MURINE SYNGENEIC MIXED LYMPHOCYTE RESPONSE

I. Target Antigens Are Self Ia Molecules

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The syngeneic or autologous mixed lymphocyte response (MLR)1 is the proliferation that occurs when T lymphocytes are cocultured with autologous or syngeneic non-T cells. An autologous MLR was first described in mice (1-3) and has since been studied in human (4-6), murine (7), and guinea pig (8) systems, although the bulk of the more recent data has been obtained using human cells. The paucity of data in the murine system stems in part from the low magnitude of the murine response in standard cultures. The best murine response to date has recently been reported by Steinman and co-workers (9) using Ia-bearing dendritic cells from mouse spleen as stimulators. Nonetheless, many of the central questions regarding the murine syngeneic MLR remain unanswered.

We utilized improved culture conditions coupled with stimulator cells obtained from the first step of the method used for dendritic cell purification (9) to obtain a murine syngeneic MLR that is comparable in magnitude to an allogeneic MLR or secondary antigen-induced proliferative response. With the aid of this assay system the identity of the target antigen in the syngeneic MLR was examined in semiallogeneic F1 → parent and fully allogeneic radiation-induced bone marrow chimeras. The response of the T cells in the syngeneic MLR was found to be determined by the environment in which the cells matured. These T cells did not appear to be directed at any foreign antigens present in the culture system but required the presence of "learned" self Ia molecules to proliferate, as determined both by mapping studies using recombinant strains and by blocking studies using monoclonal anti-Ia antibodies. The experiments suggest that the syngeneic MLR does indeed represent a T cell anti-self reaction.

Materials and Methods

Animals. C57BL/10, B10.A, B10.A(5R), A/J, and C57BL/6 strains were purchased from The Jackson Laboratory, Bar Harbor, Maine. The B10.A(4R) strain was purchased from Simonsen Laboratories, Gilroy, Calif., and the B10.A(2R) were obtained from Sprague Dawley Laboratories, Madison, Wis. The B10.A(18R) B10.A(3R), B10.BR, and (B10 × B10.A)F1 [(B × A)F1] strains were bred in our own colony (Laboratory of Immunology, National Institutes of Health, Bethesda, Md.). Conventional and germ-free (GF) BALB/c AnN, C3H/HeN, and

1 Abbreviations used in this paper: APC, antigen-presenting cells; BSA, bovine serum albumin; C, complement; CFA, complete Freund's adjuvant; FCS, fetal calf serum; GF, germ-free; LN, lymph node; MHC, major histocompatibility complex; MLR, mixed lymphocyte response; NMS, normal mouse serum; PPD, purified protein derivative; (T,G)-A--L, poly(Tyr,Glu)-poly D, l-Ala--poly Lys.
B10.D2/nSnN strains were obtained from the Division of Research Services, National Institutes of Health.

**Preparation of Responder Cells.** Responder T cells were purified from axillary, inguinal, and mesenteric lymph nodes (LN) or spleen cells by passage over nylon wool columns (10). The nylon nonadherent population contained <1% surface Ig-bearing cells by direct immunofluorescence and <1-2% macrophages by the criterion of latex ingestion. These cells cultured alone always gave <5% of the response obtained when stimulator cells were added.

**Preparation of Stimulator Cells.** Splenic stimulator cells enriched for macrophages were isolated on a bovine serum albumin (BSA) gradient by a modification of the technique of Belier and Unanue (11) and Nussenzweig and Steinman (9). BSA (Sigma Chemical Co.) was obtained as a sterile 35% solution, and dilutions were made with RPMI 1640. The gradient was prepared in nitrocellulose acetate tubes by layering 5 ml of 11% BSA over 5 ml of 35% BSA containing $4 \times 10^8$ to $6 \times 10^8$ spleen cells and then centrifuging in an SW27 rotor in a Beckman L8-70 centrifuge (Beckman Instruments, Inc., Fullerton Calif.) at 4°C for 30 min. Cells banding between 35% and 11% were used as the stimulator cell population. They comprised roughly 5% of the starting population and were found to be ~50% macrophages by the criterion of latex ingestion. Stimulators were also prepared using a Percoll gradient by layering 2-4 ml of $2 \times 10^8$ to $4 \times 10^8$ spleen cells in RPMI 1640 over 2-3 ml of a 50% Percoll solution and spinning at 3,000 rpm for 12 min at 4°C. In some experiments, whole spleen cell populations were also used as a source of stimulator cells. All stimulators were irradiated with 2,500 rad using a 137Cs irradiator.

**Preparation of Radiation-induced Bone Marrow Chimeras.** The study of the effects of the developmental milieu on T cell function has been facilitated by using radiation-induced bone marrow chimeras (12). Such animals are prepared by injecting bone marrow cells depleted of mature T cells into mice that have been lethally irradiated. When the chimeras recover, all their lymphocytes and hematopoietic cells are of bone marrow donor origin, whereas all other tissues are of host genotype. Experiments with such radiation-induced bone marrow chimeras have shown that the T cell specificity for self major histocompatibility complex (MHC) gene products is acquired in the thymus during development. For example, type A cells maturing in an (A × B)F1 animal develop the ability to interact with cells bearing both B and unique F1 MHC products in addition to cells bearing A MHC products, whereas (A × B)F1 cells maturing in a type A animal only develop the ability to interact with cells bearing A MHC products. Thus, the donor T cells maturing in the chimeric host develop receptors with specificity for the MHC antigens of the host.

We used the above experimental design to examine the effects of the developmental milieu on the specificity of the T cells responding in the syngeneic MLR. To do this, bone marrow donors were depleted of mature T cells by in vivo treatment with antithymocyte serum and cortisone acetate and in vitro treatment of bone marrow cells twice with monoclonal anti-Thy-1.2 (New England Nuclear, Boston, Mass.) and complement (C) (13). Mice irradiated with 954 rad from a 137cesium source were reconstituted with $10^7$ T cell-depleted bone marrow cells given intravenously. Mice were not used until at least 3 mo after reconstitution. Greater than 95% of their spleen cells were confirmed to be of bone marrow donor origin at the time they were tested for their restriction specificity in the syngeneic MLR.

