EFFECT OF RECENT ANTIGEN PRIMING ON ADOPTIVE IMMUNE RESPONSES

IV. Antigen-Induced Selective Recruitment of Recirculating Lymphocytes to the Spleen Demonstrable with a Microculture System

BY J. SPRENT* AND I. LEFKOVITS

(From the Basel Institute for Immunology, 487 Grenzacherstrasse, Postfach 4005, Basel 5, Switzerland, and the ICRF Tumour Immunology Unit, Department of Zoology, University College London, London, England)

Within one day of injecting mice intravenously with heterologous erythrocytes, the capacity of thoracic duct lymphocytes (TDL)1 to respond to the injected antigen on adoptive transfer is specifically abolished (1). This period of unresponsiveness, which affects both IgM and IgG antibody production, lasts for only 1–2 days. By 3 days the reactivity of TDL returns to normal and by 5 days enhanced responses are obtained. These data have been interpreted in terms of antigen-induced selective recruitment of recirculating lymphocytes (ASRL) from the recirculating lymphocyte pool to the lymphoid organs.

A priori, the spleen would seem the most likely site for ASRL since 51Cr-labeled heterologous erythrocytes lodge predominantly in this region after intravenous injection (2). Paradoxically, however, spleen cells from mice given heterologous erythrocytes 1 day previously were found to be unable to respond to the injected antigen within 1 wk of adoptive transfer (3). The unresponsiveness did not appear to reflect absence or deletion of specific precursor cells since normal or above normal responses were observed during the 2nd wk post-transfer. One possibility put forward was that the migratory properties of the transferred cells were temporarily impaired as the result of their antigen-activated state. If so it would follow that the cells should express normal function if assayed in vitro.

The present paper examines this prediction by using a microculture system to estimate the relative frequency of specific B-lymphocyte precursors in TDL and spleen cell suspensions from mice recently injected with antigen. It will be shown that, in marked contrast to adoptive transfer in vivo, in vitro culture of

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* Present address: Department of Zoology, University College London, Gower Street, London WC1E 6BT, England. Holder of a C. J. Martin Travelling Fellowship.

1 Abbreviations used in this paper: ASRL, antigen-induced selective recruitment of recirculating lymphocytes; HRC, horse erythrocytes; PFC, plaque-forming cells; 19s PFC, direct plaque-forming cells; 7a PFC, indirect (developed) plaque-forming cells; SRC, sheep erythrocytes; TDL, thoracic duct lymphocytes.
spleen cells from mice given antigen 1 day previously results in rapid and above normal responses to the injected antigen. TDL, by contrast, show almost total and specific unresponsiveness.

Materials and Methods

Animals. Male CBA/J mice aged 6-10 wk were used in all experiments unless stated otherwise. C57BL/6 mice were employed during the preparation of helper factors. The nude (athymic) mice used in Table I were partly backcrossed to C57BL/6 and bred locally.

Cell Suspensions. TDL were collected as described elsewhere (4), and spleen suspensions were prepared by a standard technique (1). In each experiment the lymphoid cells used were pooled from 2-4 donors.

Antigens. Erythrocytes were obtained from the jugular vein of sheep and horses and stored in Alsever’s solution. Before use they were washed three times in saline. In general, \( 5 \times 10^6 \) erythrocytes were calculated to be equivalent to 0.1 ml of a 25% solution, \( 10^6 \) erythrocytes as 0.2 ml of a 25% solution, and \( 10^7 \) erythrocytes as 1 ml of a 50% solution.

Injections. All suspensions of lymphoid cells and heterologous erythrocytes were injected intravenously via the tail vein.

Microcultures. The procedure used has been described in detail elsewhere (5). Briefly 10-\( \mu \)l aliquots of cell suspensions (2 \( \times \) 10\(^7\)/ml) containing 50 \( \mu \)l/ml of a 1% solution of the test erythrocyte suspension were dispensed with a Hamilton multisyringe dispenser into each of the 60 wells of 2-3 tissue culture trays (Falcon Plastics Type 3034; Falcon Plastics, Oxnard, Calif.). The medium used (Eagle’s minimal essential medium) contained 2-mercaptoethanol (5 \( \times \) 10\(^{-5}\) M) as well as HEPES (20 mM) and fetal calf serum (5%).

Unless stated otherwise, each well of the microcultures contained 2 \( \times \) 10\(^7\) cells. For the limiting dilution experiments shown in Figs. 1 and 2 in which graded numbers of lymphocytes were added to the cultures, the cell density was made up to 2 \( \times \) 10\(^6\) by adding a corresponding number of X-irradiated (1,200 R) lymphocytes; the latter were aliquots of the test lymphoid cell suspension. Microcultures were incubated under standard conditions for 5-6 days before assay.

