 IMMUNOLOGICAL MEMORY IN VITRO*

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A suitable initial exposure of an individual to an antigen typically results in the development of a specific and persisting altered reactivity manifested by facilitation of subsequent responses to that antigen. Components of such facilitation characteristically include a heightened response, earlier appearance of a detectable response, more rapid development of peak values, the capacity to respond in the presence of specific antibody, and, in the case of antibody responses, parallel production of IgM and IgG immunoglobulins (1-5). These features have been chiefly delineated by in vivo studies and the purpose of the present work was to examine the extent to which they occur in vitro.

Materials and Methods

Mice.—Female DBA/2 mice (Jackson Laboratories, Bar Harbor, Maine) 10 wk of age or older provided mouse spleen cell suspensions (MSC).† Mice receiving in vivo immunization were matched for age with nonimmunized controls. Swiss white mice were the source of mouse sera.

Antigen.—Sterile, washed sheep erythrocytes (SRBC) from one male gelding were used throughout.

Sera.—Late immune anti-SRBC serum was pooled from 200 mice receiving $3 \times 10^8$ SRBC intraperitoneally 8 and 3 wk before sacrifice. The pooled serum had a 2-mercaptoethanol-insensitive hemagglutinin titer of 1:640. Early (IgM) anti-SRBC serum was pooled from 50 mice sacrificed 3 days after receiving $3 \times 10^8$ SRBC intraperitoneally. The serum had a hemagglutinin titer of 1:32 which was abolished by 2-mercaptoethanol. Normal mouse serum was pooled from 50 nonimmunized mice. All mouse sera were warmed to 56°C for 30 min, sterilized by ultrafiltration, and then stored in frozen portions. Fetal calf serum was supplied by Reheis Chemical Co. (Division of Armour Pharmaceutical), Chicago, Ill. Guinea pig serum was lyophilized when fresh and used after reconstitution at a dilution of 1:8 as the source of complement. Rabbit anti-mouse IgG serum was prepared from rabbits immunized with immuno-

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Abbreviations used in this paper: MSC, mouse spleen cells; NMS, normal mouse serum; PFC, plaque-forming cells; SRBC, sheep erythrocytes.
electrophoretically-pure mouse IgG. Lyophilized portions were reconstituted and used at a working dilution of 1:20.

**Immunization.**—(a) In vivo: SRBC dose was $1.5 \times 10^8$ intravenously. Passive immunization comprised 0.2 ml of intravenous late-immune serum. (b) In vitro: Routinely cultures (except negative controls) received $2.5 \times 10^6$ SRBC. Cultures passively immunized received 5 μl of late-immune serum.

**Culture Technique.**—Each culture (1 ml total volume) contained $1 \times 10^7$ mouse spleen cells prepared, maintained, and given daily nutritional additive, in the manner described by Mishell and Dutton (6). Media were supplied by Grand Island Biological Company, Grand Island, N. Y.

**Assay.**—Cultures were assayed for direct and, using the anti-mouse IgG developing serum, for indirect plaque-forming cells (PFC) by a slide-modification of techniques previously described (7, 8). Slides were incubated for 1 hr before exposure to complement or complement-developing-serum mixture for a further 1½ hr.

## RESULTS

**Characterization of the Immune State.**—Spleen cell cultures were prepared from matched groups of donors at various times after injection of $1.5 \times 10^8$ SRBC intravenously. The 4 day in vitro response to SRBC was then assayed. The results, for both direct (IgM-producing) and indirect (IgG-producing) PFC are summarized in Fig. 1.