**Proliferation Assay.** T lymphocytes were mixed with syngeneic irradiated stimulator cells in varying concentrations in RPMI 1640 containing L-glutamine (300 µg/ml), penicillin-streptomycin, 2-mercaptoethanol (5 × 10^{-5} M), Hapes buffer (10 mM), and either 2.5% human AB serum (obtained from a single donor) or in certain experiments with 0.75% fresh syngeneic normal mouse serum (NMS). The cells were cultured in 0.2 ml of solution in round-bottomed microtiter plates at 37°C in 5% CO2. After 4-5 d of culture and 16-24 h before harvesting, 1 µCi of [3H]thymidine (New England Nuclear) was added to each well, and the amount of radioactivity incorporated into macromolecules was measured. Data are expressed as the arithmetic mean cpm ± SEM.

**Secondary Syngeneic MLR.** Preliminary experiments indicated that an optimal secondary response was produced by first culturing $3 \times 10^9$ lymph node T responder cells with $6 \times 10^8$ BSA gradient purified stimulator cells in human serum for 14-17 d. The surviving cells were treated with a monoclonal anti-Ia antibody plus C (see below) to ensure removal of any residual...
stimulator cells, washed two times, and then replated at $5 \times 10^4$ to $10 \times 10^4$ viable cells per well with or without stimulator cells.

**Treatment of Cells with Antisera and C.** A monoclonal anti-I-$A^b$ ascites (25-5-16) reactive with the private specificity Ia.20 (titer 1/8,000) was kindly provided by Dr. David Sachs, National Cancer Institute, National Institutes of Health, Bethesda, Md. Each $5 \times 10^6$ cells were treated with 1 ml of the ascites diluted 1:250 at $4^\circ$ for 30 min, washed, and then treated with 1 ml of rabbit C (Lo-Tox, Cedar Lane) diluted 1:15 at $37^\circ$ for 40 min.

**Blocking Studies.** A monoclonal anti-I-$A^b$ reagent (clone 10.2.16) reactive with the public specificity Ia.17 was kindly provided by Dr. Richard Hodes, National Cancer Institute. Another monoclonal antibody, the 17.3.3 reagent, which is directed at the A/E combinatorial molecule (Ia specificity 22), has recently been described (14) and was the kind gift of Dr. David Sachs, National Cancer Institute. Both reagents were culture supernatants and were used at a 1:10 or 1:15 dilution in the culture system in the absence of C.

**Results**

**A Substantial Syngeneic MLR Can Be Obtained in the Murine System Using BSA Gradient Fractionated Spleen Cells as Stimulators.** While performing unrelated experiments in this laboratory, it was observed that spleen cells harvested from the top interface of a discontinuous BSA gradient could serve as powerful antigen-presenting cells (APC) for conventional in vitro proliferative responses to foreign antigen. It was noticed, however, that if the culture period was extended past the usual 4 d used in antigen-induced proliferation assays, a very significant proliferative response occurred by day 5 or 6 in the absence of added antigen. We therefore sought culture conditions that would maximize this apparent syngeneic MLR.

Table I shows the kinetics of the proliferative response obtained by coculturing $3 \times 10^5$ nylon wool-passed LN cells with varying numbers of BSA gradient-separated stimulators. The peak proliferative response occurred on day 6 and was of comparable magnitude to an allogeneic MLR. The magnitude of the response also increased with increasing numbers of stimulator cells up to a dose of $6 \times 10^6$ cells. In additional experiments, it was found that human AB serum gave better responses than fetal calf serum (FCS), although both human serum and FCS from several sources were effective. A titration performed with human AB serum from a single source revealed that optimum responses occurred at a final concentration of 2–2.5% (vol/vol) serum in the culture medium.

**The Responding T Cells in the Syngeneic MLR Exhibit Both Memory and Specificity.** To determine whether the T cells responding in a syngeneic MLR have the classic immunologic characteristics of memory and specificity, we tested these populations in a secondary in vitro response. The kinetics of proliferation were accelerated compared

| Number of days in culture | 0 | 0.75 | 1.5 | 3 | 6 | 12 |
|---------------------------|---|------|-----|---|---|----|
| Number of stimulator cells/well $\times 10^5$ | $900 \pm 19$ | $9,324 \pm 299$ | $20,704 \pm 1,286$ | $19,174 \pm 4,209$ | $20,704 \pm 1,286$ | $12,640 \pm 737$ |

* $3 \times 10^5$ nylon wool column-passed C57BL/6 LN cells were cocultured with varying numbers of stimulator cells in 2-2.5% human serum.
to the primary response with a peak response occurring on day 4 as opposed to day 6 (Figs. 1 and 2), suggesting a memory component similar to what has been observed for allogeneic MLR (15). In addition to memory, T cells cultured with syngeneic stimulators in a primary culture displayed specificity because a greatly diminished proliferation to allogeneic stimulators was observed in the secondary culture (Fig. 2).

The Response of T Cells in a Syngeneic MLR Is Determined by the Environment in Which the Cells Mature. Evidence has accumulated over the past few years (12) to suggest that the helper, cytotoxic, proliferative, and delayed-type hypersensitivity T cell repertoires are influenced by the environment in which these T cells mature. To determine whether the responding T cells in the syngeneic MLR acquire their particular specificity during development, T cells from radiation-induced bone marrow chimeras of the F1 → parent type were examined for their syngeneic MLR against stimulators from both parental strains. The results of five experiments using H-2 congenic strains are shown in Table II. In three of the five experiments, the same population of stimulators was tested with the cells from the reciprocal chimeras. The (B10 × B10.A)F1 chimeric T cells that matured in a B10 environment responded predomi-

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![Figure 1](image1.png)

Fig. 1. A secondary syngeneic MLR can be obtained in normal mouse serum with syngeneic unfractionated spleen cells as stimulator cells. The cells from a primary syngeneic MLR culture performed with BSA-gradient separated stimulator cells in the presence of human serum were harvested at day 14, treated with a monoclonal anti-Ia reagent plus C to remove any residual stimulator cells, and replated at 8 × 10⁴ cells/well in the presence of 0.75% NMS either alone (○) or with BSA-gradient separated spleen cells (●) or unfractionated spleen cells (△) as stimulators.