Assays for Antibody Production

HEMOLYTIC SPOT TEST. As described in detail elsewhere (5), aliquots of unwashed cells from the microcultures were dispensed with a replicator (8) on to agar plates containing the test erythrocytes. After adding complement (guinea pig serum) the plates were incubated for 45 min at 37°C. The proportion of wells containing (19s) antibody-forming cells was then established by counting the number of hemolytic zones on the plates. Developed (7a) plaque-forming cells (PFC) (1) were searched for in some experiments, but none were detected.

As considered elsewhere (7), each “positive” well can be regarded as containing a single clone of antibody-forming cells when, during limiting dilution analysis, the cell density is such that 37% \( (F_0 = 0.37) \) of the cultures are negative. Strictly speaking, this only applies when the dose response curve obtained is linear.

It should be emphasized that the spot test detects not only antibody-forming cells per se, but also antibody that has accumulated since the time of initiating the cultures. The assay can thus be performed at a stage when most of the cells have ceased antibody secretion.

PFC ASSAY. For enumerating antibody-forming cells in the spleen after adoptive transfer, numbers of 19s and 7s PFC to sheep erythrocytes (SRC) and horse erythrocytes (HRC) were measured as described previously (1).

Allogeneic Factor. As detailed elsewhere (8), TDL or spleen cells from CBA/J and C57BL/6 mice were cultured in vitro at 10\(^6\) cells/ml mixed at a ratio of 1:1 for 24 h at 37°C. The factor was then obtained by centrifuging the cells and retaining the supernates. In most experiments 5 \( \mu \)l of neat supernate was added to each well of the microcultures.

Results

Conditions for Obtaining Antibody Responses to Heterologous Erythrocytes by TDL Cultured In Vitro. When TDL from normal CBA/J mice or nude mice...
were placed in microcultures for 6 days with SRC, virtually no antibody response was obtained, i.e., the number of wells producing anti-SRC 19s antibody as measured by the hemolytic spot assay (see Materials and Methods) approached zero \( (F_o = 0.99) \) (Table I). This was observed despite the presence of 2-mercaptoethanol in the cultures. Antibody responses were obtained, however, if the TDL were supplemented with either (a) irradiated syngeneic spleen cells or (b) supernates ("allogeneic factor") from mixed cultures of H-2-incompatible spleen cells or TDL (see Materials and Methods) (Table I). Reconstitution was particularly pronounced with the allogeneic factor (though not with supernates from cultures of syngeneic lymphocytes). Since reconstitution with this factor applied to nude TDL (which contain <1% T cells [4]), as well as to normal TDL (80% T cells), the allogeneic factor apparently provided a combination of T-cell help and a macrophage-like function. On the basis of these findings, TDL were routinely cultured with antigen in the presence of the allogeneic factor. The results to be considered below refer to 19s antibody production. Indirect (7s) antibody-forming cells were searched for in some experiments but were not detected.

**Specific Reduction in the Response of TDL by Preinjection of Antigen 1 Day Previously.** Groups of normal CBA/J mice were injected intravenously with 10⁹ SRC. The mice were cannulated 24 h later, and TDL were collected between 28 and 40 h. When these cells were placed in microcultures in the presence of SRC and HRC, antibody responses measured at 6 days were very low to SRC but high to HRC (Fig. 1). TDL from untreated mice, by contrast, responded well to both antigens.

Another such experiment is illustrated in Table II. Here the TDL were taken from donors injected 1 day previously with either 10⁹ SRC or 10⁹ HRC. With both groups it is evident that the response of the TDL (measured at 5 days) was either absent or very low to the injected antigen but high to the "third-party" antigen;

| Cells cultured (10⁶/well) | Material added to cultures together with SRC | Fraction \( (F_o) \) of cultures failing to produce antibody to SRC* |
|--------------------------|---------------------------------------------|----------------------------------------------------------|
| CBA/J TDL                | -                                           | 0.99                                                     |
| CBA/J TDL                | 10⁶ irradiated CBA/J spleen†                 | 0.76                                                     |
| CBA/J TDL                | 10⁶ irradiated CBA/J spleen†                 | 1.00                                                     |
| CBA/J TDl                | Allogeneic factor§                           | 0.22                                                     |
| CBA/J TDL                | Conditioned medium†                          | 0.99                                                     |
| CBA/J spleen             | -                                           | 0.09                                                     |
| Nude TDL                 | -                                           | 0.98                                                     |
| Nude TDL                 | Allogeneic factor                            | 0.12                                                     |

* Each value derived from hemolytic spot assay on 120-180 microcultures; cultures assayed on day 6.
† Cells exposed to 1,200 in vitro.
§ Supernate from cultures of C57BL/6 and CBA/J TDL incubated together for 24 h (see Materials and Methods).
† Supernate from cultures of CBA/J TDL incubated alone for 24 h.

