EFFECT OF RECENT ANTIGEN PRIMING ON ADOPTIVE IMMUNE RESPONSES

III. Antigen-Induced Selective Recruitment of Subsets of Recirculating Lymphocytes Reactive to H-2 Determinants

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For 1-2 days after injection of mice (1, 2) or rats (3) with heterologous erythrocytes or hapten-protein conjugates, thoracic duct lymphocytes (TDL)† become specifically depleted of lymphocytes reactive to the injected antigen. The spleen at this time, by contrast, contains normal or increased levels of specifically-reactive cells (ref. 4 and unpublished data of J. Sprent and I. Lefkowitz). These data are interpreted in terms of antigen-induced selective recruitment of recirculating lymphocytes (ASRL) from the circulation to regions of antigen concentration, e.g., the spleen.

Major histocompatibility determinants also appear to cause ASRL. Thus the capacity of TDL from parental-strain mice (1) or rats (3) to induce graft-versus-host (GVH) reactions is specifically reduced or abolished 1-2 days after injection of F₁ hybrid spleen cells. Similar results have been obtained by "filtering" parental-strain TDL through irradiated F₁ hybrid rats (5).

A variety of assay systems are used to measure T-cell responses to major histocompatibility determinants. These include the mixed-lymphocyte reaction (MLR), GVH reaction, allograft rejection, and cell-mediated lympholysis (CML). In mice, it is generally considered that the H-2 determinants which control the MLR and GVH reactions are coded for predominantly by the I region of the H-2 complex, whereas allograft rejection and CML are controlled largely by the K and D regions (6-8). This has led to the proposal that lymphocytes producing MLR and GVH reactions have a different specificity from those controlling CML and rejection of allografts (9). The present studies were designed to investigate whether all of these H-2-reactive populations undergo selective recruitment

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† Abbreviations used in this paper: ASRL, antigen-specific selective recruitment of recirculating lymphocytes; CML, cell-mediated lympholysis; C57BL → CBA TDL, TDL from CBA mice injected with (CBA × C57BL)F₁ spleen cells; GVH, graft-versus-host; ³HThdR, tritiated thymidine; MLN, mesenteric lymph node; MLR, mixed-lymphocyte reaction; TDL, thoracic duct lymphocytes; TxBM, adult-thymectomized, irradiated, bone-marrow-protected.
during allostimulation in vivo. It will be shown that TDL from mice injected 1 day previously with semiallogeneic spleen cells are almost totally devoid of lymphocytes controlling CML, allograft rejection, and GVH reactions toward determinants on the injected cells, but show only a limited depletion of cells reactive in MLR.

Materials and Methods

Mice. The following highly inbred strains of mice were used: CBA/J (H-2k), CBA/Ca (H-2k), DBA/2 (H-2d), C57BL/6 (C57BL) (H-2b), and F1 crosses between CBA/J or CBA/Ca and C57BL or DBA/2 and between C57BL and DBA/2. The experiments shown in Table I and Figs. 1, 2, and 4 (using CBA/J mice) were performed at the Basel Institute for Immunology using mice bred locally and derived originally from The Jackson Laboratories, Bar Harbor, Maine. The experiments shown in Tables II, IV, V, and Fig. 3 (using CBA/Ca mice) were performed at University College London with mice derived originally from The Jackson Laboratories. The experiment shown in Table III was carried out at the Walter and Eliza Hall Institute; the origin of the mice used in this experiment has been described elsewhere (4). All mice were bred and housed under conventional conditions.

Cell Suspensions. Suspensions of thymus and spleen cells were prepared as described in the first paper of this series (4). TDL were collected by the method of Sprent (10). Cell viability was measured by dye exclusion.

Injections. All cell suspensions and radioisotopes were injected intravenously via the tail vein unless stated otherwise.

Media. For experiments not involving culture in vitro, Dulbecco's solution (11) was used.

Irradiation. Mice were exposed to whole body X-irradiation as previously described (4). A dose of 800 R was used unless stated otherwise. Spleen cells were irradiated (1,000 R) at 4°C.

Injection of Irradiated Spleen Cells. After irradiation, spleen cells were cultured for 5 h in vitro at 37°C in HEPES-buffered RPMI 1640 medium with 10% added fetal calf serum in 5% CO2. Despite irradiation, the cells showed little loss of viability when harvested after this period of incubation; viability was usually in the range of 50-60%. In most experiments, 6 × 10^8 viable cultured spleen cells were injected intravenously; 3 × 10^6 cells were injected immediately after harvest and the remainder 20-30 min later. The mice tolerated these injections surprisingly well and deaths were rare. In some experiments, a similar dose of nonirradiated noncultured spleen cells was injected. Deaths after intravenous injection of these cells were common, and the cells had to be given in three divided doses over 90 min or more.

Antisera. CBA anti-C57BL and CBA anti-DBA/2 alloantisera were raised by injecting CBA/J mice with C57BL and DBA/2 spleen cells, respectively. The spleen cells were injected intraperitoneally in a dose of 2 × 10^7 cells at fortnightly intervals. Serum was obtained 10-14 days after the last of six injections. A similar regime was used to prepare C57BL anti-CBA serum. Anti-o C3H antiserum was raised by the method of Reif and Allen (12). Serum was obtained after the 10th injection of thymus cells.

Cytotoxic Testing. The dye exclusion method of Boyse et al. (13) was used employing guinea pig serum as a source of complement.

Skin Grafting. Mice were grafted with skin according to the method of Billingham and Medawar (14).

