CELLS IN BONE MARROW AND IN T CELL COLONIES GROWN FROM BONE MARROW CAN SUPPRESS GENERATION OF CYTOTOXIC T LYMPHOCYTES DIRECTED AGAINST THEIR SELF ANTIGENS

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The mechanisms involved in the induction and maintenance of self-tolerance remain unclear. The traditional point of view is that self-reactive cells are eliminated by clonal deletion (1) and many specific mechanisms, such as receptor blockade (2), have been proposed for achieving this. A more recent, contrasting point of view (3, 4) is that self-tolerance is achieved through regulatory procedures which produce active suppression. A hybrid of the two points of view is also possible. Thus, antigen may be presented in a form such that the effector arm of the immune response is not activated (and may even be specifically deleted) but at the same time, specific suppressive mechanisms are activated (5).

It is still unresolved whether clones that recognize self antigens actually exist in normal animals. There is good evidence, however, that such clones exist for certain abnormal states. Thus, it is reasonably well established that the humoral immune system can produce self-reactive antibody leading to autoimmune disease (6). In the cellular immune system, cytotoxic T lymphocytes (CL) directed against the major histocompatibility complex of syngeneic targets have been reported in NZB mice (7), a strain with pronounced autoimmune disease (8). Lethally irradiated mice injected with syngeneic bone marrow (BM) cells and heat-killed bacille Calmette-Guérin appear to contain self-reactive T cells (9).

It has long been hypothesized that the thymus plays a central role in the development of T cells and the T cell specificity repertoire. Recent experiments suggest that the self major histocompatibility complex (MHC) antigens of the thymus epithelium play a major part in structuring this repertoire (10, 11). Further, mice homozygous for the nu mutation lack a thymus, contain few, if any, mature T cells, and are severely deficient in T cell function. Yet mature functional T cells can be obtained in vitro from athymic nu/nu mice. Experiments from this lab (12) have shown that colonies containing Thy-1-bearing cells can be grown in vitro from nude spleen cells and that

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1 Abbreviations used in this paper: B6, C57BL/6; B6C3F1, (C57BL/6 × C3H)F1; B6D2F1, (C57BL/6 × DBA/2)F1; B6RF1, (C57BL/6 × RNC)F1; BM, bone marrow; C3, C3H/He; C3D2F1, (C3H × DBA/2)F1; CL, cytotoxic T lymphocyte; CLP, cytotoxic T lymphocyte precursor cell; Con A, concanavalin A; D2, DBA/2; LN, lymph node; α-MEM, α-minimum essential medium; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; PHA, phytohemagglutinin; RAMB, rabbit anti-mouse brain; TNP, trinitrophenyl.

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these Thy-1-positive cells can provide an accessory cell required for the production of CL from cytotoxic T lymphocyte precursor cells (CLP). More recently, Gillis et al. appear to have obtained CLP from nude mouse spleen cells in an in vitro culture system (13). These CLP can produce CL against allogeneic cells and have apparently acquired this ability in the absence of a thymus.

We have previously reported that the spleen of nude mice contains a subpopulation of cells which can specifically prevent the activation of CLP recognizing the MHC antigens of the nude mouse, and have speculated that such cells might play a role in eliminating self-reactive CLP in normal mice (14). Such cells would be particularly useful in a model in which the T cell repertoire is generated by varying the receptors of cells initially reactive to MHC determinants as proposed by Jerne (15). Such cells should thus also be found in normal mice. We have previously demonstrated and confirm here that they are not present in normal spleen. However, it is shown here that they are present both in normal BM and in colonies containing T cells grown from normal BM.

Materials and Methods

Mice. The inbred strains C57BL/6J Oci (B6) (H-2b), RNC (H-2a), and the F1 hybrid of the two (B6RF1) were bred at the Ontario Cancer Institute, Ontario, Canada; DBA/2J (D2) (H-2a), C3H/HeJ (C3) (H-2k), and the F1 hybrids (C57BL/6J × DBA/2)F1 (B6D2F1) (H-2b/k), (C57BL/6J × C3H/HeJ)F1 (B6C3F1) (H-2b/k), and (C3H/HeJ × DBA/2)F1 (C3D2F1) (H-2k/a) were purchased from The Jackson Laboratory (Bar Harbor, Maine). Mice were used at 12-16 wk of age. All male or all female mice were used for the mixed lymphocyte cultures of a given experiment.

BM Lymphoid Colonies. These were grown as previously described (12). Briefly, 1 × 10⁵ BM cells obtained from femur and tibia were suspended in 1 ml of 0.8% (wt:wt) methylcellulose (Dow Chemical Co., Midland, Mich.) in α-minimum essential medium (α-MEM) supplemented with 20% fetal calf serum (lot 4055979; Flow Laboratories, Inc., Rockville, Md.), 40% (vol/vol), phytohemagglutinin (PHA)-stimulated human leukocyte-conditioned medium (16) kindly provided by Dr. G. B. Price, and 5 × 10⁻⁵ M 2-mercaptoethanol. This suspension was spread in a 35-mm plastic culture dish (5221-R; Lux Scientific Corp., Newbury Park, Calif.). Three such dishes, two containing cell suspension and one containing only water, were put in a 100-mm plastic petri dish and incubated for 5 d at 37°C in an atmosphere of 5% CO₂. About 100 lymphoid colonies containing Thy-1-positive cells grew per dish. These appeared as tight spheres which floated in the methylcellulose.