Cultures did not produce detectable numbers of indirect PFC until the donor animals were more than 1 wk post-in vivo immunization. (This was in contrast to the indirect PFC activity induced by the in vivo immunization in the donor spleens, where peak values occurred on about the 6th day of the in vivo primary response and had fallen to less than 10% of this level by day 10.) In vitro responses were greatly augmented about 3 days after in vivo immunization. This degree of augmentation declined over the next 5 wk to a fairly stable level, only slightly above responses of cultures from nonimmunized mice. Mice which were 8 or more wk post-SRBC immunization had passed into this stable phase and were termed "late-immune." Such mice no longer produced detectable numbers of indirect PFC in vitro (Fig. 1). The in vivo response of these mice was however characterized by an early and predominant appearance of indirect PFC as well as by resistance to suppression by antibody, and was invariably distinguishable from the initial in vivo response of previously nonimmunized mice, as seen in a typical experiment whose results are shown in Table I. The suggestion that the in vitro response of late-immune mice resembled the response of nonimmune mice was further evaluated.

**Effect of Varying In Vitro Antigen Dose.**—Routinely $2.5 \times 10^6$ SRBC were added to each culture. Over a 64-fold range around this dose, responses of both late-immune- and nonimmune-derived cultures were similarly and relatively slightly augmented in the presence of increasing doses of antigen (Fig. 2).

This parallel behavior of the two groups suggested that the consistently
greater responses of late-immune-derived cultures do not reflect a significant change in the composition or properties of the responding cell populations.

![Graph](image)

**Fig. 1.** Effect of in vivo immunization (1.5 × 10⁸ SRBC by vein) on the in vitro response. Each point represents the mean of two to four experiments in each of which the mean of 6-12 cultures was determined. The first point (left) represents responses after no SRBC in vivo. Days indicated are those on which the cultures were started.

**TABLE I**

*Primary and Secondary In Vivo PFC Response of DBA/2 Mice. Effects of Passive Immunization with Late Immune Serum*

| SRBC (Days before assay)* | Anti-SRBC | PFC/spleen§ | Direct | Indirect |
|---------------------------|-----------|-------------|--------|----------|
| 4                         | 0         | 67,550      | 18,250 |
| 4                         | +         | 650         | NIL    |
| 60                        | 0         | 400         | NIL    |
| 60 and 2                  | 0         | 1,200       | 5,800  |
| 60 and 4                  | 0         | 9,100       | 136,350|
| 60 and 4                  | +         | 2,700       | 63,400 |

* Groups of eight mice; each immunization 1.5 × 10⁸ SRBC intravenously.
† 0.2 ml of late-immune serum 6 hr before last injection SRBC; 0 indicates no passive immunization.
§ Group means rounded to the nearest 50.

**Effect of Varying Spleen Cell Concentration.**—At the MSC concentration routinely employed (1 × 10⁷/ml) efficiency of the response (expressed as PFC/10⁶ MSC cultured) was close to maximum and unaffected by small variations of MSC concentration. Considerable reduction in the MSC concentration re-
sulted in a rapidly declining efficiency of response. The regression curves obtained for the variation of efficiency with MSC concentration were similar for late-immune- and nonimmune-derived cultures, showing a consistent and fairly uniform vertical displacement over a wide range of concentration (Fig. 3). This

Fig. 2. Comparative in vitro dose-response curves for spleen cell cultures derived from late-immune and nonimmune donor mice. Assays are for direct PFC and each point represents the mean of five cultures.

Fig. 3. Relative efficiency of responses from late-immune and nonimmune derived cultures over a range of spleen cell concentrations. Assays enumerate direct PFC and each point represents the mean of five cultures.
again suggested that the higher responses of late-immune–derived cultures were not based on a significant change in the composition or properties of the responding system.

