NATURE OF THE STIMULATING CELL IN THE SYNGENEIC AND THE ALLOGENEIC MIXED LYMPHOCYTE REACTION IN MICE

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Recently we have reported that mouse thymus cells can be stimulated by syngeneic spleen cells in a "one-way" mixed lymphocyte reaction (MLR)1 (1). The stimulating cell is a lymphocyte found in lymph nodes and in blood as well as in spleen. Throughout this paper the stimulating cell is referred to as a target cell since it might be the cell which is attacked by the stimulated lymphocytes. The ability to stimulate thymus cells appeared to be due to some stimulus, other than the H-2 complex, that developed on the lymphocytes as the animal matured.2 This syngeneic action was highest when responding thymocytes from newborn animals were cultured with adult spleen cells, although a significant response was also obtained when thymus cells and spleen cells from the same adult animals were used. In contrast to the results obtained in the syngeneic reaction, the response of thymus cells to allogeneic spleen target cells did not decline with age, thymocytes from adult mice and from newborn mice both responding with high values of thymidine incorporation in MLR.

The aim of this paper is to compare the ability of lymphocytes from different lineages and different sources to stimulate syngeneic and allogeneic thymus cells. The experiments provide further information about the nature and the specificity of the MLR in mice.

Materials and Methods

Cells.—Cell suspensions of thymus, spleen, and bone marrow from CBA/H/Wehi, C57BL Ka Lw Bradley Wehi, and BALB/c strains were prepared and cleared of debris and cell clumps as described previously by Shortman, Williams, and Adams (2). The medium for all cell suspensions was a N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES)-buffered

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‡ Abbreviations used in this paper: ATXBM, adult thymectomized, X-irradiated, bone marrow-protected; B cell, bone marrow-derived cell; BG, background; DPM, disintegrations per minute; MLR, mixed lymphocyte reaction; PHA, phytohemagglutinin; T cell, thymus-derived cell.

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balanced salt solution isosmotic with mouse serum (2) and supplemented with 10% fetal calf serum (Commonwealth Serum Laboratories, Parkville, Victoria).

Neonatal Thymectomy.—Neonatal mice less than 24 hr old were thymectomized according to the procedure described by Mitchell (3). When thymectomized animals were sacrificed, the mediastinum was examined and animals found to have thymus remnants were discarded. The relative depletion of thymus-derived (“T”) cells in peripheral lymph organs was tested functionally by the complete or almost complete elimination of a response to phytohemagglutinin (PHA) (4). Adult thymectomized, X-irradiated, and bone marrow-protected mice were kindly provided by J. Schrader (The Walter and Eliza Hall Institute).

Cell Counts.—Nucleated cells were counted in an hemacytometer using 1% acetic acid as hemolyzing and diluting fluid. Percentage of viable cells was estimated by the eosin dye exclusion method (5). For albumin gradient fractions, cell counts were performed with a Coulter Model B cell counter (Coulter Electronics, Hialeah, Fla.), with the lower threshold set to exclude erythrocytes and cell debris.

Fractionation of Spleen Cells on Continuous Bovine Serum Albumin Gradients (6).—Cells from 6-wk old CBA mouse spleens were separated into 15 fractions on the basis of buoyant density by centrifugation to equilibrium in continuous gradients of albumin according to the procedure of Shortman (6). Briefly the cells were dispersed directly into a continuous 15 ml linear 16-30% w/w albumin gradient, the albumin medium being at pH 5.1 and isosmotic with mouse serum. The gradient was centrifuged at 4000 g for 30 min in the swing-out head of a refrigerated (4°C) centrifuge. Fractions were collected by upward displacement out the top of the tube. The precise density of each fraction was determined. The albumin in each fraction was then diluted, and the cells were recovered by centrifugation and resuspended in a fixed volume of standard suspension medium. Samples of each fraction were removed for total cell counts. Fixed numbers of cells for each fraction were mitomycin C-treated and assayed for ability to serve as targets in allogeneic and syngeneic MLR. 10^7 separated cells were incubated with 10 ml of tissue culture medium containing 50 μg mitomycin C/ml.

Preparation of Purified T Lymphocytes.—Purified T lymphocytes from normal CBA spleen were prepared by a two-stage physical adherence column method. 90% of these cells were killed by incubation with anti-0 serum and complement. They respond well to PHA and restore the depleted antibody response to sheep erythrocytes of bone marrow-derived (B) lymphocytes from spleens of neonally thymectomized and athymic (nude) mice (7).