Colony Expansion Cultures. Before use in mixed lymphocyte cultures, BM lymphoid colonies were individually picked under a microscope, pooled in groups of 7-10, and recultured for a further 3 d in flat bottom microtiter trays (76-002-05; Linbro Chemical Co., Hamden, Conn.). The medium was identical with that used for colony formation except that methylcellulose was omitted. The end of this culture period, 5,000-10,000 nonadherent cells were recovered from each well.

Antiserum Treatment. Three different anti-T cell reagents were used: an anti-Thy-1 antiserum raised against purified mouse brain Thy-1 glycoprotein (17) provided by Dr. M. Letarte, a rabbit anti-mouse brain serum (lot 270; Cedarlane Laboratories, Hornby, Ontario), and an anti-Thy 1.2 hybridoma antibody (F7D5) provided by Dr. P. Lake (Department of Zoology, University College London, London, England). Cells were incubated with antiserum for 30 min at 4°C, washed once in phosphate-buffered saline, and incubated with rabbit complement (low tox, lot 4030; Cedarlane Laboratories) at a final dilution of 1:10 or 1:15 for 45 min at 37°C in a 5% CO₂ incubator. Cells were washed three times in culture medium and viability assessed by eosin exclusion. For each experiment, controls established that the antiserum dilutions chosen lysed at least 98% of the appropriate strain of thymocytes.

Adherence Separation. Adherent cells were removed from a BM cell suspension by incubating
them in a 10-ml tissue culture flask (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) for 4 h at 37°C in an atmosphere of 8% CO₂ in air.

Mixed Lymphocyte Reaction (MLR) Cultures. These were set up in 96-well V-bottom microtiter trays as previously described (14, 18). The medium was α-MEM supplemented with 10% fetal calf serum (lot 3059; International Biological Laboratories, Inc., Rockville, Md.), 5 × 10⁻⁵ M 2-mercaptoethanol, and 10 mM Hepes buffer. Each microculture contained 3 × 10⁴ lymph node (LN) cells as responder cells, 3 × 10⁵ irradiated (1,500 rad delivered at 80 rad/min from a 137Cs source) spleen cells as stimulator cells, and varying numbers of either BM cells or BM lymphoid colony cells in 0.2 ml of medium. The cultures were incubated for 5 d at 37°C in an atmosphere of 8% CO₂ in air.

Cytotoxicity Assay. MLR cultures were assayed for cytotoxic activity using ⁵¹Cr-labeled concanavalin A (Con A) spleen cell blasts derived from the stimulator strain precisely as described previously (14). Specific ⁵¹Cr release, p, defined as (observed counts minus spontaneous counts)/(total releasable counts minus spontaneous counts) was reexpressed as 100 Nat = -100 × ln(1 - p) (19). Nat is directly proportional to the number of cytotoxic lymphocytes produced (19). 100 Nat = 10 is equivalent to 10% specific ⁵¹Cr release; 100 Nat = 100 is equivalent to 63% specific ⁵¹Cr release. Each experimental group contained eight replicate cultures, and the results are given as mean ± SEM.

Limiting-Dilution Assay. The frequency of CLP was measured by limiting-dilution analysis as described elsewhere (18, 20). Briefly, replicate cultures containing varying numbers of B6 nu/ + LN responder cells and 3 × 10⁵ irradiated spleen stimulator cells were set up as described above. All cultures also contained 10⁴ B6 nu/nu spleen cells as a source of accessory cells required for CL production. These have been lost through dilution from the LN responder cells (18). All cultures were assayed using ⁵¹Cr-labeled spleen Con A blast targets derived from the same strain as the stimulator cells. ⁵¹Cr release from each culture was compared with the release from a control group of cultures from which LN responder cells were omitted. A culture was scored as responding if the ⁵¹Cr release value was more than 2 SD from the mean of the control group. The fraction of nonresponding cultures as a function of the number of responder cells cultured was fitted to \( P(O) = e^{-fcLPN} \) according to the method of Porter and Berry (21) where \( P(O) \) is the probability of no response, \( fcLP \) is the frequency of CLP, and N is the number of responder cells cultured. The Porter and Berry analysis also yields 95% confidence limits and a \( \chi^2 \) value for judging the goodness of fit.

Results

BM Contains Anti-Self-Suppressor Cells. In the experiments reported here, we measure the effect of added BM cells on the cytotoxic activity produced in MLR cultures containing LN responder cells from one mouse strain and irradiated stimulator spleen cells from an H-2different mouse strain. The basic observation is that less cytotoxic activity is produced if the added BM cells are syngeneic or semisyngeneic to the stimulator cells. Fig. 1 shows one such experiment. Adding B6RF₁ BM cells to a B6 LN anti-B6RF₁-irradiated spleen MLR reduced the cytotoxic activity produced against B6RF₁ targets; addition of B6 BM cells (syngeneic to the responder LN cells) produced little or no suppression. This effect was observed whether unfractionated or nonadherent BM cells were used.