![Figure 2](image2.png)

Fig. 2. Spleen cells from germ-free mice stimulate a secondary syngeneic MLR. The cells from a primary BALB/c syngeneic MLR culture were restimulated in a secondary syngeneic MLR with BSA-gradient separated spleen or unfractionated spleen from BALB/c mice raised in a germ-free environment or under routine environmental conditions in the presence of mouse serum from the germ-free animals. BSA germ-free, ○; BSA normal, ■; unfractionated germ-free, △; unfractionated normal, (△—△). Also included in this experiment is the proliferation elicited by allogeneic (C3H) spleen cells (○). No stimulator cells, (●).
Table II
Syngeneic MLR Is MHC Restricted

| Source of stimulator cells | Proliferative response (cpm ± SEM)* | Experiment 1 | Experiment 2 | Experiment 3 | Experiment 4 | Experiment 5 |
|---------------------------|-------------------------------------|-------------|-------------|-------------|-------------|-------------|
| Source of stimulator cells |                                     | B10         | B10 A       | B10         | B10 A       | B10         |
| B10                       | 285,249 ± 25,351                    | 42,267 ± 4,185 | 134,549 ± 7,994 | 140,077 ± 6,506 | 52,472 ± 184 |
| B10 A                     | 8,319 ± 1,296                       | 2,817 ± 733  | 16,421 ± 462 | 3,519 ± 584 | 52,472 ± 184 |
| F1 → B10 chimeric T cells |                                     | B10         | B10 A       | B10         | B10 A       | B10         |
| B10                       | 4,140 ± 2,962                       | 12,697 ± 1,675 | 6,507 ± 967 | 104,114 ± 3,996 | ND | ND |
| B10 A                     | 40,904 ± 4,183                      | 52,351 ± 6,457 | ND | ND | ND |
| F1 → B10.A chimeric T cells |                                     | B10         | B10 A       | B10         | B10 A       | B10         |
| B10                       | 30,041 ± 1,964                      | 30,041 ± 1,964 | 30,041 ± 1,964 | 30,041 ± 1,964 | 30,041 ± 1,964 |
| B10 A                     | 27,061 ± 811                        | 27,061 ± 811 | 27,061 ± 811 | 27,061 ± 811 | 27,061 ± 811 |

* 3 × 10^5 nylon wool column-passed F1 → P chimeric spleen cells were cocultured with varying numbers of parental stimulator cells in 2.5% human serum. Only the maximum response obtained by each parental stimulator cell is shown.

† Not done.

nantly to B10 as compared to B10.A stimulator cells. Conversely, (B10 × B10.A)F1 chimeric T cells that matured in a B10.A environment responded predominantly to B10.A as compared with B10 stimulators. The host preference in the series of experiments presented in this paper ranged from 40-fold to 4-fold, with a geometric mean of 13-fold. In contrast, these same T cells exhibited no host preference when a mitogen-induced response was obtained in the presence of the same BSA gradient-separated spleen cells (not shown). Responder cells from normal (B10 × B10.A)F1 animals proliferated equally well to stimulator cells from either parental strain (Table II). Thus, it appears that the MHC haplotype of the host environment determines the response pattern of the responding T cells in the syngeneic MLR.

The T Cells That Proliferate in the Syngeneic MLR Recognize K and/or I-Region-encoded Determinants. The identity of the target antigen(s) on the syngeneic stimulator cells that provokes T cell proliferation has been unclear, although the presence of an Ia-bearing cell has been shown to be necessary for proliferation to occur (16, 17). In the preceding section, it was shown that T cells respond only to stimulator cells sharing the MHC genes of the environment in which the T cells matured. Knowing this, it was possible to use stimulators from parental H-2 recombinant strains along with F1 → P chimeric responder T cells to map the genes that encode the antigen required for proliferation.

Table III shows the results of three separate mapping experiments using the (B10 × B10.A)F1 → B10 chimeras. Stimulator cells obtained from B10, B10.A(5R), B10.A(18R), and B10.A(3R) strains, all of which are K^b, I-A^b, elicited good responses with F1 → B10 chimeric T cells. Stimulators from B10.A(4R) and B10.A(2R) strains, which only have portions of the H-2^b haplotype to the right of the I-A subregion, did not elicit a substantial response. These results indicate that antigens encoded in the K region and/or the I-A subregion of the H-2^b haplotype are involved in stimulating a syngeneic MLR. In all three experiments B10.A(3R) and B10.A(5R) spleen cells, although having b alleles in the I-A subregion, consistently gave lower stimulations than B10 and B10.A(18R) spleen cells. The reason for this is not clear.

The results obtained in four separate experiments with (B10 × B10.A) F1 → B10.A chimeras are shown in Table IV. None of the MHC recombinant stimulators tested
TABLE III

Ability of Splenic APC from I-Region Recombinant Strains to Stimulate a Syngeneic MLR in F1 → B10 Chimeric T Cells

| Source of stimulator cells* | MHC alleles† | Proliferative response (cpm ± SEM)‡ |
|-----------------------------|--------------|-----------------------------------|
|                             |              | Experiment 1 | Experiment 2 | Experiment 3 |
| B10                         | bbbbbbbb     | 47,267 ± 4,185 | 41,171 ± 3,194 | 82,472 ± 184 |
| B10.A(5R)                   | bbbkkddd     | 2,817 ± 733   | 5,072 ± 1,287 | 7,337 ± 1,386 |
| B10.A(4R)                   | kkkkkkk     | 35,738 ± 4,519 | 27,764 ± 4,238 |
| B10.A(3R)                   | bbbkkkdd    | 1,915 ± 217   | 25,201 ± 5,304 | 55,132 ± 352 |
| B10.A(18R)                  | bbbkbbbd    | 63,630 ± 1,146 | 51,799 ± 6,073 |
| (B10 × B10.A)F1            | bbbbbbddd   | 82,472 ± 184 | 7,337 ± 1,386 | 55,132 ± 352 |

* 3 × 10⁶ nylon wool column-passed F1 → B10 chimeric spleen cells were cocultured with varying numbers of stimulator cells from the recombinant strains listed, and the maximum response obtained by each type of stimulator cell is shown.
† Letters represent the haplotype source of origin of the K, I-A, I-B, I-J, I-E, I-C, S, and D regions and subregions of the murine MHC.
‡ Counts per minute of nylon wool-passed chimeric T lymphocytes cultured alone was 152 ± 17 for experiment 1, 1,749 ± 62 for experiment 2, and 322 ± 39 for experiment 3.