Preparation of Thymectomized, Irradiated, and Marrow-Protected (TxBM) Mice. CBA mice were thymectomized at 6-8 wk of age by the method of Miller (15). The mice were irradiated (900 R) 3-5 wk later and protected with 3-5 × 10^6 bone marrow cells treated with anti-o serum and complement before intravenous injection. Skin grafts were applied 4-6 wk after irradiation.

Mixed Lymphocyte Reaction. (a) In vivo: The method of Sprent and Miller (16) was used. This technique involves measuring DNA synthesis in the spleen of irradiated F1 mice injected with parental strain T cells. Briefly, CBA TDL were transferred intravenously into irradiated semiallogeneic or syngeneic mice. An intravenous injection of 25 μCi tritiated thymidine (3HTdR) was given at various intervals after cell injection, and the mice were killed 45 min later. Total radioactivity in the spleen was then measured as described elsewhere (16). (b) In vitro: The method used was essentially that described by Nabholz et al. (17). TDL were washed three times,
and aliquots of $2.5 \times 10^8$ cells (responders) were cultured with $10^8$ mitomycin C-treated (18) spleen cells as stimulators in RPMI 1640 medium supplemented with 5% normal human serum, glutamine, 2-mercaptoethanol ($3 \times 10^{-3}$ M), crystallisin (200 U of penicillin/ml), and bicarbonate (3.9 g/liter). The cells were cultured at 37°C in Linbro microtubes 1S-FB-96 (Linbro Medlabs Ltd., Dublin, Eire) in 10% CO$_2$ in air. $^3$HThD incorporation was measured by washing the harvested cells through filter papers and extracting radioactivity with 0.5 ml Soluene (Packard Instrument Co., Downers Grove, Ill.) After adding scintillation fluid, radioactivity was measured in a liquid scintillation spectrometer (Packard Instrument Co.).

GVH Assay. The ability of parental strain cells to induce splenomegaly in neonatal F1 mice was determined according to the method of Simonsen (19). Intraperitoneal injections were given within 1-2 days of birth, and the spleen weights determined 10 days later. Splenic indices exceeding a value of 1.5 were considered significant.

CML. After washing three times by centrifugation, aliquots of $3 \times 10^7$ TDL were cultured with mitomycin C-treated (18) (C57BL x DBA/2)$F_1$, spleen cells for 6 days at 37°C in 10 ml Falcon 3013 plastic tubes. The medium used was identical to that for the in vitro MLR assay except that it contained fetal calf serum (10%) instead of human serum. After harvest, the cultured cells were washed twice and incubated for 4 h at various ratios with $3 \times 10^4$ ~Cr-labeled target cells in small plastic tubes at a final volume of 0.3 ml.

Tumor Cells. DBA/2 mastocytoma cells (PS15-X2) and C57BL EL4 cells were passaged in vivo through DBA/2 and C57BL mice, respectively. To prepare target cells, tumor cells ($10^7$) were incubated with 200 mCi Na$_2$ ~CrO$_4$ in 1 ml for 30 min at 37°C and washed three times before use.

Results

Reactivity of TDL from Mice Recently Injected with Irradiated Semiallogeneic Spleen Cells

In previous studies, ASRL of GVH-reactive cells was induced by injecting mice with normal (nonirradiated) semiallogeneic spleen cells (1). An objection to this approach is that a proportion of the injected cells presumably recirculate, at least initially, and thereby contaminate the host TDL. Since this contamination might interfere with the response of the TDL, it was considered important in the present study to ensure that the injected spleen cells did not recirculate. The procedure adopted was to irradiate spleen cells in vitro and then culture the cells at 37°C for several hours before intravenous injection (see Materials and Methods). This treatment has previously been shown to allow the cells to localize in the spleen but to prevent migration to lymph nodes, i.e., to regions traversed by recirculating lymphocytes (20).

In initial experiments (data not shown), CBA mice were injected with $2 \times 10^8$ irradiated semiallogeneic spleen cells. This cell dose specifically abolished the capacity of TDL collected 1 day later to produce a GVH reaction against determinants present on the injected spleen cells. The response of the TDL in MLR, however, was reduced by only two to threefold. Since complete ASRL to antigens such as heterologous erythrocytes is observed only when the antigen is injected in large doses (2), it was decided to increase the number of spleen cells injected by threefold.

Unless stated otherwise, all the results considered below were obtained with TDL from CBA mice injected intravenously with $6 \times 10^8$ viable irradiated (1,000 R) semiallogeneic or syngeneic spleen cells cultured in vitro at 37°C for 5-6 h before intravenous injection. In most experiments, thoracic duct fistulas were established in the recipients 1 day later and TDL collected between 28 and 40 h. The identity of the TDL was investigated by incubating the cells with appropri-
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Table I
Identity of TDL from CBA Mice Injected with Irradiated (CBA × C57BL)F₁ or (CBA × DBA/2)F₁ Spleen Cells 1 Day Previously

| Source of TDL                        | Cytotoxic indices with:* |
|--------------------------------------|-------------------------|
|                                      | CBA anti-DBA/2 serum    | CBA anti-C57BL serum | C57BL anti-CBA serum |
| CBA mice given irradiated            |                         |                      |
| (CBA × C57BL)F₁, spleen              | –                       | 1                    | 98                   |
| CBA mice given irradiated            |                         |                      |
| (CBA × DBA/2)F₁, spleen              | 0                       | –                    | 100                  |
| Normal (CBA × C57BL)F₁, mice         | –                       | 99                   | 99                   |
| Normal (CBA × DBA/2)F₁, mice         | 100                     | –                    | 100                  |

* TDL collected 28–40 h after intravenous injection of 6 × 10⁶ viable irradiated spleen cells. TDL were incubated with antisera (1:4 dilution) at 5 × 10⁶ cells/ml at 37°C for 30 min, washed twice, and then incubated with complement (1:8 dilution); viability was assessed with eosin. Lysis in the presence of antiserum alone, complement alone, or with normal mouse serum and complement was <10%.