Table I shows partial results from four other experiments of similar design. Experiment 1 is another example in which BM cells syngeneic to the stimulator produced suppression, whereas BM cells syngeneic to the responder did not: RNC BM cells suppressed cytotoxic activity production in a B6 anti-RNC response but not in an RNC anti-B6 response. Conversely, B6 BM cells suppressed cytotoxic activity production in an RNC anti-B6 response but not in a B6 anti-RNC response. In experiment 2, BM cells allogeneic to both responder and stimulator did not suppress: fully allogeneic B6 BM cells did not suppress a C3 anti-C3D2F₁ response; C3D2F₁
B6RF1 BM cells suppress the development of cytotoxic activity in B6 anti-B6RF1 cultures. Replicate cultures (eight per group) containing 3 × 10^6 B6 responder LN cells and 3 × 10^5 irradiated (1,500 rad) B6RF1 stimulator spleen cells were set up. Cultures also contained varying numbers of BM cells: ○, normal B6RF1 BM; □, nonadherent B6RF1 BM; ●, B6 BM; ■, nonadherent B6 BM. Cultures were incubated for 5 d and assayed for cytotoxic activity using 3 × 10^6 3^HCr-labeled spleen Con A blast cells from the stimulator strain, B6RF1. Results are expressed as (mean ± SEM) cytotoxic activity as described in Materials and Methods.

BM cells, syngeneic to the stimulator cells, did suppress. In experiment 3, BM cells semisyngeneic to the stimulator produced suppression: B6RF1 BM cells suppressed a D2 anti-B6D2F1 response which was not suppressed by either (fully allogeneic) RNC or (syngeneic to responder) D2 BM cells; B6D2F1 BM cells (syngeneic to stimulator) also produced suppression. Experiment 4 is a variation of experiment 1. In all of the above experiments, control groups were included containing BM cells and irradiated stimulator cells but no responder LN cells. No cytotoxic activity was obtained (data not shown).

Assuming that the above results are of general validity and do not just apply to the particular strains tested, the following generalizations can be drawn. (a) Suppression is produced by BM cells which are syngeneic or semisyngeneic to the stimulator. Whether the BM cells are semiallogeneic or allogeneic to the responder cells appears irrelevant. In the case of an A anti-(A × B)F1 response, B BM cells produce suppression, whereas A BM cells do not. (b) No suppression is produced by BM cells syngeneic to the responder. (c) No suppression is produced by BM cells which are allogeneic to the stimulator. Whether the BM cells are syngeneic, semisyngeneic, or allogeneic to the responder cells appears irrelevant. In those groups in which the
### Table I

*Suppression of CL Production by Added BM Cells*

| Experiment | Response          | Added BM cells | Cytotoxic activity$^\dagger$ |
|------------|-------------------|----------------|----------------------------|
| 1          | B6 anti-RNC       | 0              | 120 ± 23                   |
|            |                   | 6 × 10⁴ B6     | 206 ± 75                   |
|            |                   | 6 × 10⁴ RNC    | 78 ± 18§                   |
|            | RNC anti-B6       | 0              | 33 ± 10                    |
|            |                   | 6 × 10⁴ RNC    | 33 ± 7                     |
|            |                   | 6 × 10⁴ B6     | 12 ± 79§                   |
| 2          | C3 anti-C3D2F₁     | 0              | 35 ± 5                     |
|            |                   | 3 × 10⁴ B6     | 84 ± 11                    |
|            |                   | 3 × 10⁴ C3D2F₁ | 7 ± 2§                     |
| 3          | D2 anti-B6D2F₁     | 0              | 35 ± 18                    |
|            |                   | 6 × 10⁴ D2     | 27 ± 9                     |
|            |                   | 6 × 10⁴ RNC    | 56 ± 14                    |
|            |                   | 6 × 10⁴ B6RF₁  | 15 ± 6§                    |
|            |                   | 6 × 10⁴ B6D2F₁ | 3 ± 1§                     |
| 4          | C3 anti-B6C3F₁     | 0              | 25 ± 4                     |
|            |                   | 3 × 10⁴ C3     | 16 ± 3                     |
|            |                   | 3 × 10⁴ B6     | 4 ± 2§                     |
|            | B6 anti-B6C3F₁     | 0              | 139 ± 23                   |
|            |                   | 3 × 10⁴ C3     | 84 ± 14§                   |
|            |                   | 3 × 10⁴ B6     | 170 ± 29                   |

* Replicate groups of eight cultures containing 3 × 10⁴ responder LN cells and 3 × 10⁴ irradiated (1,500 rad) spleen stimulator cells of the strains indicated were set up with varying numbers of BM cells of the strains indicated, incubated, and assayed for cytotoxic activity as in Fig. 1. Results are shown for only one BM cell dose, the smallest number tested producing significant suppression in any of the groups of the experiment.

$^\dagger$ This was calculated as described in Materials and Methods. The entries are proportional to the number of CL present.

$§$ Indicates that the cytotoxic activity is significantly below ($P < 0.05$) that of the control group without added BM cells as assessed by the Wilcoxon rank sum test.