Tissue Culture.—Cells were cultured in Eagle’s minimal essential medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 25 mg of penicillin, 20 mg of streptomycin, 127 ml of 2.8% sodium bicarbonate, and 100 ml of fetal calf serum/liter. Plastic Falcon tubes 2001 (Falcon Plastics, Div., B-D Laboratories, Inc., Los Angeles, Calif.) contained 1 ml of medium. All mixed lymphocyte cultures were rendered “one way” by treating target cells with mitomycin C before culture (4). Test and mitomycin C-treated target cells were cultured at different ratios at a total concentration of 5 X 10^6 viable cells/ml. After incubation for 42 hr in a humidified atmosphere of 10% CO2 in air, 1 μCi tritiated thymidine (Radiochemical Centre, Amersham, England; specific activity 25.0 Ci/mM) was added, and the cultures were harvested 8 hr later. DNA was extracted as described by Byrd, v. Bohmer, and Rouse (4). The thymidine incorporation into DNA was determined by scintillation counting technique. Counts per minute were converted into disintegrations per minute (DPM) by quench correction. Statistical treatment for triplicate cultures was employed using an IBM 7044 computer. Presented values are arithmetic means with standard deviation.

To determine background values, DPM were first obtained from separate incubations of both 5 X 10^4 test cells alone and 5 X 10^4 target cells alone. The background value for a particular culture was then calculated on a proportional basis considering the numbers of test or target cells in the mixed culture.

Adams, P. 1972. A physical adherence column method for the preparation of T and B lymphocytes from normal mouse spleen. Manuscript submitted to Cell. Immunol.
RESULTS

Age of Target Spleen Cells.—Our previous studies had indicated that the age of the animal determined the ability of CBA mouse splenic lymphocytes to act as target cells in a syngeneic reaction. We therefore made a close comparative study of the influence of animal age on the ability of spleen cells to stimulate both allogeneic and syngeneic thymus cells in MLR. To study the syngeneic MLR, 4-day old thymus cells (which give a high syngeneic response) were used as reacting cells with CBA spleen target cells of different ages. A comparison with the allogeneic reaction was made in two ways: In the first comparison 4-day old C57 thymocytes were used as reacting cells against the same series of CBA spleen targets. In the second comparison the same 4-day old CBA thymus cells were used as reacting cells against allogeneic C57 spleen cells as targets. Thus both the test cells and the target cells in the syngeneic MLR were checked in allogeneic combinations. The results are given in Fig. 1 and Table I.

4-day old CBA thymus cells did not proliferate at all in response to 4-day old syngeneic spleen cells (Fig. 1). Only a small response was observed when 1-wk old CBA spleen cells were used as targets and optimal stimulation was not seen until 3 wk or more of postnatal life. In contrast to this syngeneic reaction, Fig. 1 shows that C57 thymus cells responded with significant thymidine in-

![Graph showing DNA synthesis in syngeneic and allogeneic MLR.](image)

**Fig. 1.** Ability of CBA target spleen cells obtained from mice of different ages to induce DNA synthesis in syngeneic and allogeneic 4-day old thymus cells. Values represent specific stimulation above background.
corporation to the same samples of 4-day old CBA spleen targets. However even in the allogeneic reaction the degree of stimulation also increased with aging of the animals from which the target spleen cells were obtained. The optimal stimulation in the allogeneic reaction was very similar to that of the syngeneic reaction.

In the second comparison it was shown that the 4-day old CBA thymus cells responded to 4-day old allogeneic C57 spleen targets even though they were unresponsive against the syngeneic 4-day old spleen target cells (Table I).

The conclusion from this data is that the failure to obtain a syngeneic response between 4-day old thymus cells and 4-day old spleen cells was specific and due to absence of some stimulating antigen in the 4-day old spleen, since 4-day old thymus cells were capable of both syngeneic and allogeneic responses, and 4-day old spleen cells served as effective allogeneic targets.

**Thymus Cells as Target Cells.**—The next question was whether the adult mouse lymphocytes able to stimulate syngeneic thymus cells were of the B or the T lineage. As a first test cells from primary lymph organs were studied.