It can be seen from Table I that, in the case of TDL from recipients of (CBA × C57BL)F₁ spleen (henceforth referred to as "C57BL → CBA TDL"), virtually no cells were susceptible to lysis by CBA anti-C57BL antiserum. Similarly DBA/2 → CBA TDL were entirely resistant to lysis by CBA anti-DBA/2 serum. These data indicated that the injected irradiated spleen cells did not reach the central lymph of the host.

GVH Reaction. TDL were transferred to neonatal F₁ hybrid mice and splenic indices measured 10 days later. CBA → CBA TDL (TDL from recipients of syngeneic spleen) gave high splenic indices in both (CBA × C57BL)F₁ and (CBA × DBA/2)F₁ mice, significant splenomegaly being observed with as few as 0.3 × 10⁶ TDL (Fig. 1). By contrast, C57BL → CBA TDL, though evoking strong responses in (CBA × DBA/2)F₁ mice, failed to produce splenomegaly in (CBA × C57BL)F₁ mice even in doses as high as 3 × 10⁶ TDL. Similarly, DBA/2 → CBA TDL responded well against C57BL determinants, but not against DBA/2 determinants. Two other experiments gave similar results.

MLR. The capacity of TDL to produce an MLR was tested in vivo by transferring graded numbers of cells to irradiated (800 R) mice of different strains. The recipients were killed 5 days later, 45 min after intravenous injection of ³HThdr, and levels of radioactivity were measured in the spleen (see Materials and Methods). CBA → CBA TDL gave high proliferative responses MLR both against C57BL and DBA/2 determinants, i.e., when transferred to (CBA × C57BL)F₁ and (CBA × DBA/2)F₁ mice, respectively (Fig. 2). These responses were directly proportional to the number of TDL injected. Significantly, C57BL → CBA TDL also responded against C57BL determinants, although the magnitude of the response was reduced by 55% (compared to that of CBA → CBA TDL). There was no reduction in the response of C57BL → CBA TDL against third-party (DBA/2) determinants. Reciprocal results were obtained with DBA/2...
FIG. 1. Splenic indices in (a) (CBA × C57BL)F₁ and (b) (CBA × DBA/2)F₁, mice injected 10 days previously (at birth) with varying numbers of C57BL → CBA TDL (●), DBA/2 → CBA TDL (○), or CBA → CBA TDL (×). The three TDL populations were collected at 28-40 h after injecting CBA mice with $6 \times 10^6$ viable irradiated semiallogeneic or syngeneic spleen cells. Each point represents arithmetic mean (±SE) of data from six to eight mice.

FIG. 2. $^3$HtdR incorporation in spleens of irradiated (a) (CBA × C57BL)F₁ and (b) (CBA × DBA/2)F₁, mice injected 5 days previously with varying numbers CBA → CBA TDL (●), C57BL → CBA TDL (○), DBA/2 → CBA TDL (□), or normal syngeneic TDL (■). The three CBA TDL populations were collected at 28-40 h after intravenous injection of $6 \times 10^6$ viable irradiated semiallogeneic or syngeneic spleen cells. Radioactivity measured in spleens removed 45 min after intravenous injection of 25 μCi $^3$HtdR. Each point represents arithmetic mean (±SE) of data from 4-5 mice.

→ CBA TDL, i.e., there was a significant though reduced response against the specific (DBA/2) determinants and a near normal response against third-party (C57BL) determinants. Transfer of the three TDL populations to syngeneic (CBA) mice produced no response, i.e., $^3$HtdR incorporation was no higher than in mice not given cells (data not shown).

Four other experiments gave essentially similar findings. In no experiment was there more than a 70% reduction in the response against the determinants on the injected spleen cells. Responses against third-party determinants were
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Fig. 3. MLR measured in vitro of (a) CBA → CBA TDL, (b) C57BL → CBA TDL, and (c) DBA/2 → CBA TDL; TDL populations collected at 28-40 h after intravenous injection of 6 × 10⁹ viable irradiated semiallogeneic or syngeneic spleen cells. The stimulating cells (mitomycin C-treated spleen cells) were from (CBA × C57BL)F₁ (x), (CBA × DBA/2)F₁ (○), and CBA (●) mice. *HTdR was added to cultures 18 h before harvest. Each point represents mean levels of radioactivity (±SE) from triplicate cultures.

generally slightly reduced, although this was statistically significant in only one experiment.

Essentially similar results were obtained when MLR was measured in conventional mixed lymphocyte culture in vitro. As shown in Fig. 3, C57BL → CBA TDL gave a definite proliferative response against C57BL determinants when measured at either day 3, 4, or 5 culture. At 3 and 4 days the response was three to fourfold less than to third-party (DBA/2) determinants. At 5 days no reduction was observed; this was probably an artifact inasmuch as the response against DBA/2 determinants declined between day 4 and day 5, presumably as a consequence of overcrowding of the cultures. DBA/2 → CBA TDL gave reciprocal results, the response against DBA/2 determinants being three to fourfold less than that against C57BL determinants on day 3 and 4, but virtually identical on day 5.

The experiment illustrated in Fig. 4 examines both the kinetics and the magnitude of MLR given by TDL collected at various times after spleen cell injection. TDL were collected over 16 h from groups of the recipients at 1, 2, 3, or 6 days later, and MLR were measured in vivo.

