated spleen cell cultures (control primary response). See the text for experimental details. The symbols represent the average of two experiments at each interval after priming. SRBC = sheep erythrocytes; BRBC = burro erythrocytes.

Effect of Macrophage-Rich Population Cells on the Response of Separated Lymphoid Cells from Primed Mice.—The response in cultures of recombined macrophage-rich + lymphoid cell-rich populations in the first few days after priming was always greater than the response in the separated lymphoid cell populations. Clearly, the macrophage-rich population performed some function

which enhanced the response of the lymphoid cell population. If the few phagocytic cells contaminating the separated lymphoid cell population were performing a function essential for the response by the lymphoid cells, cells from the macrophage-rich population might enhance the response by increments as the number of macrophages was increased.

Mice were primed with sheep erythrocytes 1, 3, 5, and 7 days before the preparation of spleen cell suspensions and separated cell populations. The separated lymphoid cell-rich population was prepared as usual. Since about 10% of the cells in spleen cell suspensions attach to plastic (Table III), culture dishes containing 1×10^6 , 5×10^5 , 1×10^5 , 5×10^4 , and 1×10^4 macrophages were prepared from spleen cell suspensions containing 1×10^7 , 5×10^6 , 1×10^6 , 5×10^5 , and 1×10^5 cells/ml. Cells were eluted from some of the dishes with culture medium containing EDTA to confirm the numbers of macrophages in the dishes. To each dilution of macrophages, 1×10^7 separated lymphoid cells and 10^7 sheep erythrocytes were added, and the cultures were assayed for direct PFC after 4 days.

The PFC responses in Table IV are expressed both as PFC per 10^6 recovered cells and as a percentage of the response of unseparated spleen cells from primed mice. The responses in cultures of both the separated lymphoid cell-rich populations and recombined cell populations (1×10^6 macrophages + 1×10^7 lymphoid cells) were similar to the responses shown in Fig. 3 at the same intervals after priming. Of significance was the fact that reducing the number of macrophages in the system to 5×10^5 essentially reduced the response to that of the separated lymphoid cells to which no macrophages had been added. Approximately 1×10^5 phagocytic cells contaminated the separated lymphoid cell populations; addition of this number of macrophages to the system did not increase the response above that of the separated lymphoid cells alone. This experiment indicated that between 5×10^5 and 1×10^6 macrophages were required to increase the response of separated lymphoid cells from primed mice above the response of these cells in a macrophage-poor culture.

Effect of Erythrocyte Dose on the PFC Response of Spleen Cells and Separated Lymphoid Cells from Primed Mice In Vitro.—The antigen dose required to stimulate a maximum response in primed animals is usually less than the antigen dose required to stimulate a maximum primary response. In the preceding experiments, the erythrocyte dose was 10^7 erythrocytes/culture, a level which probably presented excess antigen to the system. The following experiment investigated the effect of decreasing the erythrocyte dose on the response of primed and nonprimed spleen cells in vitro.

Mice were primed with sheep erythrocytes 1, 3, 5, and 7 days before the preparation of spleen cell suspensions and separated cell populations. Similar cell suspensions were prepared from nonprimed mice. Cultures of unseparated spleen

cells and separated lymphoid cells were incubated with 10^7 , 10^6 , 10^5 , 10^4 , or 10^3 sheep erythrocytes and assayed for direct PFC after 4 days in culture.

The response in cultures of nonprimed spleen cells was maximum at 10^7

TABLE IV

Effect of Macrophage-Rich Population Cells on the Response of Separated Lymphoid Cells from Primed Mice