Dependence on Separable Cell Types.—The primary in vitro immune response to SRBC is dependent on the interaction of at least two behaviorally distinct cell populations. Mosier (9) settled MSC suspensions on plastic surfaces and was able to separate a subpopulation of cells adherent to the surface from one containing the nonadherent cells. Such subpopulations were singly essentially incapable of producing in vitro PFC responses but regained this ability when recombined. We found the response of MSC suspensions from late-immune mice was similarly dependent on adherent and nonadherent subpopulations (Table II).

| Cells cultured* | SRBC+ | PFC/culture† |
|-----------------|-------|--------------|
| L.R. + M.R.     | +     | 5540         |
| L.R. + M.R.     | 0     | 290          |
| L.R.            | 0     | 4            |
| M.R.            | 0     | 0            |
| M.R.            | +     | 290          |

* L.R. indicates nonadherent (lymphocyte-rich) cells at 1 × 10⁷ cells/culture. M.R. indicates adherent (macrophage-rich) cells at 1 × 10⁶ cells/culture.
† 0 indicates no SRBC added to cultures.
§ Mean of duplicate assays of three to six cultures.

Effect of Anti-SRBC Antibody.—MSC suspensions from nonimmune mice differed from immune suspensions in not having been exposed to anti-SRBC antibody in vivo. The effect of such exposure was therefore tested by comparing the in vitro responses of normal nonimmune suspensions with that of suspensions from nonimmune donors which had been given 0.2 ml of late-immune anti-SRBC serum 24 hr before sacrifice. The results (Table III) indicated that in vivo exposure to a quantity of antibody sufficient to prevent the in vivo primary response did not affect a subsequent response in vitro.

The addition of either early (IgM) mouse anti-SRBC serum (diluted to give a final hemagglutinin titer in the culture of 1:1) or late-immune mouse anti-SRBC (predominantly IgG, 5 μl added to 1 ml of culture for a final hemagglutinin titer of 1:2) inhibited the PFC responses of both nonimmune and late-immune spleen suspensions. Normal mouse serum (NMS) absorbed with SRBC was similarly diluted and added to other cultures as a control. Such NMS, as well as late-immune serum absorbed with SRBC and added to cultures in the
### TABLE III

**In Vivo Passive Immunization. Effects on In Vivo and In Vitro Primary Responses Compared**

| In vivo anti-SRBC* | SRBC‡ | PFC/10^6 spleen cells§ |
|--------------------|-------|------------------------|
|                    | In vivo | In vitro | In vivo | In vitro |
| 0                  | +      | +        | 871     | 262      |
| 0                  | +      | +        | 12      |          |
| +                  | +      | +        | 215     |          |

* + indicates 0.2 ml of late immune serum intravenously 24 hr before active immunization. 0 indicates no passive immunization.

‡ 1.5 × 10^8 intravenously or 2.5 × 10^8 in vitro.
§ Direct PFC. Means of five mice or duplicate assays of five cultures; all assays 4 days after active immunization.

### TABLE IV

**Effect of In Vitro Passive Immunization and In Vivo Active Immunization on In Vitro Responses**

| In vivo SRBC* at time before culture | In vitro anti-SRBC‡ | In vitro SRBC§ | Duration of culture (days) | In vitro response (PFC/culture) | Direct | Indirect |
|-------------------------------------|---------------------|----------------|-----------------------------|---------------------------------|--------|----------|
| None given                          | 0                   | +             | 4                           | 1,869                           | 0      | 0        |
| None given                          | +                   | +             | 4                           | 12                              | 0      | 0        |
| None given                          | 0                   | 0             | 4                           | 14                              | 0      | 0        |
| 3                                   | 0                   | +             | 4                           | 68,700                          | 0      | 0        |
| 3                                   | +                   | +             | 4                           | 592                             | 0      | 0        |
| 3                                   | 0                   | 0             | 4                           | 1,215                           | 0      | 0        |
| 8                                   | 0                   | +             | 4                           | 29,718                          | 0      | 0        |
| 8                                   | +                   | +             | 4                           | 987                             | 0      | 0        |
| 8                                   | 0                   | 0             | 4                           | 706                             | 0      | 0        |
| 25                                  | 0                   | +             | 2                           | 954                             | 2,345  |          |
| 25                                  | 0                   | +             | 4                           | 4,606                           | 9,824  |          |
| 25                                  | +                   | +             | 4                           | 239                             | 2,566  |          |
| 25                                  | 0                   | 0             | 4                           | 132                             | 355    |          |
| 60                                  | 0                   | +             | 4                           | 2,393                           | 0      | 0        |
| 60                                  | +                   | +             | 4                           | 108                             | 0      | 0        |
| 60                                  | 0                   | 0             | 4                           | 13,690                          | 24,912 |          |
| 60 and 3                            | 0                   | +             | 4                           | 24,912                          | 0      | 0        |
| 60 and 3                            | +                   | +             | 4                           | 1,119                           | 11,280 |          |
| 60 and 3                            | 0                   | 0             | 4                           | 1,202                           | 5,201  |          |