CBA → CBA TDL collected at either 1 or 3 days after spleen cell transfer gave a strong MLR both against C57BL and DBA/2 determinants (Fig. 4). Responses were not detectable on day 2 of assay, but rose steeply thereafter and reached a peak on day 6. Similarly, a high response of normal kinetics was observed when C57BL → CBA TDL collected at 1, 2, 3, or 6 days posttransfer were exposed to third-party (DBA/2) determinants. Different results were obtained when these cells were exposed to the specific (C57BL) determinants. With C57BL → CBA TDL collected on day 1, the MLR against C57BL determinants, though reduced in magnitude by about 50%, showed normal kinetics. With C57BL → CBA TDL collected at 2, 3, or 6 days, however, both the magnitude and kinetics of the response changed. All three populations gave definite responses on day 2 of assay. Maximal responses were observed on day 4 of assay rather than on day 6,
FIG. 4. 3HTdR incorporation in spleens of irradiated (CBA × C57BL)F, and (CBA × DBA/2)F, mice injected 2, 4, or 6 days previously with CBA → CBA TDL or C57BL → CBA TDL (5 \times 10^6 TDL given intravenously); 25 μCi 3HTdR was given 45 rain before removing spleens. The C57BL → CBA TDL were collected at 1, 2, 3, or 6 days (a, b, c, d) after injecting CBA mice with 6 \times 10^6 irradiated (CBA × C57BL)F, spleen cells; CBA → CBA TDL were collected at 1 or 3 days (a, c) after injecting CBA mice with syngeneic irradiated spleen cells. Data shown: response of C57BL → CBA TDL in (CBA × C57BL)F, (O) and (CBA × DBA/2)F, (O) mice; response of CBA → CBA TDL in (CBA × C57BL)F, (□) and (CBA × DBA/2)F, (□) mice; response of syngeneic TDL in (CBA × C57BL)F, (▲) and (CBA × DBA/2)F, (▲) mice. Each point represents arithmetic mean of data from 4-6 mice; SE were within 10-15% of mean.

particularly with later TDL collections. In the case of TDL collected at day 3, the response against C57BL determinants was higher at each stage of assay than that of CBA → CBA TDL.

The above data showed that the reduction in the capacity of TDL to produce an MLR against determinants on the injected spleen cells was maximal at day 1 posttransfer. The fact that this reduction was incomplete might have been related to the irradiation of the injected spleen cells. To investigate this possibility, similar experiments were performed with mice given 6 \times 10^6 nonirradiated spleen cells; the cells were not cultured in vitro before injection. Among the TDL collected from CBA mice 1 day after injection of the above dose of nonirradiated (CBA × C57BL)F, spleen cells, 12–25% were susceptible to lysis by CBA anti-C57BL alloantisemur. This proportion of the TDL was therefore derived from the injected spleen cells. Table II shows the MLR measured in vivo of TDL collected at 28–40 h; the cells were treated with CBA anti-C57BL serum and complement before injection. It is evident that the response against C57BL determinants was reduced by only 41% compared to TDL from recipients of syngeneic spleen cells. Both populations of TDL gave comparable responses against DBA/2 determinants. Similar results were obtained in two other experi-
TABLE II

Proliferative Response In Vivo of TDL from CBA Mice given Nonirradiated CBA or (CBA × C57BL)F₁ Spleen Cells 1 Day Previously

| Group | Viable cells transferred* (4 × 10⁶) | Irradiated CBA | Irradiated (CBA × C57BL)F₁ | Reduction compared to group 2 | Irradiated (CBA × DBA/2)F₁ | Reduction compared to group 2 |
|-------|---------------------------------|----------------|-----------------------------|-------------------------------|-----------------------------|-------------------------------|
| 1     | -                               | 2.1 ± 0.2      | 2.6 ± 0.2                   | 2.7 ± 0.2                     |                             |                               |
| 2     | TDL from CBA mice given CBA spleen 1 day before | 1.9 ± 0.1 | 25.4 ± 0.8 | 34.0 ± 1.2 |                             |                               |
| 3     | TDL from CBA mice given (CBA × C57BL)F₁, spleen 1 day before | 2.0 ± 0.1 | 16.1 ± 0.6 | 41 | 33.3 ± 1.0 | 2 |

* TDL collected 28-40 h after intravenous injection of 6 × 10⁸ viable spleen cells; spleen cells were not cultured in vitro before injection. Before transfer to irradiated mice, both TDL populations were treated with CBA anti-C57BL alloantiserum and complement before injection, as described in footnote to Table I.
† Responses measured at 5 days after intravenous transfer of 4 × 10⁶ TDL. Each value represents the arithmetic mean levels of radioactivity in the spleen removed 45 min after intravenous injection of 25 μCi [³H]Tdr; 4-6 mice per group.

ments; in one, the responses were also measured in vitro. In neither experiment were MLR against the injected determinants reduced by more than 60%.