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added BM cells were allogeneic to both responder and stimulator cells, significant enhancement of cytotoxic activity was observed. This phenomenon has not been investigated.

We conclude that BM contains a cell population capable of suppressing a response against its own antigens, i.e., BM contains anti-self-suppressor cells.

* Spleen Does Not Contain Anti-Self-Suppressor Cells. Fig. 2 shows an experiment comparing the ability of B6RF₁ BM and spleen cells to suppress the anti-B6RF₁ response. The cytotoxic activity produced in the cultures with added spleen cells, whether irradiated (1,500 rad) or not, was equal to or higher than in the control cultures without added cells. The added BM cells produced significant suppression. We conclude that spleen cells do not contain the anti-self-suppressor cells found in BM.

* BM Cells Stimulate LN Cells Poorly. When unirradiated BM cells were used as
stimulator cells in place of 1,500 rad-irradiated spleen cells, the cytotoxic activity was markedly lower (Fig. 3). If the BM cells were irradiated, more cytotoxic activity was obtained but it was still significantly less than that obtained using irradiated spleen stimulator cells. Thus, as well as suppressing the response against irradiated spleen cells syngeneic or semisyngeneic to themselves, BM cells cannot serve as effective stimulator cells for the development of a cytotoxic response to their own histocompatibility antigens.

Characterization of BM Cells Having Anti-Self-Suppressive Activity. The BM suppressor cells are not strongly adherent cells. After adherence separation (Materials and Methods), ~60% of B6RF1 BM cells were recovered as nonadherent cells. These nonadherent BM cells were equivalent or slightly better at suppressing the B6 anti-B6RF1 cytotoxic response than untreated B6RF1 BM cells (Fig. 1).

The BM suppressor cells are sensitive to 900 rad-irradiation. BM cells from B6RF1 mice were irradiated before being added to B6 anti-B6RF1 cultures (Fig. 4). BM cells exposed to 900 rad-irradiation lost all or nearly all their suppressive activity, and even 200 rad significantly removed suppressive activity.

The BM suppressor cells are not sensitive to anti-T cell serum treatment. B6RF1 BM cells were treated with three different anti-T cell antisera plus complement before being added to B6 anti-B6RF1 MLR (Table II). None of these treatments significantly removed anti-self-suppressive activity from BM cells.

T Cell Colonies Grown from BM Contain Anti-Self-Suppressor Cells. The extent of anti-
Fig. 3. Failure of BM cells to act as effective stimulator cells. Replicate cultures (12/group) were set up and assayed as in Fig. 1. Responder cells were $3 \times 10^6$ B6 LN cells. Stimulator cells, of the strains indicated, were $3 \times 10^5$ irradiated spleen cells, or $3 \times 10^5$ BM cells irradiated or not as indicated.

| Exp. | Added BM cells | Relative cytotoxic activity |
|------|----------------|-----------------------------|
| 1    | B6RF$_1$ (0 rods) |                             |
|      | B6RF$_1$ (900 rods) |                             |
| 2    | B6 (0 rods)      |                             |
|      | B6RF$_1$ (0 rods) |                             |
|      | B6RF$_1$ (200 rods) |                           |
|      | B6RF$_1$ (900 rods) |                           |

Fig. 4. The suppressive ability of BM cells is destroyed by irradiation. Replicate B6 anti-B6RF$_1$ cultures were set up and assayed as in Fig. 1. The added BM cells ($3 \times 10^5$/culture) were irradiated as indicated before addition. The cytotoxicity is expressed relative to control cultures without added BM cells. Cytotoxic activities in these control cultures were 53 ± 11 (experiment 1) and 22 ± 6 (experiment 2).

self-suppressive activity of whole BM was variable from experiment to experiment, and large doses of BM cells often produced nonspecific suppression (Fig. 1). We therefore looked for a more active, less nonspecific subpopulation of cells derived from BM. Previous work (12) had shown that T cell-containing lymphoid colonies could be grown from BM, and we tested whether such colonies had anti-self-suppressive activity.

As shown in Fig. 5 (top panel), 300 lymphoid colony cells grown from B6 BM

| Stimulator cells | Cytotoxic activity |
|------------------|--------------------|
| C3 spleen (1500 rods) | ![Graph](#) |
| C3 BM (0 rods) | ![Graph](#) |
| C3 BM (1500 rods) | ![Graph](#) |
| B6C3F$_1$ spleen (1500 rods) | ![Graph](#) |
| B6C3F$_1$ BM (0 rods) | ![Graph](#) |
| B6C3F$_1$ BM (1500 rods) | ![Graph](#) |
significantly suppressed a C3 anti-B6C3F1 cytotoxic response but the same colony cells did not significantly suppress a B6 anti-B6C3F1 response up to a dose of 1,000 cells. Slight nonspecific suppression is seen at a dose of 6,000 cells. This is much more active than whole BM; at least 3 × 10^4 B6 BM cells were required to suppress the C3 anti-B6C3F1 response (Table I). Note that the results of Fig. 5 are expressed relative to the control without added colony cells to facilitate comparing the effects of adding the same colony cells to MLR between different responder-stimulator pairs. As a second example (Fig. 5, bottom panel), as few as 300 colony cells from B6D2F1 BM suppressed a B6 anti-D2 response but did not suppress a B6 anti-C3 response except for the largest dose tested, 6,000 cells. From these results, BM lymphoid colony cells appear to be about 100 times more effective than whole BM cells for the suppression of a specified cytotoxic response.