[Table I](#)
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FIG. 1. Anti-SRC (●) and anti-HRC (○) 19s antibody response by TDL placed in microcultures for 6 days with SRC and HRC. In Fig. 1a the TDL were from mice injected intravenously with 10⁷ SRC 24 h before cannulation; TDL collected at 28-40 h. In Fig. 1b the TDL were from normal mice. The horizontal axis shows the number of TDL placed in each microculture well; the final cell density was made up to 2 × 10⁵ cells/well by addition of irradiated (1,200 R) TDL (See Materials and Methods). The vertical axis represents the fraction of microcultures which did not produce antibody to the test erythrocytes suspension by the hemolytic spot test. 120 microcultures per point. Vertical bars represent 95% confidence limits.

TABLE II

In Vitro Anti-SRC and Anti-HRC Response of TDL from Mice Injected with SRC or HRC 1 Day before Cannulation

| Antigen given to TDL donors 1 day before cannulation* | Antigen added to microcultures | Fraction of cultures failing to produce antibody to:† |
|-----------------------------------------------------|--------------------------------|-----------------------------------------------------|
| SRC                                                 | SRC                            | SRC                                                 |
|                                                     | HRC                            | HRC                                                 |
|                                                     | SRC + HRC                      | SRC + HRC                                           |
|                                                     | 1                              | 1                                                   |
|                                                     | 0.97                           | 0.52                                                |
|                                                     | 0.94                           | 0.47                                                |
| HRC                                                 | SRC                            | SRC                                                 |
|                                                     | HRC                            | HRC                                                 |
|                                                     | SRC + HRC                      | SRC + HRC                                           |
|                                                     | 0.41                           | 1                                                   |
|                                                     | 1                              | 1                                                   |
|                                                     | 0.43                           | 1                                                   |

* 10⁷ erythrocytes given intravenously; mice cannulated 24 h later and TDL collected at 28-40 h.
† Each value derived from hemolytic spot assay on 120 microcultures; cultures assayed on day 5; cell density 10⁵ cells/well.

this applied irrespective of whether the TDL were cultured with each antigen separately or with both antigens combined.

It is apparent from these data that preinjection of the TDL donors with antigen caused at least a 10-fold reduction in the frequency of precursor (B) cells capable of producing clones of antibody-forming cells specific for the injected antigen. Indeed, in the case of TDL from mice preinjected with HRC, precursors for HRC were undetectable. Two other experiments gave comparable results. In general, responses to the third-party antigen (the antigen not used for preinjection) were no lower than with TDL from untreated mice (see Fig. 1). As an aside, it may be pointed out the curves of the responses obtained from limiting dilution analyses (Fig. 1) were generally of the “multihit” type, i.e., they were not linear.
TABLE III

In Vitro Anti-SRC and Anti-HRC Response of TDL from Mice Primed to HRC and Boosted with HRC 24 h before Cannulation

| Antigen given to TDL donors before cannulation* | Total numbers of 19s PFC to:† |
|-----------------------------------------------|-----------------------------|
| 6 wk before cannulation                       |                             |
| 5 × 10⁸ HRC                                  | 10¹⁰ HRC                    |
| 5 × 10⁸ HRC                                  | Saline                      |

* Antigen given intravenously; mice cannulated 24 h after injection of 10¹⁰ HRC or saline and TDL collected at 28–40 h.
† Both SRC and HRC were added to microcultures (10³ TDL/well). The cultures (a total of 60 wells) were harvested on day 5, pooled, and assayed for both direct (19s) and indirect (7s) PFC (no indirect PFC detected).

In this situation we do not attempt to calculate the precise frequency of precursor cells (see reference 7).

In a further experiment TDL were taken from donors that had been primed with 5 × 10⁸ HRC 6 wk previously. These mice were divided into two groups. One group was boosted with 10¹⁰ HRC 1 day before cannulation, and the other received saline. The TDL were then placed in microculture with both SRC and HRC. Antibody-forming cells were assayed after 5 days, not as in previous experiments by estimating the number of "positive" wells, but by pooling the cells from each well and estimating the total number of PFC to each antigen. It can be seen from Table III that TDL from the saline-injected group gave a good response to HRC and a somewhat lower response to SRC (indicative of the preimmunization with the former antigen). Boosting the TDL donors with a high dose of HRC 1 day before cannulation, however, virtually abolished the response to HRC, but did not reduce the response to SRC.