SKIN ALLOGRAFT REJECTION. CBA mice injected with 6 × 10⁸ irradiated and in vitro-culture (CBA × C57BL)F₁, spleen cells were cannulated 1 day later, and TDL were collected over the next 16 h. From 1-20 × 10⁶ TDL were injected into TxBM CBA mice. Within 2 h the recipients were grafted with skin from both C57BL and BALB/c mice. In control experiments, skin-grafted TxBM mice were injected with TDL from normal CBA mice. No rejection of either C57BL or BALB/c grafts was observed with TxBM mice not injected with TDL (Table III). TDL from normal CBA mice caused rapid rejection of both grafts even when low cell doses were used. Thus, 50% of the grafts were rejected within 21 days when as few as 10⁶ TDL were given. C57BL → CBA TDL had only a limited capacity to reject C57BL grafts. Even when as many as 2 × 10⁷ TDL were injected, grafts on three out of eight mice survived for at least 40 days; the grafts at this stage were healthy and carried luxuriant tufts of hair. From the data in Table III it is evident that the pattern of rejection of C57BL grafts by 10⁶ normal CBA TDL was similar to that shown by 1-2 × 10⁷ C57BL → CBA TDL, i.e., a 10-20-fold difference in cell numbers. In contrast to C57BL grafts, C57BL → CBA TDL were effective at rejecting BALB/c grafts. With cell doses exceeding 10⁶ TDL, all grafts were rejected within 21 days. The tempo of rejection of BALB/c grafts, however, was slightly slower with C57BL → CBA TDL than with normal CBA TDL (e.g., with transfer of 10⁵ cells) (Table III).

CML. TDL collected 28-40 h after injection of 6 × 10⁸ irradiated and in vitro cultured (CBA × C57BL)F₁, spleen cells were cultured for 6 days in vitro with mitomycin C-treated (C57BL × DBA/2)F₁, spleen cells as stimulators (see Mate-
### Table III

**Capacity of TDL to Cause Rejection of Skin Allografts on TxBM CBA Mice: Reactivity of TDL from CBA Mice Injected with Irradiated (CBA × C57BL)F₁, Spleen Cells 1 Day Previously**

| TDL transferred | Number of TDL injected | Number of TxBM recipients | C57BL | BALB/c |
|-----------------|------------------------|--------------------------|-------|-------|
|                 |                        |                          | 10-15 days | 16-20 days | >21 days | 10-15 days | 16-20 days | >21 days |
| Normal CBA      | -                      | 5                        | -      | -      | 5       | -      | -      | 5       |
| TDL            | 10⁶                   | 7                        | 1      | 3      | 3       | 1      | 2      | 4       |
|                 | 5 × 10⁶              | 5                        | 3      | 2      | -       | 3      | 2      | -       |
|                 | 10⁷                   | 5                        | 5      | -      | -       | 5      | -      | -       |
| TDL from CBA   | 10⁶                   | 14                       | -      | 2      | 12      | 4      | 4      | 9       |
| mice given     | 5 × 10⁶              | 10                       | -      | 2      | 8       | 3      | 7      | -       |
| (CBA × 10⁷      | 9                     | 2                        | 3      | 4      | 4       | 5      | 4      | -       |
| C57BL(F₁) spleen | 2 × 10⁷              | 8                        | 2      | 3      | 3       | 4      | 4      | -       |

* TDL collected 24-40 h after intravenous injection of 6 × 10⁶ viable irradiated spleen cells.

**Skin grafts applied within 2 h of injecting TDL.**

The cultured TDL were then harvested and incubated for 4 h in vitro at various ratios with ⁵¹Cr-labeled EL4 (C57BL) tumor cells or P815 (DBA/2) mastocytoma cells. "Activated" CBA → CBA TDL caused effective destruction of both types of target cells, significant lysis being detected with lymphocyte:target cell ratios as low as 2:1 (Table IV). C57BL → CBA TDL were also effective at lysing the DBA/2 tumor, but did not cause detectable lysis of the C57BL targets even at ratios as high as 50:1. Mixing the two cultured populations together during incubation with the target cells did not impair the capacity of the CBA → CBA TDL to lyse the C57BL tumor. Results similar to those shown in Table IV were obtained in two other experiments (data not shown); in one experiment, phytohemagglutin-transformed spleen cells, as well as the two tumor cell populations, were used as targets.

**Reactivity of TDL from Irradiated F₁ Mice Recently Injected with Parental Strain Lymph Node Cells.** The preceding data raised the question of whether irradiated cells under any circumstances could induce complete ASRL of cells involved in the MLR. Accordingly, it was decided to investigate the reactivity of parental strain T cells filtered from blood to lymph through irradiated F₁, mice. 10 (CBA × C57BL)F₁ mice were exposed to 1,000 R and then, within 1 h, injected intravenously with 10⁷ mesenteric lymph node (MLN) cells from normal CBA mice; 72% of these cells were θ-positive. The mice were cannulated 12 h later, and TDL was collected at various intervals over the next 3 days. Between 18 and 40 h after injection the lymph contained very few cells (3–5 × 10⁶/12 h per mouse). Of these cells <0.5% were blasts, <5% were of host origin, i.e. were susceptible to lysis by CBA anti-C57BL antiserum, and nearly all were T lymphocytes (98% θ-positive). Blast cells were first detected in the lymph at 44–48 h and by 60–72 h accounted for nearly all of the lymph-borne cells. At this stage, total cell outputs reached as high as 3 × 10⁷/12 h per mouse. Of these cells, <2% were of host origin and >98% were θ-positive.

Testing the proliferative capacity of the cells in vivo showed that pooled TDL collected at intervals between 18 and 48 h gave no detectable response to host-
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Table IV
Capacity of TDL to Differentiate into Cytotoxic Lymphocytes during Culture in vitro with C57BL and DBA/2 Determinants: Reactivity of TDL from CBA Mice Injected with Irradiated (CBA × C57BL)/F1, or CBA Spleen Cells 1 Day Previously

| CBA TDL* cultured in vitro for 6 days with mitomycin C-treated (C57BL × DBA/2)/F1, spleen cells | Lymphocyte:target cell ratio | Specific 51Cr-release with:† |
|---|---|---|
| | | EL4 (C57BL) targets | P815 mastocytoma (DBA/2) targets |
| TDL from mice given CBA spleen | 50:1 | 72.3 | 42.1 |
| | 25:1 | 54.9 | 31.7 |
| | 10:1 | 41.8 | 17.2 |
| | 2:1 | 13.8 | 12.0 |
| TDL from mice given (CBA × C57BL)/F1 spleen | 50:1 | 0.5 | 37.2 |
| | 10:1 | −3.3 | 13.1 |
| | 2:1 | −0.3 | 5.4 |
| TDL from mice given CBA spleen + (CBA × C57BL)/F1 spleen | 25 | 51.5 | 37.9 |
| TDL from mice given (CBA × C57BL)/F1 spleen | 10 | 30.1 | 14.7 |