**TABLE I**

*Allogeneic and Syngeneic Responses of 4-Day Old Mouse Thymus Cells*

| Test cell | Mitomycin C-treated target cell | Calculated background | MLR    |
|-----------|--------------------------------|-----------------------|--------|
| 4-day old CBA thymus | 4-day old CBA spleen | 1348                  | 1079 ± 170 |
| " "        | 4-day old C57 spleen   | 1366                  | 2851 ± 161 |

Thymus cells from 10-wk old CBA mice were tested as targets for induction of DNA synthesis in syngeneic 4-day old CBA and allogeneic 4-day old BALB/c thymus cells. The effect of thymus target cells was compared with that of spleen target cells. Different ratios of test cells to target cells were studied, since optimal ratios differed when using thymus targets as compared with spleen targets. As shown in Fig. 2 no stimulation at all was obtained in the syngeneic reaction with 10-wk old thymus cells as targets although 10-wk old spleen cells elicited a good response. However 4-day old BALB/c thymus cells showed a good response in culture to 10-wk old allogeneic CBA thymus targets (Fig. 3). Thus the failure to obtain a syngeneic reaction was due to the specific absence of the syngeneic stimulus on thymus cells.

**Bone Marrow Cells as Target Cells.**—Experiments similar to those with target thymus cells were carried out with bone marrow obtained from 10-wk old CBA mice, since this may be the primary lymphoid source of B cells. As test cells 4-day old syngeneic CBA and 4-day old allogeneic C57 thymus cells were used. The results obtained with bone marrow targets were similar to those obtained with thymus targets. Bone marrow cells could induce only a very poor response in syngeneic 4-day old thymus cells but a quite significant stimulation in 4-day old allogeneic C57 thymus cells (Figs. 4 and 5).
Background

Figs. 2 and 3. Comparison of spleen cells and thymus cells from 10-wk old CBA mice to serve as targets for syngeneic or allogeneic thymocytes.

Figs. 4 and 5. Comparison of bone marrow cells and spleen cells from 10-wk old CBA mice to serve as targets for syngeneic or allogeneic thymocytes.
Peripheral T Cells as Targets.—Since lymphoid cells from primary lymph organs such as bone marrow and thymus did not stimulate syngeneic thymocytes and since we showed earlier that a relatively pure fraction of splenic lymphocytes was able to induce DNA synthesis in syngeneic thymus cells, it was of interest to study whether peripheral B or T lymphocytes initiated the syngeneic reaction. A preparation of purified CBA T cells obtained by a physical adherence glass bead column separation was used as targets for syngeneic and allogeneic 4-day old thymus cells. This preparation consisted of 90-92% T cells both by the criteria of anti-θ serum cytotoxic assay and by the antibody-antigen complex binding assay for B lymphocytes described by Basten et al. (8). As shown in Fig. 6 there was a definite but reduced response of 4-day old CBA thymus cells to this target. However the degree of stimulation decreased with increasing ratios of test to target cells. This was reverse to the situation observed with normal spleen cells. By comparison 4-day old C57 thymus cells responded to the allogeneic CBA T cell preparation with higher values of thymidine incorporation than 4-day old CBA thymus cells and in addition the degree of stimulation showed no decrease with increasing test cells to target cell ratios as observed in the syngeneic reaction (Figs. 6 and 7).

Figs. 6 and 7. Comparison of column purified splenic T lymphocytes and normal spleen cells from 10-wk old CBA mice to serve as targets for syngeneic or allogeneic thymocytes.
The results show that those peripheral T cells stimulate either poorly or not at all in the syngeneic reaction. The small syngeneic response could be due to some B cell contamination. The comparison of the T cell targets at different ratios of test cells to target cells in the allogeneic and syngeneic MLR suggests that the stimulation obtained in the syngeneic reaction is limited by a different target cell type than that in the allogeneic reaction.

**Peripheral B Cells as Targets.**—Since peripheral T cells were poor targets in the syngeneic MLR, a comparative study was made on the ability of spleen cells from neonatally thymectomized mice, of normal spleen cells and of purified peripheral T cells, to act as targets in the syngeneic reaction. These sources in this order should represent decreasing levels of B and increasing levels of T cells. Fig. 8 shows that spleen cells from 6-wk old neonatally thymectomized CBA mice stimulated syngeneic thymus cells better than both normal spleen cells and purified T cells at all ratios of test to target cells tested. Hence the main source of the syngeneic target stimulus in CBA spleen appeared to be a peripheral B lymphocyte. This suggestion was strengthened by the finding that spleen cells from adult thymectomized, X-irradiated, and bone marrow-protected (ATXBM) mice induced marked DNA synthesis in syngeneic 4-day old thymus cells (Fig. 9).