Inability of 1-Day-Primed TDL to Inhibit the Response of Normal TDL.

The experiment illustrated in Table IV was designed to investigate the claim that short-term "priming" with SRC leads to the rapid production of suppressor cells (9). It can be seen from Table IV that the high in vitro response to SRC given by TDL from normal mice was not inhibited when these cells were cultured in the presence of TDL from donors given 10⁶ SRC 1 day before cannulation. (The latter gave negligible responses to SRC when cultured alone [Table IV] although they responded well to HRC [data not shown]). Indeed there was some enhancement of the response. This appeared to be a nonspecific effect reflecting the fact that, within certain limits, increasing the cell density in microcultures facilitates the response (7). Thus it is evident that the response of 5 × 10⁴ normal TDL was increased to approximately the same extent by the addition of irradiated (1,200 R) normal syngeneic TDL as with irradiated "1-day-primed" TDL (Table IV).

Response of Spleen Cells from Mice given Antigen 1 Day Previously: Specific Hyperreactivity Detected In Vitro Despite Hyporeactivity on Adoptive Transfer.

To study the effect of short-term priming with antigen on the response of spleen cells the following experiment was performed: Groups of normal mice
were injected with either $10^9$ SRC or $10^8$ HRC. 1 day later, half of the mice in each group were killed and the spleens removed; the remainder were cannulated to provide TDL. Responses of the spleen cell suspensions and TDL were assayed (a) by placing aliquots of cells in microcultures with SRC and anti-SRC responses determined 6 days later, and (b) by adoptively transferring cell aliquots to irradiated syngeneic mice together with SRC and HRC and measuring responses to both antigens 7 days later.

In the case of spleen cells, it can be seen that when responses were measured in vitro (Fig. 2 b) the response to SRC was appreciably higher with cells from mice given SRC 1 day before than with cells from the HRC-injected group or from untreated mice; two other experiments gave similar results; in one, the cells were assayed on day 5 culture (data not shown). These findings were in marked contrast to the effect of transferring spleen cells in vivo (Table V). In this situation, cells from SRC-injected mice gave only extremely low responses to SRC while responding well to HRC; reciprocal results were obtained with spleens from HRC-injected mice.

In contrast to spleen cells, 1-day-primed TDL were specifically unresponsive to the injected antigen irrespective of whether responses were measured in vitro (Fig. 2 a) or in vivo (Table V).

Discussion

The observation that TDL from mice given $10^9$ heterologous erythrocyte 1 day previously were specifically unresponsive to the injected antigen when assayed

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**Table IV**

| Lymphocytes placed in each microculture well with SRC | Fraction of cultures failing to produce antibody to SRC† |
|-----------------------------------------------------|----------------------------------------------------------|
| $5 \times 10^4$ SRC-primed TDL                      | 1                                                        |
| $10^5$ SRC-primed TDL                              | 1                                                        |
| $1.5 \times 10^4$ SRC-primed TDL                    | 0.92                                                     |
| $5 \times 10^4$ normal TDL                          | 0.86                                                     |
| $10^5$ normal TDL                                   | 0.34                                                     |
| $1.5 \times 10^4$ normal TDL                        | 0.12                                                     |
| $5 \times 10^4$ SRC-primed TDL + $5 \times 10^4$ normal TDL | 0.63                                                     |
| $7.5 \times 10^4$ SRC-primed TDL + $7.5 \times 10^4$ normal TDL | 0.32‡                                                   |
| $5 \times 10^4$ SRC-primed TDL + $10^5$ normal TDL  | 0.22                                                     |
| $10^4$ SRC-primed TDL + $5 \times 10^4$ normal TDL  | 0.43                                                     |
| $5 \times 10^4$ SRC-primed TDL + $5 \times 10^4$ irradiated SRC-primed TDL | 1                                                        |
| $5 \times 10^4$ normal TDL + $5 \times 10^4$ irradiated normal TDL | 0.70                                                     |

* $10^8$ SRC given 24 h before cannulation; TDL collected at 28-40 h.
† Each value derived from hemolytic spot assay on 120 microcultures.
‡ 60 microcultures only.
§ Cells exposed to 1,200 R in vitro; these cells produce no antibody when cultured alone with SRC.
in microcultures is consistent with previous findings that the cells were similarly unresponsive on adoptive transfer (1, 2). A priori, the failure of the cells to respond to antigen in vitro could reflect (a) depletion of specific T cells or B cells, (b) a nonspecific lack of macrophages (accessory cells), or (c) the presence of specific suppressor cells.