* TDL collected 28–40 h after intravenous injection of 6 × 10⁸ viable irradiated spleen cells. For conditions of culturing TDL with mitomycin C-treated stimulators, see Materials and Methods.
† After activation in vitro, lymphocytes were cultured with 3 × 10⁴ target cells for 4 h. Each value represents mean of triplicate cultures; SE were within 10% of mean. Spontaneous release of ⁵¹Cr from EL4 was 9% and from P815, 11%.

Type (C57BL) determinants at either day 2 or 5 of assay (Table V). High responses, however, were observed against third-party (BALB/c) determinants (demonstrable on day 5 though not on day 2). With TDL collected at 64–72 h, i.e. cells consisting almost entirely of blast T cells (vide supra), low but significant responses were observed against both sets of determinants. The responses were higher on day 2 of assay than day 5 and higher against C57BL than BALB/c determinants. No responses were obtained when the cells were transferred to syngeneic (CBA) mice (data not shown).

TDL from normal CBA mice gave high late responses against both sets of determinants, definite responses being observed with as few as 10⁵ cells. With respect to C57BL determinants, it is to be noted that the "filtered" MLN cells were totally unresponsive even with cell numbers as high as 10⁶.

Discussion
By parameters other than the MLR, TDL collected from mice injected 1 day previously with 6 × 10⁸ irradiated and in vitro cultured semiallogeneic spleen cells were specifically depleted of lymphocytes reactive to the H-2 determinants on the injected cells. For GVH reactivity the depletion was >10-fold, for skin
allograft rejection 10-20-fold, and for CML >25-fold (calculated from data in Fig.
1, Tables III and IV, respectively). Reactivity against third party determinants
was usually not significantly reduced. Whether the use of such massive doses of
spleen cells is essential to achieve the degree of depletion observed has yet to be
investigated. It may be added that the cell dose used caused no detectable
decrease in TDL outputs (unpublished data).

With regard to GVH reactivity, recent experiments have shown that the
unresponsiveness affecting TDL does not apply to cells recovered from the
spleen (unpublished data of the authors). This, together with previous evidence
that TDL regain their reactivity after 3 days (1), is consistent with the hypothe-
sis that the transient unresponsiveness observed with TDL at 1-2 days in the
present study was the result of ASRL to organs such as the spleen. ASRL would
also seem the most likely explanation for the unresponsiveness detected towards
determinants controlling skin allograft rejection and CML. In this respect,
preliminary studies have indicated that the unresponsiveness towards CML
determinants does not apply to (a) spleen cells removed on day 1 after allogeneic
cell transfer or (b) TDL collected on day 5. It should be emphasized that in the
case of CML there was no evidence that the unresponsiveness of TDL collected
on day 1 resulted from active suppression (Table IV).

The failure to produce in TDL more than a partial depletion of cells reactive to
MLR determinants was unexpected. In no experiments did the degree of depletion exceed fourfold, and in most experiments it was only two to threefold. The possibility that the restriction in the degree of depletion observed reflected destruction and loss of immunogenicity of the injected spleen cells due to their prior irradiation is unlikely, since similar results were obtained with nonirradiated spleen cells (Table II). The present findings contrast with reports of other workers. In sheep, it was found that within 2–3 days of injecting allogeneic lymphocytes into the draining area of the popliteal lymph node, cells collected from the efferent lymphatics of the injected node and also from the contralateral node were completely devoid of cells reactive to the injected determinants in MLR (21). In rats, peripheral blood lymphocytes taken shortly after intravenous injection of low doses of allogeneic spleen cells (e.g. \(3 \times 10^7\)) were profoundly unresponsive in MLR against the injected determinants (22, 23). In contrast to the present findings and those in sheep (21) (both using lymph-borne cells), the unresponsiveness observed in rats lasted for several weeks; this was shown to be due in part to the presence of blocking factors (23).

Our observation that the residual activity of 'selected' TDL populations was directed solely towards MLR determinants is consistent with evidence that lymphocytes reactive to MLR determinants have a different specificity from those controlling CML and skin allograft rejection (6, 7, 9, 24, 25). To account for the dichotomy in the ability of "selected" TDL to respond in MLR but not to evoke GVH reactions, however, is more difficult since, as mentioned in the introduction, the (I-region) determinants which control these two reactions are generally considered to be identical. One possibility is that the residual MLR exerted by the TDL was directed not against \(H-2\) but M-locus (Mls) determinants (26), i.e., determinants that do not evoke GVH reactions by the assay used in the present paper. This is unlikely because in the case of the response of CBA TDL to C57BL determinants shown in Table II and Fig. 3, CBA/Ca mice were used (see Materials and Methods), CBA/Ca and C57BL being M-locus compatible (Mls b) (ref. 26 and unpublished data of the authors).