| Cell population* | Direct PFC/ 10^6 recovered cells after 4 days of culture; cultures initiated on the following days after priming: | | | |
|---|--|---------------|--------------|--------------|
| | 1 | 3 | 5 | 7 |
| 1×10^7 unseparated primed spleen cells | 3,457 (100)† | 70,330 (100) | 5,299 (100) | 8,383 (100) |
| 1×10^7 separated primed lymphoid cells | 795 (23.0) | 33,021 (47.0) | 3,507 (66.1) | 5,691 (67.9) |
| 1×10^6 macrophages + 1×10^7 lymphoid cells | 2,650 (76.7) | 49,358 (70.2) | 4,002 (75.5) | 6,357 (75.8) |
| 5×10^5 macrophages + 1×10^7 lymphoid cells | 927 (26.8) | 40,982 (58.3) | 3,155 (59.5) | 5,486 (65.4) |
| 1×10^5 macrophages‡ + 1×10^7 lymphoid cells | 729 (21.1) | 28,583 (40.6) | 3,413 (64.4) | 5,442 (64.9) |
| 5×10^4 macrophages + 1×10^7 lymphoid cells | 753 (21.8) | 28,710 (40.8) | 3,668 (69.2) | 5,700 (68.0) |
| 1×10^4 macrophages + 1×10^7 lymphoid cells | 776 (22.4) | 29,650 (42.2) | 3,522 (66.5) | 5,522 (65.9) |

* All cultures were incubated with 10^7 sheep erythrocytes.

† The response of the cell populations at varying ratios of macrophages and lymphoid cells is expressed as a percentage of the response in cultures of unseparated spleen cells from primed mice and is given in parentheses. Data are from a reproducible experiment.

‡ The numbers of macrophages added to these cultures approximates the 2% phagocytic cells which contaminated the separated lymphoid cells.

erythrocytes/culture; at lower erythrocyte doses the response was markedly reduced (Table V).

In contrast, the response in cultures of unseparated spleen cells from primed mice was usually maximum at 10^6 erythrocytes/culture. The responses at 10^5 erythrocytes/culture were reduced, but not to the degree that a similar decrease in erythrocyte dose reduced the response in nonprimed spleen cell cultures.

The responses in cultures of separated lymphoid cells from primed mice showed a decrease similar to that in cultures of primed, unseparated spleen

cells with decreasing erythrocyte dose. However, the response in the separated lymphoid cells at each erythrocyte dose was about the same fraction of the response of unseparated spleen cells at the same erythrocyte dose.

These experiments indicated that the primed spleen cells were more sensitive to antigen than nonprimed spleen cells, since the maximum response could be

TABLE V
Effect of Erythrocyte Dose on the Direct PFC Responses of Unseparated Spleen Cells and Separated Lymphoid Cells from Nonprimed and Primed mice

| Cell population | Sheep erythrocyte dose | PFC/10 ⁶ recovered cells*; cultures initiated on following days after priming: | | | | |
|--------------------------|------------------------|--|-------------|---------------|--------------|--------------|
| | | Nonprimed spleen cells | 1 | 3 | 5 | 7 |
| Unseparated spleen cells | 10 ⁷ | 1,070 | 3,861 | 44,060 | 6,032 | 7,390 |
| | 10 ⁶ | 666 | 4,195 | 40,140 | 8,701 | 6,892 |
| | 10 ⁵ | 206 | 2,393 | 13,688 | 5,860 | 4,793 |
| | 10 ⁴ | 110 | 1,572 | 3,958 | 2,298 | 2,941 |
| | 10 ³ | 46 | 583 | 1,369 | 770 | 1,030 |
| | 0 | 35 | 229 | 976 | 404 | 611 |
| Separated lymphoid cells | 10 ⁷ | 7 (0.6) | 832 (21.5)† | 25,304 (57.4) | 3,496 (58.0) | 5,213 (70.5) |
| | 10 ⁶ | 7 (1.1) | 743 (17.7) | 19,516 (48.6) | 5,252 (60.4) | 4,574 (66.4) |
| | 10 ⁵ | 6 (2.9) | 518 (21.6) | 7,018 (51.3) | 3,280 (56.0) | 3,219 (67.2) |
| | 10 ⁴ | 5 (4.5) | 466 (29.6) | 2,008 (50.7) | 1,292 (56.2) | 1,910 (64.9) |
| | 10 ³ | 5 (10.9) | 113 (19.4) | 725 (53.0) | 421 (54.7) | 710 (68.9) |
| | 0 | 7 (20.0) | 25 (10.9) | 633 (64.9) | 215 (53.2) | 408 (66.8) |

* Data shown are from a representative, reproducible experiment assayed after 4 days of culture. Cell recoveries in the experimental groups were comparable.