TABLE IV

Ability of Splenic APC from I-Region Recombinant Strains to Stimulate a Syngeneic MLR in F1 → B10.A Chimeric T Cells

| Source of stimulator cells | MHC alleles* | Proliferative response (cpm ± SEM)‡ |
|----------------------------|--------------|-----------------------------------|
|                             |              | Experiment 1 | Experiment 2 | Experiment 3 | Experiment 4 |
| B10.A(5R)                  | bbbkkkddd    | 52,391 ± 6,457 | 104,114 ± 3,996 | 46,904 ± 4,183 | 13,534 ± 831 |
| B10                        | bbbbbbddd    | 12,697 ± 1,675 | 6,507 ± 962 | 4,140 ± 2,982 |
| B10.A(3R)                  | kkkkkddd     | 47,554 ± 6,897 | 48,115 ± 1,125 | 17,635 ± 1,893 | 7,297 ± 915 |
| B10.A(18R)                 | bbbkbbbd    | 13,502 ± 2,999 | 20,907 ± 3,741 | 4,960 ± 2,001 |
| B10.A(3R)                  | bbbkkkddd    | 13,573 ± 994 | 20,907 ± 3,741 | 4,960 ± 2,001 |
| (B10 × B10.A)F1           | bbbbbbddd   | 14,337 ± 344 |

* See footnote to Table III.
† The proliferative response of the chimeric responder T cells cultured alone was 176 cpm in experiment 1, 216 cpm in experiment 2, 124 in experiment 3, and 273 in experiment 4. See footnote to Table III for details of the experimental conditions.

Consistently produced a response of equal magnitude to that elicited by the fully MHC-matched B10.A stimulators. Nonetheless, B10.A(4R) stimulators, which share the K region and I-A subregion with B10.A, did produce substantial proliferation, whereas B10.A(5R) or B10.A(3R) stimulators, with B10.A homology only to the right of I-A, did not stimulate significant proliferation except in one case (experiment 2). These results indicate that Kk- and/or I-Ak-encoded antigens are involved in stimulating a syngeneic MLR. However, the better stimulation by B10.A than B10.A(4R) spleen cells (seen in 3 of 4 experiments) suggested that an additional effect was determined by gene products mapping to the right of I-Ak. Further analysis of this issue was undertaken by performing blocking studies of the syngeneic MLR in normal B10.A animals with selected monoclonal anti-Ia reagents.
Both the I-A and I-E Subregion Gene Products Participate in the Syngeneic MLR in the B10.A Strain. The results obtained with F1 → B10.A chimeras suggested the possibility that I-E-subregion-encoded determinants, in addition to K/I-A-region-encoded determinants, might be involved in the syngeneic MLR. Data obtained from anti-Ia blocking studies (Fig. 3) suggest that this is indeed the case. Inclusion in the syngeneic MLR culture of a monoclonal anti-I-A\(^\kappa\) reagent (10.2.16) reactive with the public specificity, Ia.17, at 10% or 6.7% decreased the proliferative response by 37–74%. Inclusion at 10% or 6.7% of a monoclonal reagent 17.3.3, which reacts with an A/E combinatorial antigen (specificity Ia.22) on the \(\beta_{AE} \alpha_{I}Ia\) molecule, suppressed proliferation by 63–76%. When both reagents were present in the culture system at 6.7% each, a more profound suppression of proliferation (97%) was achieved. This effect was specific for the presence of the target antigen on the stimulator cells because the inclusion of both of these reagents in the culture medium of a B6 syngeneic MLR did not significantly affect the response obtained. Furthermore, as seen in Fig. 3, the 10.2.16 reagent did not inhibit the proliferation obtained with F1 → B10 chimeric T cells and (B10 × B10.A)\(^F\)\(_1\) stimulators, although the 10.2.16 reagent could interact with the non-stimulating I-region products on the stimulator cells. The blocking experiments

![Fig. 3. Effect of I subregion-specific monoclonal antibodies on the syngeneic MLR. Two monoclonal anti-Ia reagents, one (10.2.16) directed against a determinant in the I-A subregion and the other (17.3.3) directed against the A/E combinatorial molecules, were included at a dilution of 1:10 or 1:15 in the culture system of a B10.A or B6 syngeneic MLR. The effect of the 10.2.16 reagent on the syngeneic MLR of responder F1 → B10 chimeric T cells and F1 stimulator cells is also shown.](image-url)
suggest that antigenic determinants encoded in both the I-A and I-E subregions are involved in stimulating T cell proliferation in the syngeneic MLR.

A Secondary Syngeneic MLR Can Be Obtained in the Presence of NMS Using Unfractionated Spleen Cells as Stimulator Cells. The results obtained with chimeras indicated that T cells in the syngeneic MLR proliferate in response to I-region-encoded antigens homologous to the environment in which the T cells mature. This finding suggested either that the syngeneic MLR represents a response to these antigens alone or that it is a response to these antigens in association with other foreign antigens in a manner similar to the proliferation obtained by ourselves and others using in vivo primed T cells and soluble antigens such as (T,G)-A--L or pigeon cytochrome c. To differentiate between these possibilities, we attempted to identify a foreign antigen in our system that might be responsible for the syngeneic MLR. Two obvious candidates for foreign antigen were antigenic substances present in the human serum in the culture medium and the BSA used in the preparation of the stimulator cell population. To test the possible role of these two sources, we performed secondary syngeneic MLR in the absence of either human serum or BSA.

The results in Fig. 1 indicate that when a primary response is performed in the presence of human serum, a secondary response of considerable magnitude (156,844 cpm) occurs in the presence of NMS. Thus, the presence of human serum antigens in the secondary culture medium does not appear necessary to obtain proliferation. Furthermore, when a primary syngeneic MLR is performed with BSA-gradient separated stimulators, a secondary syngeneic MLR can be obtained using unfractionated spleen cells never exposed to BSA, although the maximum response (79,842 cpm) was not as good as that obtained using BSA-gradient purified cells.