Table III shows the results of testing various strain combinations of responder, stimulator, and BM lymphoid colony cells as in Table I. Experiment 1 is an example of lymphoid colony cells producing suppression when they are syngeneic to the stimulator but not when they are allogeneic: D2 colony cells, but not B6 colony cells, suppressed a C3 anti-D2 response; the same D2 colony cells did not suppress a C3 anti-B6 response. Experiment 2 is an example showing that the colony cells must be syngeneic to that parent of an F1 stimulator which actually produces the stimulation: B6 colony cells suppressed a C3 anti-B6C3F1 response but not a B6 anti-B6C3F1 response. Experiment 3 shows the same thing in another way: B6RF1, but not B6, colony cells suppressed a B6 anti-B6RF1 response. The same B6RF1 colony cells do not suppress a third-party B6 anti-B6D2F1 response. Experiments 4 and 5 are further variations on the same theme. In all of the above experiments, control groups were included containing BM colony cells and irradiated stimulator cells but not responder LN cells. No cytotoxic activity was obtained (data not shown). The genetic restrictions on the conditions required to obtain suppression appear identical with those deduced above for suppression by BM cells, and we conclude that BM lymphoid colonies also contain a cell population capable of suppressing a response against their own antigens.

Characterization of Colony Cells Having Anti-Self-Suppressive Activity. The anti-self-
Suppression of the development of cytotoxic activity in MLR cultures by added BM lymphoid colony cells. (Top) Cells from pooled B6 BM lymphoid colonies were added in the numbers indicated to replicate (eight per group) cultures of either B6 anti-B6C3F1 (△) or C3 anti-B6C3F1 (○), set up and assayed as in Fig. 1. The cytotoxic activities are expressed relative to control cultures without added BM lymphoid colony cells. These were 141 ± 18 for B6 anti-B6C3F1 and 151 ± 57 for C3H anti-B6C3F1. (Bottom) Cells from pooled B6D2F1 BM lymphoid colonies were added as above to either B6 anti-C3 (○) or B6 anti-D2 (△) cultures. The cytotoxic activities in control cultures without added BM lymphoid colony cells were 36 ± 5 for B6 anti-C3 and 329 ± 82 for B6 anti-D2.

Suppressor cells in BM lymphoid colonies are sensitive to anti-T cell serum treatment. In Table IV, BM lymphoid colony cells were treated with the same three kinds of anti-T cell serum and complement as used in Table II for the treatment of whole BM. The recovery figures indicate that about one-half of colony cells are Thy-1-positive cells. The three anti-T cell reagents, with the possible exception of rabbit anti-mouse brain (RAMB), removed the suppressive ability of the BM lymphoid colony cells. We conclude that the suppressor cell in BM lymphoid colonies carries Thy-1 antigen.

The anti-self-suppressor cells in BM lymphoid colonies are resistant to an irradiation dose of 1,500 rad. Colony cells from D2 BM were exposed to 1,500 rad-irradiation before addition to C3 anti-D2 MLR cultures. The D2 colony cells, whether irradiated or not, produced suppression at all cell doses tested (Fig. 6). However, the suppression seen at the highest cell dose, 6,000 cells, is probably not specific as suppression was also produced at this dose by syngeneic-to-responder C3 colony cells (Fig. 6). The suppressive activity of colony cells from two other strains (B6D2F1 in a B6 anti-D2 response, and C3D2F1 in both a C3 anti-D2 and C3 anti-C3D2F1 response) was also completely resistant to an irradiation dose of 1,500 rad.
### Table III

*Suppression of CL Production by Added BM Lymphoid Colony Cells*

| Experiment | Response          | Added BM lymphoid colony cells | Cytotoxic activity |
|------------|-------------------|-------------------------------|-------------------|
| 1          | C3 anti-D2        | 0                             | 280 ± 59          |
|            |                   | 600 B6                        | 239 ± 40          |
|            |                   | 600 D2                        | 157 ± 24*         |
|            | C3 anti-B6        | 0                             | 92 ± 9            |
|            |                   | 600 D2                        | 112 ± 19          |
| 2          | C3 anti-B6C3F1    | 0                             | 146 ± 21          |
|            |                   | 300 B6                        | 62 ± 11*          |
|            | B6 anti-B6C3F1    | 0                             | 82 ± 11           |
|            |                   | 300 B6                        | 100 ± 14          |
| 3          | B6 anti-B6RF1     | 0                             | 277 ± 118         |
|            |                   | 1,000 B6RF1                   | 128 ± 23*         |
|            |                   | 1,000 B6                      | 257 ± 141         |
|            | B6 anti-B6D2F1    | 0                             | 124 ± 27          |
|            |                   | 1,000 B6RF1                   | 140 ± 66          |
| 4          | C3 anti-D2        | 0                             | 215 ± 50          |
|            |                   | 300 C3D2F1                    | 134 ± 14*         |
|            | C3 anti-B6        | 0                             | 91 ± 8            |
|            |                   | 300 C3D2F1                    | 111 ± 19          |
|            | B6 anti-D2        | 0                             | 151 ± 18          |
|            |                   | 300 C3D2F1                    | 114 ± 11*         |
| 5          | C3 anti-C3D2F1    | 0                             | 73 ± 10           |
|            |                   | 1,000 D2                      | 47 ± 7*           |
|            | B6 anti-C3        | 0                             | 35 ± 5            |
|            |                   | 1,000 D2                      | 35 ± 7            |
|            |                   | 1,000 C3D2F1                  | 16 ± 2*           |