† The responses in the cultures of separated lymphoid cells are expressed as a percentage of the responses of unseparated spleen cells at the same sheep erythrocyte dose and are given in parentheses.

attained at a lower antigen dose. Also, the constant fraction of the response in the separated lymphoid cell cultures suggests that these cells were as sensitive to antigen as unseparated spleen cell suspensions.

DISCUSSION

Lymphoid cells after experimental exposure to antigen differ in immunological reactivity from experimentally "virgin" lymphoid cells. Separated lymphoid cells from primed mice can respond to the priming antigen in vitro in a macrophage-poor culture; separated lymphoid cells from nonprimed mice cannot. Other studies have shown the importance of macrophages for the development of the primary immune response (10, 20-22, 28-33). In the first 7-10 days after

priming, the response in these separated lymphoid cells progressively increases relative to the response in cultures of unseparated spleen cells. This indicates that the response of lymphoid cells from primed mice becomes more independent of whatever function macrophages perform with time after priming. This increasing macrophage independence will be discussed later with reference to the development of immunological memory.

Either the lymphoid cells in the separated lymphoid cell-rich population can respond directly to a second contact with antigen, or the few phagocytic cells in this population provide the effective stimulus for the response by the lymphoid cells.

Direct proof for the first alternative was not possible in these experiments because of the contaminating phagocytic cells in the separated lymphoid cells. Primed lymphoid cells might respond directly to a second contact with antigen (34, 35), the response being triggered by binding of the antigen by antibody-like antigen receptors on the cell surface (36).

The contaminating phagocytic cells may be a different type of cell from those phagocytic cells shown to be necessary for the development of a primary type of response *in vitro*. These contaminating phagocytic cells do not readily and firmly attach to plastic. Although about 2% of the cells in the separated lymphoid cell-rich population were phagocytic, this estimate may be erroneously high, since any cell having titanium dioxide on the cell surface or in the cytoplasm was classified as phagocytic. Furthermore, these cells may be from the lymphocytic series and may have obtained the ability for phagocytosis *in vitro* (37, 38). These phagocytic cells clearly cannot support a primary type of response in the separated lymphoid cells from primed mice to a second erythrocyte (Fig. 4), but this may be due to the reduced number of phagocytic cells in the system. The possibility that these contaminating phagocytic cells may provide the essential stimulus for the response by primed lymphoid cells therefore cannot be eliminated.

Priming an animal changes the immunological status of its lymphoid cells; as the interval after priming increases, lymphoid cells from these animals exhibit an increasing ability to respond in a culture lacking the majority of the type of macrophage necessary for a primary type of response.

That this type of macrophage can enhance the response of the lymphoid cells is clear, since the cultures of the recombined macrophage-rich + lymphoid cell-rich populations always had higher responses than the separated lymphoid cells alone in the first days after priming. However, between 5×10^5 and 1×10^6 macrophages were required to increase the response above that in the separated lymphoid cells alone. Adding macrophages to the separated lymphoid cells in numbers approximating those which contaminated the separated lymphoid cells was without significant effect (Table IV).

As the erythrocyte dose per culture was reduced, the response in both the

unseparated spleen cells and the separated lymphoid cells decreased. However, at each interval after priming, the response in the separated lymphoid cells was the same proportion of the response in the unseparated spleen cells at each erythrocyte dose.

These experiments document some of the results of the changes in immunological status of the lymphoid cells induced by priming. These changes could be of a qualitative or quantitative nature.

A quantitative or proliferative change, stimulated by *in vivo* priming, would provide an increased number of specifically responsive lymphoid cells susceptible to a second antigenic stimulus *in vitro* provided by the same pathway as in the primary type of response. As the number of these cells increases after priming, it is conceivable that fewer macrophages would be required to provide the essential stimulus to the responding lymphoid cells. When macrophages are in excess, as in cultures of unseparated spleen cells, the magnitude of the response would be related to the number of these lymphoid cells present when the culture is initiated. Hence, the greatest response *in vitro* is found in cultures established just prior to the time of maximum *in vivo* response, when these specifically responsive lymphoid cells are present in maximum numbers. As the interval after priming increases, the magnitude of the response decreases because of the disappearance of these lymphoid cells. However, the fraction of the response in the unseparated spleen cell cultures found in cultures of the separated lymphoid cells decreases much more slowly than the absolute magnitude of the response.