The weaker response obtained with unfractionated spleen cells might reflect their less potent presentation of I-region-encoded antigens. An alternative explanation, that this weaker response reflects the absence of BSA in the secondary culture system, proved not to be the case because in four of five experiments the addition of soluble BSA to unfractionated stimulator spleen cells did not increase the secondary response above the levels obtained with unfractionated spleen cells alone (not shown). Furthermore, stimulator cells enriched for antigen-presenting cells by fractionation of whole spleen on a Percoll density gradient were equally potent stimulators of a primary syngeneic MLR as compared to stimulators obtained from a BSA density gradient (not shown). These results taken together make it unlikely that the proliferation occurring in the syngeneic MLR is directed primarily against human serum proteins or BSA determinants present in the culture system.

Spleen Cells from Mice Raised in a GF Environment Stimulate a Secondary Syngeneic MLR. Another set of foreign antigens that might stimulate proliferation in the syngeneic MLR are environmental antigens that could be present in the spleens of mice because the animals are undergoing reactions against them. To address this issue, secondary cultures were performed using stimulators from the spleens of mice raised in a GF environment and mouse serum obtained from these GF animals. As shown in Fig. 2, unfractionated spleen cells or BSA-gradient-separated stimulator cells from BALB/c mice raised under GF conditions provided stimulation equivalent to that produced by similar populations of cells from BALB/c mice raised under routine laboratory environmental conditions.

 Fully Allogeneic Chimeras Produce an MHC-restricted Syngeneic MLR. The results
strongly suggested that the proliferation generated in a syngeneic MLR was not the result of stimulation by xenogeneic serum antigens present in the culture system or by environmental antigens carried by mice. However, it was still possible that the syngeneic MLR represented a secondary response to some unknown antigen present in our system (e.g., a latent virus). To resolve this issue, we took advantage of the finding that a secondary proliferative response to foreign antigen cannot be obtained after conventional in vivo priming in fully allogeneic chimeras. Table V shows the results of experiments to determine whether a syngeneic MLR can be obtained in full allogeneic chimeras. T cells from a B10.A → B10 chimera could not mount a secondary proliferative response to PPD, (T,G)-A--L, or pigeon cytochrome c despite priming in the footpads with these foreign antigens in CFA (footnote to Table V). This was the expected result because the chimeric T cells should have acquired the ability to recognize host B10 MHC gene products as self, but because all the APC of the chimera are of B10.A donor type, no T cell priming could occur. In contrast to the inability to respond to foreign antigens, in both of these experiments an excellent and host-restricted syngeneic MLR was obtained from the spleen cells of the chimeras. This response was of comparable magnitude to a normal syngeneic MLR (compare with data of Table I).

As a control to show that the allochimeric T cells were capable of responding to foreign antigens when primed in the right environment, B10 APC (4 × 10⁷ irradiated BSA-gradient separated spleen cells) were adoptively transferred into an allogeneic chimera at the time of in vivo antigen priming. Under these circumstances, as shown in Table VI, B10.A → B10 allogeneic chimeras were competent to mount a good antigen-induced proliferative response to PPD and (T,G)-A--L, antigens to which the B10 host is a responder, but not to pigeon cytochrome c, an antigen to which the B10 host is a nonresponder. In the case of PPD, only B10 host-type irradiated spleen cells were capable of presenting the antigen; B10.A donor-type irradiated spleen cells were ineffective. This same animal also produced a substantial and host-restricted syngeneic MLR (data not shown).

These results demonstrate that the T cells from allochimeras are MHC-restricted to and express the Ir phenotype of the irradiated host. Furthermore, without addition of host-type APC, the chimeric T cells could not express their recognition potential in a

**Table V**

| Source of stimulator cells | Proliferative response (cpm ± SEM)* | Experiment 1† | Experiment 2
|---------------------------|-----------------------------------|---------------|---------------
| B10                      | 148,286 ± 6,769                   | 60,780 ± 4,531|
| B10.A                    | 25,415 ± 1,821                    | 11,389 ± 218  |
| B10.S                    | 182,818 ± 292                     | 160,306 ± 7,144|

*In experiment 1, the cpm of nylon column-passed spleen T cells when cultured alone was 3,558 ± 211, and in experiment 2, T alone = 2,910 ± 201
† In experiment 1, the chimeric animal was primed in the footpads with PPD, (T,G)-A--L, and pigeon cytochrome c in CFA. The proliferative responses (cpm ± SEM) obtained were as follows: PPD, 133 ± 63; (T,G)-A--L, 140 ± 68; pigeon cytochrome c 281 ± 199; and medium alone, 142 ± 63. In experiment 2, the chimeric animal was not antigen primed.
BI10.A T Cells Maturing in a B10 Host Are Restricted to Host MHC and Ir Phenotype

| T cells | APC | Soluble antigen | [3H]Thymidine incorporation (cpm ± SEM) |
|---------|-----|----------------|----------------------------------------|
| 4 × 10^5 BI10.A → B10 chimeric T cells | 10^6 BI10.A + 10^5 | 0 | 1,533 ± 154 |
| 4 × 10^5 BI10.A → B10 chimeric T cells | 10^6 BI10.A + 10^5 | 20 µg/ml PPD | 12,588 ± 1,825 |
| 4 × 10^5 BI10.A → B10 chimeric T cells | 10^6 BI10.A + 10^5 | 100 µg/ml (T,G)-A-L | 15,222 ± 1,046 |
| 4 × 10^5 BI10.A → B10 chimeric T cells | 10^6 BI10.A + 10^5 | 100 µg/ml pigeon cytochrome c | 1,373 ± 202 |

B10.A → B10 chimeras were given 4 × 10^7 irradiated BSA-gradient separated splenic APC intravenously and then immunized in the footpads and tail with 50 µg (T,G)-A-L and 100 µg pigeon cytochrome c in complete Freund's adjuvant. 8 d later, draining lymph node cells were passed over nylon wool columns and their proliferative response to the priming antigens was assessed in a 5-d assay. The cells were exposed to antigen either in soluble form (top panel) or in the form of antigen-pulsed irradiated spleen cells (bottom panel). When soluble antigen was added to the culture, 10^5 BI10.A and 10^5 B10 irradiated spleen cells were added as a source of APC.

A Primary Proliferative Response to Foreign Antigens Has Not Been Obtainable in the Mouse. However, the potent effect of our culture conditions on the generation of a syngeneic MLR raised the possibility that such culture conditions were adequate to generate a primary in vitro response to soluble foreign antigen. If this were true, then the syngeneic MLR might represent a primary response to a foreign antigen. However, we were unable to generate a primary in vitro response or a secondary response after in vitro priming to several potent soluble foreign antigens (OVA, PPD, GLb, BSA) despite using the culture conditions used for the syngeneic MLR (data not shown). These culture conditions were, however, perfectly adequate to generate a secondary antigen-induced proliferative response after in vivo priming (data not shown).