A tentative explanation for the capacity of "selected" TDL populations to respond in MLR, but not to evoke GVH reactions, is offered as follows. It is suggested that: (a) the affinity of lymphocytes for Ig-region-controlled \(H-2\) determinants varies, both high- and low-affinity cells proliferating in MLR, but only high-affinity cells being able to evoke GVH reactions (splenomegaly in newborn mice); (b) ASRL after injection of I-region-incompatible spleen cells in the present system affects only high-affinity cells, the low-affinity cells remaining in the circulation and thereby accounting for the residual capacity of TDL to proliferate in MLR, but not to evoke GVH reactions. It follows from this hypothesis that ASRL of low-affinity cells would occur only after exposure to extremely high concentrations of the selecting determinants, i.e., higher than can be administered intravenously. This prediction is consistent with the results of the experiments in which parental strain T cells were filtered from blood to lymph through irradiated F₁ hybrid mice, i.e., a situation where the hosts per se presented the selecting determinants (Table V). Thus, when CBA lymph node cells were transferred to irradiated (CBA × C57BL)F₁ mice, the cells of donor
origin, which entered thoracic duct lymph 18–48 h later, produced no detectable MLR against C57BL determinants but responded well to third party (BALB/c) determinants. This system was originally described by Ford and Atkins (5) who observed that parental strain TDL filtered from blood to lymph through sublethally irradiated AgB-incompatible F₁ rats were specifically devoid of cells capable of evoking a GVH reaction (popliteal lymph node enlargement) against host-type determinants. These findings were subsequently confirmed by Dorsch and Roser (27) who made the additional observation that the filtered TDL were specifically depleted cells capable of rejecting host-type skin allografts.

With both systems used in the present study, cells capable of responding in MLR against the sensitizing determinants re-entered the lymph after 2–3 days. This was associated with a marked alteration in the kinetics of the response, particularly with cells recovered from irradiated F₁ recipients of parental-strain lymph node lymphocytes. In this situation, TDL recovered after 48 h produced an MLR that was clearly detectable on day 2 of assay, but was very low on day 5; similar results were obtained previously with lymph-borne cells recovered from irradiated F₁ recipients of parental strain thymus cells (28). Since most of the cells in the lymph at times after 48 h were θ-positive blast cells, these findings imply that ASRL ceased after day 2, the activated progeny of the recruited cells then entering the lymph in large numbers and synthesizing DNA rapidly when re-exposed to the sensitizing determinants.

Alteration in the kinetics of MLR was also a feature of late TDL collections from parental strain recipients of irradiated F₁ spleen cells. For example, with TDL collected on day 4, the MLR against the sensitizing determinants (a) was of rapid onset, (b) peaked at 4 days (instead of 6 days with unstimulated TDL), and (c) was above normal in amplitude (Fig. 4); similar findings were reported previously for peripheral blood lymphocytes of rats sensitized subcutaneously (22). Interpreting these data concisely is clearly difficult. Perhaps the simplest explanation is that only a proportion of the cells that re-entered the lymph subsequent to ASRL at day 1 were in an activated state. Studies of Ford et al. (29) are relevant here. These workers observed that whereas 12% of lymphocytes transferred to irradiated AgB-incompatible rats were selectively recruited to the spleen, only 50% of these cells underwent blast transformation. It was suggested that the nontransformed recruited cells might have been of low affinity. Whether these cells subsequently re-entered the circulation was not investigated.

Summary

Information was sought on the reactivity of thoracic duct lymphocytes (TDL) from parental strain mice injected intravenously with large numbers of irradiated semiallogeneic spleen cells. TDL collected at 1 day after spleen cell injec-

² Of peripheral interest is the fact that whereas only 72% of the injected lymph node cells were θ-positive, θ-positive lymphocytes accounted for 98% of the cells recovered from the lymph at 18–40 h (>95% of these cells were of donor origin). This would appear to confirm previous findings that, in mice, B cells recirculate far more slowly than T cells (10).
tion were almost totally depleted of lymphocytes able to produce cell-mediated
lympholysis (CML), a graft-versus-host (GVH) reaction, and skin allograft rejection
against the H-2 determinants on the injected spleen cells. Normal or near
normal responses were observed against third-party determinants. In the case of
CML, there was no evidence that the unresponsiveness was due to suppressor
cells.

In marked contrast, the capacity of TDL to exert a specific mixed lymphocyte
reaction (MLR) against the injected determinants was reduced by no more than
two to fourfold; this applied whether MLR were measured in vivo or in vitro.
Injection of normal rather than irradiated semiallogeneic spleen cells gave
similar results. Complete and specific removal of MLR-producing lymphocytes
was achieved, however, in a different system in which parental strain T cells
were filtered from blood to lymph through irradiated F1 hybrid mice. Since this
system presumably provided a much higher concentration of H-2 determinants
to the responding lymphocytes, it is suggested that the differing results obtained
with these two systems may indicate that certain cells reactive to H-2 determi-
nants are of low affinity, their reactivity being detected in the MLR, but not by
other parameters. With both systems, MLR-producing lymphocytes reappeared
in the lymph after 2–3 days; the cells collected at this stage gave an MLR of
altered kinetics.

The present data, in toto, suggest that under certain conditions of antigen
presentation, virtually all recirculating lymphocytes reactive to a given set of H-
2 determinants can be induced to leave the circulation for a period of 1–2 days.
After responding to the injected determinants (presumably in organs such as the
spleen), the cells re-enter the circulation in an activated state after 2–3 days.