These observations suggest, rather, the appearance of qualitatively or physiologically different populations of lymphoid cells which are capable, after a second exposure to antigen in a macrophage-poor culture, of proliferating and differentiating to produce a plaque-forming cell response. The gradual appearance of these populations and their increase in effective size with time after priming suggests they represent the development of "memory cell" pools (39-41).

The memory cell pool for direct PFC appears to develop very rapidly and contains the maximum number of responsive cells 3 days after immunization. Cultures initiated 3 days after priming developed direct PFC responses of about 50,000 PFC/ 10^6 recovered cells after 4 days of culture (Fig. 2); about 50% of this response developed in the separated lymphoid cell cultures (Fig. 3). In terms of absolute numbers, the memory pool for direct PFC decreases thereafter, as judged from the magnitude of the PFC response. However, the fraction of the direct PFC response in cultures of unseparated spleen cells which is found in cultures of the separated lymphoid cells decreases only slightly. In fact, 1 year after priming, sufficient cells remained in the memory pool to produce a response in separated lymphoid cells that was approximately 25% of the response in unseparated spleen cells. This response was 20 times greater than the

response in separated lymphoid cells from nonprimed animals and approximates the size of the memory pool remaining 1 year after priming found by others (42).

When considering the development of a memory pool for indirect PFC, one must remember that only if indirect PFC were detected when the culture was initiated (4 or more days after priming) did a significant indirect PFC response develop *in vitro*. Apparently, certain factors, present only *in vivo*, are required for the stimulation, proliferation, and differentiation of precursors of indirect PFC to the degree that such cells survive and respond to a second antigenic stimulus *in vitro*. This critical developmental period appears to be between 3 and 4 days.

Hence, an indirect PFC response developed only in cultures of separated lymphoid cells established 5 or more days after priming (Fig. 3). In cultures established 7–10 days after priming, the indirect PFC response in the separated lymphoid cells was of the same magnitude as that in recombined cell population cultures. The memory cell pool for indirect PFC appears to develop more rapidly after the initial appearance of an indirect PFC response *in vitro* than does the pool for direct PFC. However, when making this comparison, one must remember the delay in the development of the indirect PFC response as a whole.

The function of the memory cell pool for indirect PFC was quite long-lived; 1 year after priming, sufficient cells remained to develop a response, which was 60% of the unseparated spleen cell response.

As the interval after priming increases, the contribution by cells stimulated by antigen *in vivo* to the *in vitro* response decreases. The largest fraction of the *in vitro* response is then by cells from memory cell pools. 1 year after priming, the indirect PFC response is largely by cells in the memory cell pool. In contrast, the direct PFC response is probably mostly a "virgin" primary type of response, with only a small contribution being made by the remaining memory cells.

SUMMARY

A cell suspension culture system combined with a procedure which separates most macrophages from lymphoid cells was used to investigate some of the cellular requirements for direct and indirect plaque-forming cell responses by nonprimed and primed mouse spleen cells *in vitro*.

The plaque-forming cell response to heterologous erythrocytes in cultures of nonprimed spleen cells required both macrophages and lymphoid cells for its development. A significant indirect plaque-forming cell response did not develop in cultures of nonprimed spleen cells.

In contrast, cultures of separated or macrophage-poor lymphoid cells from primed mice exhibited increasing responses relative to the response of unseparated spleen cells as the interval after priming increased. The cultures of

separated lymphoid cells were not entirely free of phagocytic cells. Despite some evidence which suggests that these phagocytic cells had little function in the response, one cannot ascertain whether the lymphoid cells were responding directly to a second contact with antigen or whether the few contaminating phagocytic cells were performing a function essential to the response by the lymphoid cells. Physiologically different populations of cells appear to develop after priming and are able to respond *in vitro* in a macrophage-poor culture. Some of the properties of these populations suggest that they are "memory cell" pools containing precursors of direct and indirect plaque-forming cells highly susceptible to a second antigenic stimulus.

It is a pleasure to thank Dr. Baruj Benacerraf and Dr. Richard Asofsky for invaluable advice during these experiments and in the preparation of the manuscript. The skilled technical assistance of Mrs. Barbara Johnson is gratefully acknowledged.

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