A Secondary Syngeneic MLR Can Be Obtained Using H-2-matched but Non-H-2-different Stimulator Cells. Although the syngeneic MLR does not appear to be a reaction to a foreign antigen in association with self I-region products, the possibility remains that it represents a response to polymorphic self antigens encoded outside the MHC in association with self Ia molecules (18). To address this issue, T cells from a primary
TARGET ANTIGEN IN SYNGENEIC MIXED LYMPHOCYTE RESPONSE

Syngeneic MLR were restimulated in a secondary syngeneic MLR with BSA-gradient separated spleen cells from either the original syngeneic strain or with stimulator cells from an H-2 matched but non-H-2-different strain. Table VII demonstrates that H-2-matched, non-H-2-different (B10.D2, DBA/2, B10.A, B10.BR, and C3H) stimulator cells are as effective in stimulating a secondary syngeneic MLR as the fully H-2- and non-H-2-matched (BALB/c and A/J) syngeneic stimulator cells. These results suggest that the syngeneic MLR is not the summation of a large number of responses to individual non-MHC cell surface self antigens (minor histocompatibility antigens) seen in association with self Ia molecules.

**Discussion**

We have described a system that produces a primary murine syngeneic MLR whose magnitude approaches that of a standard primary allogeneic MLR or a secondary antigen-induced proliferative response. The murine syngeneic MLR we studied resembles quite closely that which has been described by others in human, murine, and guinea pig systems (1-9). As in the studies of others, a carefully selected population of stimulator cells was required to generate a substantial response, and this population was simultaneously enriched for APC. Furthermore, a higher stimulator to responder cell ratio was required than customarily used for either an alloantigen or a soluble foreign antigen-induced response. The kinetics of the murine syngeneic MLR also resembled those described in human and guinea pig systems. A peak proliferative response was observed at day 6 of culture, and an enhanced secondary response, indicating memory, was seen on day 3-4 of culture. Finally, the murine syngeneic MLR displayed specificity in that T cells cultured with syngeneic stimulators responded well in secondary cultures to syngeneic stimulators but poorly to allogeneic stimulators. Thus, the system described in this paper appears closely analogous to both the human and guinea pig syngeneic MLR.

Having developed a murine syngeneic MLR with the properties of a specific immunologic response, the first question we asked was whether this stimulation was an in vitro artifact. The syngeneic MLR, as it is usually set up, does not have a proper control. It could be argued that when T lymphocytes are purified away from

### Table VII

| Source of responder cells | Source of stimulator cells in second culture | Proliferative response (cpm ± SEM) |
|--------------------------|---------------------------------------------|-----------------------------------|
|                          | BALB/c                                      | B10.D2                            | DBA/2  |
| a. BALB/c                |                                             |                                   |
| Experiment 1             | 98,783 ± 4,317                              | 97,087 ± 4,706                    | 104,074 ± 3,732 |
| Experiment 2             | 93,307 ± 9,514                              | 129,125 ± 1,685                  | —      |
| b. A/J                   |                                             |                                   |
|                          | 10,372 ± 927                                | 6,473 ± 783                      | 9,254 ± 1,046 | 10,298 ± 938 |

* A primary syngeneic MLR was performed using responder and stimulator cells from BALB/c animals (a) or A/J animals (b). These cells were then harvested (see Materials and Methods) and restimulated either with syngeneic cells or with H-2-identical, non-H-2-different stimulator cells, or with medium alone [1,284 ± 211 cpm and 4,592 ± 1,071 cpm in (a), and 345 ± 57 cpm in (b)].
supporting cells such as macrophages, they do not receive the proper nutritional environment for survival, and thus they rapidly die off in culture. Similarly, when the stimulator cell population is irradiated with 2,000 rad, it does not have the ability to proliferate. However, the irradiated spleen cell population can still supply its nutritional function after irradiation. Therefore, a trivial explanation for the syngeneic MLR would be that the stimulator cells provide the nutritional environment to keep the T lymphocytes alive and this allows them to complete ongoing immune responses they were carrying out in vivo.

To refute this explanation, we had to find control conditions under which we could mix the T lymphocytes with a potential stimulator population and not get a response. This was achieved through the use of radiation-induced, bone marrow chimeras. Bevan (19) and Zinkernagel et al. (20) first demonstrated that cytotoxic T lymphocytes obtained from (A × B)F₁ cells that had been allowed to mature in a parental A environment were restricted to lyse virus-infected target cells expressing H-2 K and/or D antigens of the A parental haplotype, i.e., the T cells would not lyse targets expressing H-2 K and/or D antigens of the nonhost parental B haplotype even though the APC in such chimeras were of (A × B)F₁ donor marrow origin. Subsequent studies by other investigators demonstrated that helper (21-26), DTH (27), and proliferating (12) T lymphocytes from F₁ → parent chimeras are also restricted to recognition of antigens in association with the irradiated host's MHC molecules, although for these T cells the restricting elements are encoded within the I region of the MHC. Experiments using thymic transplants have suggested that the critical host element for all types of restriction is the thymus (20, 21, 28-29).

When we examined T cells from F₁ → P chimeras for the effect of the host environment on the response to syngeneic stimulator cells, we found that F₁ cells maturing in an A environment responded predominantly to A stimulator cells, whereas these same F₁ cells maturing in a B environment responded predominantly to B stimulator cells. Thus, the T cells involved in the syngeneic MLR were subject to the same constraints imposed by the host environment as demonstrated for other T cell-mediated responses. This observation provided us with an appropriate control for the syngeneic MLR. The stimulation observed with F₁ → Pₐ T cells and A-presenting cells could now be compared with the stimulation observed with the same T cells and B-presenting cells. The B-presenting cells were shown to be functional by their ability to stimulate F₁ → B chimeric T cells. Therefore, they were capable of providing all the nutritional requirements for stimulation. The comparison between A- and B-presenting cells yielded an average of 13-fold greater stimulation by the presenting cell-bearing MHC gene products to which the T cells were restricted. This difference is what we would regard as the syngeneic MLR. A similar conclusion was reached by Yamashita and Shevach (8) using a secondary syngeneic MLR of F₁ T cells stimulated with either parental spleen cell population.