**Spleen Cells of Differing Buoyant Density as Target Cells.**—The preceding results suggested that B cells alone were the primary target for the syngeneic MLR, whereas T cells, as well as B cells, were targets for an allogeneic MLR. To see if this difference between allogeneic and syngeneic targets could be confirmed by an independent cell separation procedure, and to test if all B cells served equally as syngeneic targets, spleen cells were separated on the

![Graph showing results](image-url)
basis of buoyant density by centrifugation in a continuous albumin density gradient. This procedure gives some separation of B and T cells in CBA spleen, and separates subpopulations of B and T lymphocytes differing in biological activity and stage of differentiation (Shortman [6]; Shortman, Brunner, and Cerottini [9]; Shortman et al. [10]; Kraft and Shortman [11]). After separation, spleen cell fractions were mitomycin C-treated and samples tested for ability to act as MLR targets, using as reacting cell either 4-day old syngeneic CBA thymus cells or 6-wk old allogeneic BALB/c thymus cells. The ratio of test cells to target cells was 20:1, a level that made target cells limiting, so both increases and decreases in responsiveness could be measured. The results are illustrated in Fig. 10.

The entire density spectrum of spleen cells had some capacity to stimulate both allogeneic and syngeneic thymus cells into blast formation (Fig. 10).

However, this capacity was not evenly distributed on a cell-for-cell basis. In both allogeneic and syngeneic reactions, light density cells were more effective targets than dense cells. This may have been related to the increased size, and hence greater surface antigen content of light density cells, or it may have been due to other factors such as their higher mitotic rate or metabolic activity.
any case, selection of cells less dense than 1.074 g/cm$^3$ produces significant enrichment for cells serving as syngeneic MLR targets.

In addition, ability to stimulate syngeneic MLR did not exactly parallel ability to stimulate allogeneic MLR (Fig. 10). The distribution of syngeneic of B lymphocytes in CBA mouse spleen (12). For example, in density fractions around 1.071 g/cm$^3$, where a nearly pure peak of B lymphocytes is always located, the cells were active as syngeneic MLR targets but not as efficient as allogeneic MLR targets. Conversely, around density 1.09 g/cm$^3$, where T lymphocytes are enriched, the cells were enriched for allogeneic target activity but depleted for syngeneic target activity.

**DISCUSSION**

The mixed lymphocyte reaction is supposed to reflect blast cell transformation and proliferation after antigen recognition, since the thymidine uptake can be inhibited by antibody fragments directed specifically against antigen receptors on responding cells (13) as well as antibody fragments directed against antigenic structures on target cells (14). There seem to exist different clones of MLR reactive cells. "Suicide" experiments of Salmon et al. (15) and Zoschke and Bach (16) suggest that different clones of responding cells were stimulated by different samples of target cells from genetically different donors. The results
presented in this paper suggest that thymus cells can recognize certain antigenic structures on both allogeneic and syngeneic lymphoid cells.

Our previous studies had shown the target cell for both syngeneic and allogeneic MLR was likely to be a lymphocyte. The precise nature of the lymphocyte has been examined by comparing the ability of different lymphoid cell preparations to stimulate 4-day old syngeneic thymus cells as opposed to allogeneic 4-day old thymocytes, adult thymus cells, and spleen cells. The target cell or the target cell antigen appears to be different in allogeneic and syngeneic MLR. One important parameter was the age of the animal providing the target spleen lymphocytes with a marked quantitative difference between syngeneic and allogeneic MLR over the 4-day-3 wk age period. 4-day old CBA thymus cells gave no response to cells from 4-day old CBA spleen in contrast to the positive syngeneic reaction with older CBA spleen cells. However, the same 4-day old spleen cells were effective targets in an allogeneic reaction with 4-day old C57BL thymus cells. The stimulus for the syngeneic reaction therefore develops on splenic lymphocytes with maturation, differentiation, or aging. This antigen is unlikely to be H-2 or other antigens determining the allogeneic MLR, since these are present in 4-day old spleen.