All experiments were performed and analyzed in a manner identical to those of Table I except that BM lymphoid colony cells were used in place of BM cells.

* These entries are significantly suppressed (P < 0.05) relative to the control without added BM colony cells.

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*Site of Action of the BM Suppressor Cell.* The BM suppressor cell could reduce the cytotoxic activity per culture either by preventing the activation of CLP or by reducing the number of CL produced per activated CLP. One way to distinguish between the two possibilities is to measure the frequency of CLP in the presence and absence of added BM suppressor cells. We have previously developed a procedure for measuring CLP frequencies by limiting-dilution (18, 20). Table V summarizes the results of an experiment using this procedure to measure the frequency of CLP in a B6 anti-B6RF1 response in the presence or absence of B6RF1 BM cells. Without added
### Table IV
Effect of Anti-T Cell Antisera on the BM Lymphoid Colony Suppressive Activity*

| Antiserum treatment (dilution)‡ | Percent recovery of viable cells | Relative cytotoxic activity§ |
|-------------------------------|---------------------------------|-----------------------------|
| None                          | 100                             | 0.44 ± 0.25 || |
| C only                        | 75                              | 0.46 ± 0.23 || |
| Anti-Thy-I (1:200) plus C     | 28                              | 1.17 ± 0.61 |
| RAMB (1:25) plus C            | 33                              | 0.70 ± 0.34 |
| Anti-Thy-1.2 hybridoma (1:200) plus C | 46 | 1.21 ± 0.64 |

*Replicate C3 anti-B6C3F1 cultures containing 1,000 B6 BM lymphoid colony cells treated as indicated were set up and assayed as in Fig. 1.

‡The three anti-T cell reagents and their method of use are described in Materials and Methods.

§Cytotoxic activity is expressed relative to control cultures not containing added BM cells for which the cytotoxic activity was 151 ± 57.

|| These entries are significantly (P < 0.05) suppressed relative to control.

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**Fig. 6.** The suppressive ability of BM lymphoid colony cells is not affected by irradiation. Replicate C3 anti-D2 cultures were set up and assayed as in Fig. 1. Varying numbers of irradiated (1,500 rad,  at 250) or unirradiated ( at 250) D2 BM lymphoid colony cells were added to the cultures as indicated. As a control ( at 250), unirradiated D2 colony cells were added to C3 anti-B6 cultures. Cytotoxic activities in the absence of added colony cells were 272 ± 89 for the C3 anti-D2 response and 91 ± 8 for the C3 anti-B6 response.

BM cells, the frequency was 432/10⁶ responder cells; with added BM cells, the frequency was 53.3/10⁶ responder cells, i.e., the frequency is about eight times lower. This difference is significant (Table V, 95% confidence limits).

From the data of Table V, one can also determine whether the suppressor cell is affecting the number of CL produced per activated CLP. Both the mean cytotoxic...
TABLE V
Limiting-Dilution Analysis of B6 Anti-B6RF1 Cytotoxic Response in the Presence and Absence of B6RF1 BM Cells*

| Responder LN cells/culture | Number of responding cultures | Mean cytotoxic activity/culture | Mean cytotoxic activity/responding culture |
|---------------------------|-------------------------------|---------------------------------|------------------------------------------|
| A, no added BM            | 1,000                         | 7/20                            | 5.1 ± 1.4                                |
| \( f_{CLP} = 432/10^6 \) |                               | 3/20                            | 3.5 ± 1.3                                |
| Range = (256-731)/10⁶     | 300                           | 2/20                            | 0.5 ± 1.0                                |
| \( \chi^2 = 0.14 \)      | 0                             | 0/15                            | 0 ± 0.9                                  |

| B, with added BM          | 30,000                        | 16/20                           | 26.3 ± 3.6                               |
| \( f_{CLP} = 533/10^6 \) |                               | 9/20                            | 12.2 ± 2.9                               |
| Range = (35.7-79.5)/10⁶  | 3,000                         | 2/20                            | 2.4 ± 1.6                                |
| \( \chi^2 = 0.48 \)      | 0                             | 1/16                            | 0 ± 1.7                                  |

* Replicate cultures containing varying numbers of B6 LN responder cells as indicated and \( 3 \times 10^5 \) irradiated (1,500 rad) B6RF1 stimulator spleen cells were set up and assayed as in Figure 1. All cultures also contained \( 10^5 \) B6 nu/nu spleen cells as a source of accessory cells required for CL production (18). In part B, all cultures also contained \( 3 \times 10^5 \) B6RF1 BM cells.