The meaning of the apparent weak stimulation by the inappropriate spleen cells, F₁ → Pₐ T cells stimulated with Pₐ spleen cells, is not clear. Compared to chimeric T cells alone, the thymidine incorporation was always significantly higher. However, this could represent merely a nutritional effect of the spleen cells on the T cells because again there is no appropriate control population to compare the thymidine incorporation with. On the other hand, if the difference between T cells alone and in the presence of inappropriate spleen cells does represent immunologically meaningful
stimulation, then several interpretations are possible. One might suggest that a small portion of the chimeric T cell response is unrestricted, which is similar to what has been observed for some chimeric T cell cytotoxic responses (21, 30, 31) and helper responses (21, 23, 32). This could reflect the presence of a small number of contaminating mature F1 T cells in the original bone marrow preparation used to make the chimeras, or it could indicate that the thymic influence on the T cell repertoire is not absolute. Another intriguing possibility is that the T cell receptors involved in the syngeneic MLR are cross-reactive with MHC determinants from other strains. Currently, our data do not allow us to distinguish between these explanations.

Because B10 congenic strains differing only in their MHC genes were used in the chimeric studies, the implication of our results is that MHC-encoded antigens, at least in part, are likely to be responsible for stimulation. We therefore attempted to map to specific subregions of the H-2 complex the genes determining the target antigens being recognized. Our studies using responder T cells from (B10 × B10.A)F1 → B10.A or B10 chimeras and stimulator cells from H-2 recombinant strains suggested that self-determinants recognized by the responding T cell were determined by genes within the K and/or I-A subregion. We did not have available chimeras that split the K and I-A regions in such a way that we could formally test the role of K region products as stimulators of the syngeneic MLR. However, the finding that D-region products were not stimulatory suggested that Ia antigens encoded in the I region were probably the self antigens recognized by the responding T cell. The hypothesis that Ia antigens stimulate the syngeneic MLR is also consistent with the finding that no H-2 recombinant strains stimulated F1 → B10.A T cells as well as the B10 stimulators because none of the recombinants expresses the combinatorial Ia molecule, βεκ Δεκ, encoded in part in the I-Ak and I-Ek subregions (33).

To more directly test the hypothesis that Ia antigens stimulate the syngeneic MLR, we performed blocking studies in a normal B10.A syngeneic MLR using monoclonal antibodies directed at Ia antigens. Consistent with the hypothesis, an antibody directed at an I-Ak specificity inhibited partially, one directed at the I-Ak/I-Ek combinatorial molecule inhibited partially, and the two together inhibited nearly completely. The specificity of this blocking was demonstrated by the failure of the I-Ak antibody to inhibit the response of B6 T cells or F1 → B10 chimeric T cells stimulated with F1 spleen cells, even though in the latter situation the antibody bound to the stimulating population.

We concluded from the mapping and blocking studies that Ia antigens were intimately involved in the stimulation of the murine syngeneic MLR. However, this is also true for the secondary proliferative response to all soluble protein antigens. The T cell must see syngeneic I-region gene products on APC in association with the antigen to be stimulated, and anti-Ia antibodies will block the stimulation (34). Furthermore, Yamashita and Shevach (8) have recently observed in the guinea pig that the characteristics of the stimulator cell as well as the genetic requirements for the activation of the syngeneic MLR precisely resemble the requirements for antigen-specific T cell activation. Stobo and his co-workers have obtained similar findings in a human system (35, 36). Thus, the possibility had to be considered that the syngeneic MLR represented the response to an unidentified foreign antigen in association with syngeneic I-region gene products.

Potential sources of foreign antigen in our culture system that might have stimulated
a proliferative response included (a) the human serum in the medium, (b) the BSA used to prepare the stimulator cells, and (c) environmental antigens endemic to normal mice. To test the possible contribution to the syngeneic MLR of these antigens, secondary syngeneic MLR were performed using (a) normal mouse serum, (b) stimulators that had not contacted BSA, and (c) stimulator cells and serum from mice raised in a GF environment. Excellent proliferation was obtained under these conditions, arguing against the participation of these antigens in the syngeneic MLR. However, one could still claim that there are environmental antigens to which even GF mice might be exposed, e.g., viral antigens. Therefore, we again turned to chimeric mice, this time allogeneic A → B chimeras, to rule out the possibility of a secondary proliferative response to any and all foreign antigens.

As shown in Tables V and VI, allogeneic chimeras did not respond to soluble antigens unless they were primed in the presence of APC syngeneic to the host MHC haplotype. This is presumably because the A T cells developed in a B thymus and became restricted to recognizing antigen only in association with B MHC gene products. Because the APC in the chimera at 3 mo after creation derive almost entirely from the donor A bone marrow (13), the T cells and APC are mismatched, and thus the T cells cannot be stimulated. It is possible that a small overlap of the host-restricted T cells and APC occurs during the development of the chimera in that T cells begin to emerge from the thymus at 3 wk after reconstitution, whereas functional APC of host MHC type turn over between 1 and 6 wk after irradiation (13, 37, 38). However, even with this caveat, if the syngeneic MLR required priming to foreign antigens, one would expect to see a quantitative decrease in the syngeneic MLR. The fact that the syngeneic MLR generated with allochimeric T cells is just as strong as the syngeneic MLR generated from normal T cells argues quite forcefully that the syngeneic MLR does not represent a secondary proliferative response to a foreign antigen. In contrast to the strong proliferative syngeneic response, no cytotoxic response against host MHC antigens could be detected (unpublished observations), ruling out the trivial explanation for the results that the allochimeric donor T cells were not tolerant to the host's MHC antigens. The possibility that there was a state of partial tolerance (39) also seems unlikely because again one would have expected some quantitative decrease in the magnitude of the syngeneic MLR.