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References
1. Sprent, J., J. F. A. P. Miller, and G. F. Mitchell. 1971. Antigen-induced selective
recruitment of circulating lymphocytes. Cell. Immunol. 2:171.
2. Sprent, J., and J. F. A. P. Miller. 1974. Effect of recent antigen priming on adoptive
immune responses. II. Specific unresponsiveness of circulating lymphocytes from
mice primed with heterologous erythrocytes. J. Exp. Med. 139:1.
3. Rowley, D. A., J. L. Gowans, R. C. Atkins, W. L. Ford, and M. E. Smith. 1972. The
specific selection of recirculating lymphocytes by antigen in normal and preimmun-
ized rats. J. Exp. Med. 136:499.
4. Sprent, J., and J. F. A. P. Miller. 1973. Effect of recent antigen priming on adoptive
immune responses. I. Specific unresponsiveness of cells from lymphoid organs of mice
primed with heterologous erythrocytes. J. Exp. Med. 138:143.
5. Ford, W. L., and R. C. Atkins. 1971. Specific unresponsiveness of recirculating
lymphocytes after exposure to histocompatibility antigens in F1 hybrid rats. Nat.
New Biol. 234:178.
6. Bach, F. H., M. B. Widmer, M. L. Bach, and J. Klein. 1972. Serologically-defined and
lymphocyte-defined components of the major histocompatibility complex in the mouse. *J. Exp. Med.* 136:1430.

7. Meo, T., J. Vives, V. Miggiano, and D. Shreffler. 1973. A major role for the Ir-1 region of the mouse *H-2* complex in the mixed-leucocyte reaction. *Transplant. Proc.* 5:377.

8. Klein, J., and J. M. Park. 1973. Graft-versus-host reaction across different regions of the *H-2* complex of the mouse. *J. Exp. Med.* 137:1213.

9. Bach, F. H., M. Segall, K. S. Zier, P. M. Sondel, B. J. Alter, and M. L. Bach. 1973. Cell mediated immunity: separation of cells involved in recognitive and destructive phases. *Science (Wash. D.C.)* 180:403.

10. Sprent, J. 1972. Circulating T and B lymphocytes of the mouse. I. Migratory properties. *Cell. Immunol.* 7:10.

11. Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.* 99:167.

12. Reif, A. E., and J. M. V. Allen. 1964. The AKR thymic antigen and its distribution in leukaemia and nervous tissues. *J. Exp. Med.* 120:413.

13. Boyse, E. A., L. J. Old, and E. Stockert. 1962. Some further data on cytotoxic isoantibodies in the mouse. *Ann. N. Y. Acad. Sci.* 99:574.

14. Billingham, R. E., and P. B. Medawar. 1951. The technique of free skin grafting in mammals. *J. Exp. Biol.* 28:385.

15. Miller, J. F. A. P. 1960. Studies on mouse leukaemia. II. The role of the thymus in leukaemogenesis by cell-free leukaemic filtrates. *Br. J. Cancer.* 14:93.

16. Sprent, J., and J. F. A. P. Miller. 1972. Interaction of thymus lymphocytes with histoincompatible cells. I. Quantitation of the proliferative response of thymus cells. *Cell. Immunol.* 3:361.

17. Nabholz, M., J. Vives, H. M. Young, T. Meo, V. Miggiano, A. Rijnbeek, and D. C. Shreffler. 1974. Cell-mediated cell lysis in vitro: genetic control of killer cell production and target specificities in the mouse. *Eur. J. Immunol.* 4:378.

18. H. von Boehmer. 1974. Separation of T and B lymphocytes and their role in the mixed lymphocyte reaction. *J. Immunol.* 112:70.

19. Simonsen, M. 1962. Graft-versus-host reactions. Their natural history and applicability as tools of research. *Prog. Allergy* 6:349.

20. Anderson, R. E., J. Sprent, and J. F. A. P. Miller. 1974. Radiosensitivity of T and B lymphocytes. I. Effect of irradiation on cell migration. *Eur. J. Immunol.* 4:199.

21. Hay, J. B., R. N. P. Cahill, and Z. Trnka. 1974. The kinetics of antigen-reactive cells during lymphocyte recruitment. *Cell. Immunol.* 10:145.

22. Wilson, D. B., and P. C. Nowell. 1971. Quantitative studies on the mixed lymphocyte interaction of rats. V. Tempo and specificity of the proliferative response and the number of reactive cells from immunized donors. *J. Exp. Med.* 131:442.

23. Larner, B. J. 1973. Redistribution of mixed lymphocyte culture-reactive cells after antigen administration. *Transplantation (Baltimore).* 16:134.

24. Cantor, H., and E. A. Boyse. 1975. Functional subsets of T lymphocytes bearing different Ly antigens. II. Cooperation between subclasses of Ly + cells in the generation of killer activity. *J. Exp. Med.* 141:1391.

25. Sprent, J., H. von Boehmer, and M. Nabholz. 1975. Association of immunity and tolerance to host *H-2* determinants in irradiated F1 hybrid mice reconstructed with bone marrow cells from one parental strain. *J. Exp. Med.* 142:321.

26. Festenstein, H. 1973. Immunogenetic and biological aspects of in vitro lymphocyte allotransformation (MLR) in the mouse. *Transplant. Rev.* 15:62.

27. Dorsch, S., and B. Roser. 1974. The clonal nature of alloantigen-sensitive small lymphocytes in the recirculating pool of normal rats. *Aust. J. Exp. Biol. Med. Sci.* 52:45.
28. Cheers, C., J. Sprent, and J. F. A. P. Miller. 1974. Interaction of thymus lymphocytes with histoincompatible cells. IV. Mixed lymphocyte reaction of activated thymus lymphocytes. Cell Immunol. 10:57.

29. Ford, W. L., S. J. Simmonds, and R. C. Atkins. 1975. Early cellular events in a systemic graft-vs.-host reaction. II. Autoradiographic estimates of the frequency of donor lymphocytes which respond to each Ag-B-determined antigenic complex. J. Exp. Med. 141:681.