The frequency of CLP (\( f_{CLP} \)), range (95% confidence limits), and \( \chi^2 \) were calculated by limiting dilution analysis as described in Materials and Methods.

The mean cytotoxic activity of all cultures in the group for which the \( ^{51}Cr \) release exceeded the spontaneous release by 2 SD, as all groups are near limiting-dilution, these values should approximately equal the cytotoxic activity per clone. The entries in parentheses are the measured values corrected for the possibility of there being more than one CLP in a responding culture calculated using the measured CLP frequency.

The mean values are 10.3 and 23.2 in the absence and presence of BM cells, respectively. If anything, the cytotoxic activity per responding culture has increased in the presence of BM cells. Some responding cultures are likely to contain more than one CLP. The Poisson distribution can be used to estimate this probability and the entries in parentheses in the last column of the table represent such estimates of the true cytotoxic activity per clone for each group. The mean values of cytotoxic activities per clone are 8.8 and 16.0 in the absence and presence of BM cells, respectively. We conclude that the BM suppressor cell acts by preventing CLP activation and not by reducing the number of progeny CL per activated CLP.

If BM suppressor cells act on CLP, then they should have no suppressive effect if their addition is delayed for a sufficiently long time after culture initiation. Fig. 7 is an experiment showing this to be the case. Two doses of B6RF1 BM cells were added to B6 anti-B6RF1 cultures at varying times after culture initiation. The lower dose (\( 6 \times 10^6 \)) produced suppression at 0 and 18 h but not at 40 or 66 h; the higher dose (\( 3 \times 10^6 \)) produced suppression at 0, 18, and 40 h, but not at 66 h. The possible significance of the enhancement seen at late times has not been investigated. However, it is clear that the suppression acts at early times rather than late times, which is consistent with the conclusion that the CLP is the site of the suppressive activity.
Both BM cells and cells from T cell colonies grown from BM can, when added to an MLR, suppress the development of cytotoxic activity against the stimulator if the BM cells or colony cells are syngeneic to the stimulator cells (Figs. 1 and 6). We have extensively investigated whether semisyngeneic and allogeneic combinations will also produce suppression. Assuming that the limited number of strains tested for suppression by BM (Table I) and by T cell colonies grown from BM (Fig. 5; Table III) are representative of all strains, the combinations of responder, stimulator and BM, or BM colony cells necessary for suppression can be summarized as in Table VI. BM or colony cells from strains B, (A × B)F₁, and (B × C)₁F₁ can suppress the production of cytotoxic activity in either A anti-B or A anti-(A × B)₁F₁ cultures. BM or colony cells from strains A, C, or (A × C)₁F₁ do not suppress the production of cytotoxic activity in either A anti-B or A anti-(A × B)₁F₁ cultures. In other words, suppression is obtained so long as the added BM or colony cells are syngeneic or semisyngeneic to the stimulator cells irrespective of whether they bear all or none of the histocompatibility antigens of the responder cells. In accordance with these findings, when BM cells are used directly as stimulator cells in an MLR, very little cytotoxic activity is obtained in comparison with using spleen cells (Fig. 3). In the type of MLR studied here in which responder and stimulator strains differ at H-2, the vast majority of the CL produced are directed against H-2 determinants (22, 23). Thus one might expect that, to obtain suppression, the added BM or colony cells must share H-2 with the stimulator cells and might still produce suppression if differing at various minor histocompatibility loci. This point has not yet been
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TABLE VI
Schematic Summary of Results Obtained on Adding BM or BM Colony Cells to an MLR

| Strain of BM cells or colony cells | Cytotoxic activity |
|----------------------------------|-------------------|
|                                  | A anti-B response | A anti-(A × B)F₁ response |
| A                                | —                 | —                         |
| B                                | S                 | S                         |
| C                                | —                 | —                         |
| (A × B)F₁                        | S                 | S                         |
| (B × C)F₁                        | S                 | S                         |
| (A × C)F₁                        | —                 | —                         |

An entry of S indicates that the added BM or BM colony cells produce suppression; an entry of — that they do not. The cytotoxic activity is against a target from the same strain as the stimulator.

It has been shown previously (12) that the ability of BM to form T cell-containing colonies is unaffected by treatment with an anti-T cell antiserum and complement but is destroyed by 1,000-rad irradiation. Thus the colony-forming unit is composed of one or more Thy-1-negative (or Thy-1-weak) cells at least one of which must proliferate. During colony development, Thy-1-positive cells are produced.