We concluded from the allochimera experiments that if the syngeneic MLR were a reaction to a foreign antigen in association with self Ia antigens, it could only represent a primary in vitro response to the antigen (40). We believe that this is unlikely because a primary antigen-induced proliferative response of this magnitude or of any magnitude with the exception of the response to alloantigen has not previously been obtained in the mouse. Furthermore, we could not obtain a primary antigen response or a secondary antigen response after in vitro priming to a variety of potent soluble foreign antigens despite using the culture conditions described for a syngeneic MLR. Finally, in the guinea pig, where a primary response to soluble protein antigens has been generated, the magnitude of the actual proliferation was only twofold or threefold (41). On the basis of all these findings, we believe that the murine syngeneic MLR does not represent any kind of a response to foreign antigen.

These experiments force us to conclude that the syngeneic MLR is solely a response against self antigens. The simplest type of autoreactivity would be a response only to the Ia antigens. However, an alternative possibility suggested by Yamashita and
Shevach (8) that the target antigens represent a variety of minor self histocompatibility antigens seen in association with Ia molecules had to be considered. This possibility was tested by priming with syngeneic spleen cells and then restimulating in a secondary syngeneic MLR with H-2-identical but non-H-2-different stimulators. The experiments demonstrated that restimulation depended only on H-2 identity between responder and stimulator cells. Thus, the syngeneic MLR is unlikely to represent a response to polymorphic self antigens encoded outside of the MHC in association with self Ia molecules. The possibility still remains that a nonpolymorphic self antigen seen in association with self Ia molecules is the target antigen. Nonetheless, the implications for autoreactivity of such a response do not differ from a reaction directed to self Ia antigens alone.

The biologic significance and function of such an autoreactive T cell population is unclear. Because the target antigens appear likely to be solely I-region-encoded proteins, it seems reasonable to surmise that the responding T cells possess anti-self receptors with sufficient affinity for self Ia to be stimulated in the absence of any antigen. Why then wouldn’t these cells be continually stimulated in vivo? Essentially two classes of answers can be envisioned to account for the normal existence of such cells. One class postulates that the cells are an early differentiation state of T lymphocytes in which interaction with Ia antigens alone is sufficient to stimulate the cells in vivo. After this stimulation, the cell would differentiate to a new state in which it would be resistant to a repeat stimulation by Ia antigens alone, although it could be stimulated by antigen plus Ia. This might be accomplished by altering the threshold affinity of receptor-Ia interactions required to stimulate the cell or by uncoupling a signal transducing mechanism from the anti-self receptor and attaching it to the anti-antigen receptor.

The second category of solutions would postulate that these cells are not normally stimulated in vivo by Ia antigens alone, although they normally function in response to Ia plus certain foreign antigens. For example, one could suggest that during the development (or alteration) of T cell specificity in the thymus by either positive (42, 43) or negative selection (44, 45), a set of anti-self Ia specificities is finally expressed on all cells that leave the organ. Each T cell would express only one member of the set, and the affinities of each member for self Ia could potentially be different. None of the receptors would have sufficient affinity for self Ia alone to be stimulated in the peripheral in vivo environment; they require, in addition, stimulation with antigen. However, those T cells with anti-self receptors at the higher end of the affinity spectrum might be able to be stimulated in vitro when exposed to large numbers of Ia-bearing stimulator cells in a pellet at the bottom of a microtiter well. In essence, this model suggests that a higher Ia antigen density can be achieved in vitro than in vivo and thus, that some of the T cells normally not stimulated in the animal will be stimulated in vitro. Another way in which a cell population might be stimulated in vitro but not in vivo would be if it were down regulated by other cells or inhibitory factors circulating in the animal. These in vivo phenomena might affect the threshold for stimulation of the cell, preventing low affinity interactions from triggering proliferation. When the cells are moved to the in vitro environment, inhibitory factors might be diluted out or the balance between regulatory and responding cells might be disturbed by selective cell survival, thus allowing the cells to be stimulated.

Clearly, other models can be envisioned for both categories. However, regardless of
which model one might favor, the data in this paper strongly argue that the syngeneic MLR is not a nutritional artifact or a cryptic response to foreign antigens. Rather, it appears to be a unique response to I-region-encoded antigens. An understanding of the mechanism by which the response occurs should yield insights into T lymphocyte specificity or differentiation.

Summary

A system has been described that produces a murine syngeneic mixed lymphocyte response (MLR) comparable in magnitude to an allogeneic MLR. The responder cells in these cultures exhibit the classic immunologic characteristics of both memory and specificity. Studies using radiation-induced bone marrow chimeras of F1 → parent type indicated that, similar to many other T cell-mediated immune responses, the response of the T lymphocytes in the syngeneic MLR was major histocompatibility complex-restricted and was determined by the environment in which the T cells matured. Using responder T cells from F1 → parent chimeras and stimulator cells from H-2 recombinant strains, it was possible to map the genes involved in the stimulation to the K and/or I regions. In addition, blocking studies with monoclonal anti-Ia antibodies suggested that in the B10.A strain the critical molecules were products of both the I-Ak and I-Ek subregions.

The issue of whether the syngeneic MLR is directed solely at self I-region antigens or whether the response represents proliferation to an unknown antigen in association with self I-region determinants was also addressed. Secondary syngeneic MLR were successfully performed in normal mouse serum and with stimulator cells prepared in the absence of bovine serum albumin to rule out the possibility that xenogeneic serum antigens were involved in the stimulation. The possibility that the syngeneic MLR might represent a secondary response to environmental antigens was eliminated by using germ-free mice as a source of stimulator cells and by demonstrating that spleen cells from unimmunized, fully allogeneic chimeras (B10.A → B10) could generate a normal syngeneic MLR even though such chimeras could not be primed to respond to any foreign antigens unless supplemented in vivo with a source of antigen-presenting cells syngeneic to the B10 host. The possibility that the syngeneic MLR was a primary response to a foreign antigen was considered unlikely because by using our culture conditions we could not obtain a primary antigen response or a secondary antigen response after in vitro priming to a variety of potent foreign antigens. Finally, the possibility that the syngeneic MLR represents a response to a variety of minor histocompatibility self antigens in association with self Ia molecules was eliminated by showing that the secondary responses to H-2 compatible, non-H-2 different strains (A/J vs. B10.A and C3H, or BALB/c vs. B10.D2 and DBA/2) were comparable to the secondary responses to syngeneic stimulators. Thus, we conclude that the target antigens in the syngeneic MLR are solely determinants on self Ia molecules, although the functionally equivalent possibility of a single, nonpolymorphic, minor self antigen seen in association with self Ia molecules cannot be excluded.

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