* 1.5 × 10^8 intravenously.
‡ + indicates 5 µl of late-immune serum added to each culture at outset; 0 indicates no antiserum added.
§ + indicates 2.5 × 10^8 SRBC added to each culture at outset; 0 indicates no SRBC added.
‖ Means of duplicate assays of at least four viable cultures, each seeded with 1 × 10^7 MSC.
dilution employed with unabsorbed late-immune serum, typically had a mild enhancing effect on the response.

Routinely 5 μl of late-immune anti-SRBC mouse serum was added to the appropriate cultures to produce a final hemagglutination titer of 1:2. This invariably produced marked specific inhibition of direct PFC responses regardless of the donor's immune status (Table IV).

Indirect PFC could be shown to appear and increase in number only when the suspensions were obtained either from donor animals which received in vivo immunizations with SRBC between 1 and 6 wk before sacrifice, or from late-immune animals which had recently received second in vivo SRBC immunizations. Late-immune serum added to indirect PFC-producing cultures almost abolished the direct PFC response but had much less effect on the generation of indirect PFC (Table IV).

Insofar as cultures from both nonimmune and late-immune donors were similarly sensitive to antibody inhibition, the duration of this sensitive phase was assessed. We were able to confirm previous findings that the primary PFC response to SRBC in vitro is inhibitable by antibody only in the first 24–36 hr phase of the response (10). We also found a similar limited period of susceptibility for responses from late-immune MSC suspensions (Fig. 4).

**DISCUSSION**

Differences between immune and nonimmune animals cannot be solely due to simple quantitative changes suggested by Burnet's initial clonal selection hypothesis (11) if apparent qualitative distinctions of the secondary response are to be accounted for (1-5, 12-14). The immune state must reflect in addition some change in the composition of the cellular population responding to reimmunization, as experiments testing the performance of immune cell populations suggest (15-17).
8 wk after immunization with $1.5 \times 10^6$ SRBC the response of DBA/2 mice to SRBC in vivo is clearly distinguishable from that of closely matched mice which have not been previously given SRBC injections. In our hands three invariable and distinctive features of such a secondary response in vivo are: (a) inability to abrogate the response by administration of immune serum; (b) the simultaneous generation of direct and indirect PFC from the outset of the response; and (c) the presence of indirect PFC in numbers greatly exceeding those of direct PFC. The in vitro responses to SRBC of spleen cells from such late-immune and nonimmune mice were, however, essentially indistinguishable.

Neither late-immune nor non-immunized mice yielded spleen cell cultures in which we could detect indirect PFC responses. There is a possibility this deficiency indicates that cells responsible for producing indirect PFC did not function well in the in vitro conditions. However, good indirect PFC responses occurred in cultures set up between 10 and 30 days after in vivo immunization. This might be more compatible with the indirect PFC response in vitro being dependent on a cell type which accumulates in the spleen late in the primary response but subsequently migrates. The peak potential for indirect PFC formation in vitro was about 20 days after primary in vivo immunization. This lagged considerably behind the preceding primary in vivo indirect PFC response, which in our system has fallen to less than 10% of its peak by the 10th day after primary immunization. Thus splenic indirect PFC content did not relate to the capacity of derived cultures for indirect PFC generation. This suggests that, if poor in vitro indirect PFC responses are due to the lack of a particular cell type, the cell type concerned is not the indirect PFC per se.