Another finding which strongly suggests that H-2 antigen is not responsible for the syngeneic stimulation is that thymocytes from 10-wk old mice as targets could not induce DNA synthesis in 4-day old syngeneic thymus test cells whereas those targets stimulated well allogeneic 4-day old BALB/c thymus cells and presumably therefore presented stimulating antigen in an effective form.

In both primary lymphoid organs, bone marrow and thymus, failed to provide significant stimulation in the syngeneic MLR, although they both served as a stimulus in the allogeneic situation. The small stimulation observed with bone marrow cells as targets in the syngeneic MLR may have been due to contamination with more mature peripheral blood lymphocytes, which do induce blast transformation in syngeneic thymus cells. These results again suggest the target antigen for the syngeneic MLR arises on lymphocytes with differentiation.

The experiments with column-separated purified peripheral T cells, with spleen density fractions enriched for B and T cells, and with B cell-enriched spleen preparations led to the conclusion that the target lymphocyte for a syngeneic MLR is a B cell. This contrasts with the allogeneic MLR, where both B and T (probably mainly T cells) serve as targets. These studies do not indicate the nature of the target antigen for the syngeneic response, beyond the fact that it is not the antigen which is responsible for allogeneic stimulation. In fact, they do not even establish that stimulation in MLR is due to antigen, rather than some other form of stimulus, although soluble stimulating factors released from cells in the medium have been definitely excluded.

4 v. Boehmer, H. Direct cell contact is required in the syngeneic mixed lymphocyte reaction in mice. Manuscript submitted to Eur. J. Immunol.
A positive syngeneic reaction has also been described by Howe et al. (17, 18). However, these authors reported that lymph node cells were not able to induce DNA synthesis in syngeneic thymus cells thus suggesting that we are dealing with a different stimulating cell.

One basic question is the relationship of the syngeneic response to the mechanism of "self"/"not self" discrimination by T cells developing within the thymus.

It is pertinent to ask why a syngeneic response was apparently obtained with one self antigen, but not with another such as H-2. The MLR appears to reflect only stimulation by special antigens presented on the surface of viable lymphocytes, so the spectrum of possible responses that can be detected by this approach is limited. Self reactivity against such a basic antigen as H-2 would be lethal, and by evolutionary considerations the mechanisms for eliminating such cells would have to be rapid and efficient. In addition, since there is H-2 antigen within the thymus at the earliest stages, any proliferative response due to this stimulus might be included in the "background," and not detectable in MLR.

Thus detection of a syngeneic response in our system may be accidental, due to the antigen being on a lymphocyte, being normally absent from the thymus, and only developing slowly after birth, so a reasonable pool of reactive cells develops while the self antigen has not yet been encountered.

An important point, under current investigation, is whether the thymus cells responding to the syngeneic stimulus by the blast transformation and proliferation could then go on to produce cytotoxic killer cells reactive against the self target. If so, a detailed study of how these potentially "self"-destructive cells are eliminated would be a useful model for studies of self/not self discrimination. This elimination of the self response could be by tolerization of the cells when they reach the periphery, or could be by direct elimination within the thymus itself, perhaps by the sterile differentiation pathway we have proposed elsewhere (9).

**SUMMARY**

Thymus cells from CBA and BALB/c mice are stimulated by syngeneic peripheral lymphoid cells in a "one-way" mixed lymphocyte reaction. The stimulating cell appears to be a mature B cell.

Spleen cells from neonatal mice and thymus cells or bone marrow cells from adult mice are not able to induce DNA synthesis in syngeneic thymus cells, although they stimulate significantly allogeneic thymocytes. The ability of peripheral B cells to serve as stimulating cell in a syngeneic reaction develops with the age of the animal.

The marginal stimulation of syngeneic thymus cells when 90% pure peripheral T cells were used as stimulating cells indicated that T cells alone were ineffective in stimulating in syngeneic mixed lymphocyte reaction. However they stimulated effective allogeneic thymocytes.
On a cell-to-cell basis, light density splenic lymphocytes stimulated both syngeneic and allogeneic thymocytes better than did more dense lymphocytes. The data obtained suggest that stimuli other than those responsible for allogeneic stimulation induce proliferation of syngeneic thymus cells under identical culture conditions.

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