The fact that TDL from normal mice failed to respond to SRC in culture unless they were supplemented with either syngeneic irradiated spleen cells or with an
allogeneic factor strongly suggests that at least in mice, though not necessarily in rats (10), TDL contain suboptimal numbers of functional macrophages. The allogeneic factor also appeared to provide T-cell help since T-cell-deficient TDL (TDL from nude mice) gave good response to SRC in the presence of the factor, but not without it. This effect cannot be attributed simply to the macrophage-like activity of the factor since, as shown previously (7), nude spleen cells (which presumably contain optimal numbers of macrophages) fail to respond to SRC unless supplemented with the factor (or with allogeneic irradiated spleen cells). A detailed description of the properties of the factor used in the present study will be published elsewhere.

No evidence could be found to support the contention of McCullagh (9) that, in rats, the unresponsiveness of TDL induced by prior injection of SRC is due to the rapid generation of specific suppressor cells. In the present study, the addition of 1-day-primed TDL to cultures of normal TDL failed to inhibit the response of the latter to SRC (Table IV).

Since the unresponsiveness of the TDL could not be attributed to active suppression and was observed despite the presence of the allogeneic factor in the cultures, it would seem reasonable to conclude that the unresponsiveness reflected a specific depletion (ASRL) of precursor B lymphocytes. Whether there was a concomitant deficiency of specific T cells could not be tested with the present system. This seems highly likely, however, since the failure of T-cell-depleted normal B cells to respond to SRC on adoptive transfer cannot be overcome by addition of 1-day-primed TDL as a source of helper cells (J. S., unpublished data). Furthermore, Rowley et al. (11) reported that TDL adoptively transferred from rats injected with hapten-carrier conjugates 1 day before were specifically depleted of carrier-reactive (T) cells as well as hapten-reactive (B) cells.

Perhaps the most convincing evidence that the unresponsiveness of 1-day-primed TDL was due to ASRL was the reciprocal increase observed in numbers of specific antibody-forming cell precursors in the spleen (Fig. 2). This hyperreactivity of spleen cells is of interest in two respects. Firstly, it supports the notion that the transient marked unresponsiveness observed previously when 1-day-primed spleen cells were transferred in vivo reflects a temporary alteration in the homing properties of the cells due to their antigen-activated state (3). Secondly, it is not consistent with the report of Ford et al. (12) that although lymphocytes from rats can be selectively recruited to the spleen in response to AgB determinants, most of the recruited cells are destroyed during the preparation of single cell suspensions from the spleen. It may be relevant that the latter study employed irradiated hosts as the recipients of the allogeneic lymphocytes. Since the microarchitecture of the spleen collapses rapidly soon after irradiation, it is possible that recently-activated lymphocytes experience lethal trauma when removed manually from irradiated spleens, but not when taken from normal spleens.

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2 I. Lefkovits and J. Sprent. Allogeneic factor from thoracic duct lymphocytes: analysis in the micoculture system. Manuscript in preparation.
Summary

When thoracic duct lymphocytes (TDL) from mice given sheep erythrocytes (SRC) 1 day previously (1-day-primed TDL) were placed in microcultures with antigen for 5–6 days, the cells failed to produce antibody to SRC, but responded well to horse erythrocytes (HRC); reciprocal results were obtained with TDL from HRC-injected mice. The specific unresponsiveness of the TDL was observed despite the presence of an allogeneic factor in the cultures; this factor exerted not only a macrophage-like effect (TDL from normal mice failed to respond in the absence of this factor) but also a T-cell-replacing function. It was concluded therefore that the unresponsiveness of the TDL was the result of selective recruitment of specific B lymphocyte precursors from the recirculating lymphocyte pool to the lymphoid tissues; whether there was also recruitment of specific T lymphocytes was not investigated.

Since addition of 1-day-primed TDL to cultures of normal TDL did not inhibit the response of the latter, there was no evidence that active suppression accounted for the unresponsiveness. The spleen appeared to be the main site for recruitment since 1-day-primed spleen cells placed in microcultures contained above normal numbers of specifically-reactive precursors. This was in striking contrast to the effect of transferring 1-day-primed spleen cells in vivo where, as previously reported, the cells fail to respond to the injected antigen within 1 wk of transfer. An explanation for this paradox is discussed.

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