Anti-SRBC antibody essentially abolished the responses of cultures from late-immune mice but had little effect on the in vivo response of these animals. Such antibody also dramatically inhibits the primary in vivo response and the response of cultures from nonimmune mice. The action of administered antibody in cultures from late-immune and nonimmune mice appeared similar in both degree and timing; it was almost totally inhibiting if present in the first 24 hr of the in vitro response but was not inhibiting thereafter.

The one established difference between MSC cultures from late-immune and nonimmune donors was that the former gave higher overall responses. This quantitative difference was slight compared to that between MSC cultures from 3-day immune and nonimmunized donors. Further, we could find no evidence for any differences in the composition or properties of the in vitro-responding cell populations from these two sources. Such evidence was mainly sought by assessing the effect on the responses of late-immune and nonimmune cultures

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2 The detection of a small number of indirect PFC in primary MSC cultures has been reported with the use of heavy chain-specific antisera (18). While the sensitivity of our own assay cannot exclude the presence of a small proportion of indirect PFC, it serves to establish the presence of a deficiency in this component of the response.
when the in vitro antigen concentration and the spleen cell concentration were varied, which resulted in parallel changes in the responses of the two groups. In addition, the in vitro response of spleen cells from late-immune animals was shown to resemble that of spleen cells from nonimmune animals in its dependence on two subpopulations which could be separated by surface adherence (9).

With respect to both indirect PFC production and susceptibility to inhibition by specific antibody, responses of cultures from late-immune mice resembled the early primary in vivo response and the in vitro response of nonimmune mice. The in vitro response of early-immune mice appeared distinct from these and more akin to the in vivo secondary response. Thus spleen cell cultures taken from mice between 10 and 30 days after in vivo immunization not only generated indirect PFC but produced them concurrently with the production of direct PFC from the outset, in a response which was relatively insensitive to inhibition by the presence of anti-SRBC antibody.

An interpretation of the data is that the response to SRBC of MSC cultures from animals receiving SRBC 8 or more wk previously differs basically from the in vivo response of such mice, and reflects the characteristics of the cell system determining the response in MSC cultures from nonimmunized animals.

Passive immunization of nonimmune mice abolished their capacity to produce a primary in vivo PFC response to SRBC. However, washed spleen cells from such passively immunized animals gave a normal in vitro response. The procedure of putting spleen cells into culture appears to free them from the specific inhibiting effect of antibody which they would have had to contend with in vivo. This is compatible with the in vitro direct PFC response from late-immune spleen cells representing a response which is largely suppressed in vivo due to the continuing presence there of anti-SRBC antibody.

The in vivo secondary response of late-immune mice is characterized by the antibody-insuppressible generation of predominantly indirect PFC. In vitro, spleen cells of these mice fail to yield such a response. It is thought possible that this is due to the spleen's lacking a cell type necessary for the in vivo response of these mice. Such a deficiency is not present 2-3 wk after in vivo immunization and is rapidly corrected after in vivo reimmunization of late-immune animals.

It is concluded that the specific adaptation determining the immune state to SRBC involves development of a new pathway for an antibody response, and that the immune mouse also fully retains an unadapted preexisting pathway which had served for the original early primary response.

**SUMMARY**

The immune responses to sheep erythrocytes of mouse spleen cell suspensions from immune and nonimmune donors were compared in vitro. In vivo immunity
was only transiently reflected in vitro, and 8 wk after in vivo immunization the responses of cultures from immunized and nonimmunized mice were virtually identical. There appeared to be two mechanisms for an antibody response to sheep erythrocytes. The first was responsible for the early primary response and is unmodified in the immune animal though contributing little to subsequent in vivo responses due to its suppressibility by specific antibody. The second was expressed in the in vivo secondary response but not on in vitro challenge of spleen cells from mice immunized many weeks previously; spleen cell cultures from such immune mice, freed from the antibody of the in vivo environment, once again demonstrate a pure primary-type response.

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