There is substantial precedent for production of Thy-1-positive cells from Thy-1-negative precursors in in vitro cultures of BM. Komuro and Boyse (25) demonstrated that Thy-1 antigen can be induced in vitro on Thy-1-negative BM cells after a 2-h incubation with a thymus extract. Press et al. (26) found that BM from both normal
and nu/nu mice contains Thy-1-negative cells that can give rise to Thy-1-positive blast cell progeny in response to PHA. Schrader et al. (27) report that cells containing terminal deoxynucleotidyl transferase, a probable marker of thymocyte progenitors, are generated in long-term cultures of murine BM. Using BM cells from similar long-term cultures and a culture system for growing T cell colonies identical with that described here, Jones-Villeneuve et al. (28) obtained colonies containing Thy-1-positive cells. These colonies grew from Thy-1-negative precursors. Further, they showed that the colony cells can provide help required for the generation of CL but cannot themselves make CL. These colonies also contain anti-self-suppressive activity (S. Muraoka. Unpublished observations.).

Although the cells responsible for suppression in BM and in BM T cell colonies appear to be quite different, they might be related. We propose that the suppressive ability of BM is caused by a Thy-1-negative precursor cell that proliferates and differentiates in culture into a radiation-resistant, Thy-1-positive cell actually responsible for the suppression. This latter cell would then be the same as the cell in BM T cell colonies responsible for producing suppression. We have, however, no conclusive evidence to rule out the conclusion that the suppression produced by BM and by colony cells are completely independent processes.

The suppression produced by added BM cells appears to be a result of a decrease in the number of CLP activated rather than a decrease in the number of CL produced per activated CLP. This follows directly from the experiment of Table V in which CLP frequency and relative CL clone size were measured in the presence and absence of added BM cells. The same results are obtained using BM T colony cells as a source of suppressor cells (J. J. Rusthoven and R. G. Miller. Unpublished observations.). It is also observed that when BM cells are added at varying times after the initiation of an MLR, they produce suppression only when added early (Fig. 7). We conclude that the suppressive mechanism operates by reducing the number of CLP activated. This would be most simply accomplished by deletion of the CLP but we cannot at present exclude the possibility that some essential step in their activation has been blocked. Whether the initial block is followed by development of active suppression (4, 5) has not been investigated.

What are the steps involved in the blocking of CLP activation by the anti-self-suppressive mechanism? There are two broad classes of models depending upon the initial direction of recognition, recognition of responder cells by suppressor cells, or vice versa (29, 30). In the first class of models, the suppressor cells would have to bear receptors capable of recognizing receptors in the responding population that can recognize the self antigens of the suppressor cells, i.e., the suppressors would have to bear anti-idiotype receptors. This would require the suppressor cells to have a receptor repertoire capable of distinguishing between idiotypes directed against self and nonself. An alternative simpler model (14, 29, 30) is that the suppressor cell inactivates CLP which recognize the self antigens of the suppressor cell. Such an inactivation could take place directly, through cell contact, or indirectly through release of a short-range factor. A long-range factor would appear to be ruled out as this would have prevented us from getting a good fit to the one-hit Poisson model in the limiting-dilution experiment of Table V. In addition, experiments looking for a suppressive supernatant factor have been negative (S. Muraoka and R. G. Miller. Unpublished data.).
The spleens of athymic nude mice, but not of normal mice, contain a cell subpopulation capable of suppressing the development of cytotoxic activity with self-nonself discriminatory powers and mode of action apparently identical with those described here (14). In addition, in a completely syngeneic system, the suppressor cells in nude spleen will suppress an H-2-restricted, trinitrophenyl (TNP)-specific response against TNP-modified stimulator cells if the nude spleen cells are also TNP modified (29). This provides strong evidence in favor of the conclusion that suppression involves recognition of the suppressor cell by the CLP rather than vice versa.

Thymus also appears to contain a cell capable of suppressing a response against its own antigens in a manner similar to that seen for the suppressor cells in bone marrow and in nude spleen ([29]; Manuscript in preparation.). Assuming these cells are all related, one possibility is that the suppressor cell seeds from bone marrow to thymus in the normal mouse; the spleen would then be an ectopic site in the athymic nude mouse.

Duwe and Singhal (31, 32) have described a subpopulation of cells in mouse BM which can suppress the ability of syngeneic spleen cells to make IgM antibody-producing cells in vitro. Production of IgG antibody-producing cells is not affected. Suppression appears to be at the level of B cell activation. The suppressor cells are nonadherent, Thy-1 negative (or weak), and radiation sensitive. They hypothesize that this suppressive mechanism is involved in maintenance of self-tolerance. The parallels between their BM suppressor cell, active in humoral immunity, and our BM suppressor cell, active in cell-mediated immunity, are striking although there is no direct evidence at present that they are related.

Summary
Both normal mouse bone marrow and cells from T cell-containing colonies grown in vitro from normal bone marrow contain cells which can specifically suppress the development of cytotoxic T lymphocytes capable of recognizing alloantigens on the bone marrow or colony cells. Suppression, as assessed by reduction in cytotoxic activity, is produced by adding bone marrow or colony cells to mixed lymphocyte reactions between lymph node responder cells and irradiated histoincompatible spleen stimulator cells. The cytotoxic activity is reduced if the added bone marrow or colony cells are syngeneic or semisyngeneic to the stimulator cells but not if they are allogeneic. Suppression results from a reduction in the number of cytotoxic lymphocyte precursor cells activated in the cultures. The suppressor cells in bone marrow are radiation sensitive and Thy-1 negative; those in colonies grown from bone marrow are radiation resistant and Thy-1 positive.

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