SUPPRESSOR T CELLS ACTIVATED IN A PRIMARY IN VITRO RESPONSE TO NON-MAJOR HISTOCOMPATIBILITY ALLOANTIGENS*

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Attempts made to generate cytotoxic lymphocytes (Tc)\(^1\) against minor locus-encoded histocompatibility antigens (i.e., non-H-2) in primary in vitro cultures using responder cells from normal mouse strains have not been successful (1–3), although secondary responses after in vivo priming are readily demonstrable (4–7). In some reports (1–6), an M locus (Mls) incompatibility was included and in others (4), not. The Mls is a genetic locus (8), not linked to H-2, whose product(s) induce a strong proliferative response in incompatible responder cells in a manner analogous to the class II antigens coded for by genes in the I region of the H-2 gene complex. We have reported a low level of primary in vivo generated T cell-mediated cytotoxic activity in spleen cells of BALB/c mice injected 10–12 d previously with H-2 compatible, Mls, and other minor loci incompatible tumor cells of DBA/2 origin, which was tentatively postulated to be Mls specific (9). A number of groups (10–14) have recently described such a primary in vitro response to non-H-2 alloantigens using responder cells from the autoimmune mouse strain NZB that was postulated to be caused by hyperactive helper cells (13), which we feel is unlikely for reasons elaborated below (see Discussion) and from our observation on the role of suppressor cells in this type of response.

It was possible, using a limiting dilution assay (LDA), to obtain a primary in vitro Tc response to non-major histocompatibility complex (MHC) alloantigens using responder cells from a normal mouse strain (15). In the LDA, decreased responses were observed at the higher responder cell doses, suggesting the presence of relatively infrequent suppressor cells too dilute to exert an effect at the lower responder cell numbers. Such putative suppressor cells could also explain the lack of response observed in bulk cultures that contain five times the number of responder cells per well of the highest LDA concentration. We also showed (16) that by pretreating a population of responder cells with an anti-Ia antiserum (which exhibited strong anti-I-J\(^k\) activity) and complement (C) before stimulation in a bulk MLR culture with H-2-compatible non-H-2-incompatible stimulator cells, a primary in vitro Tc response was obtained. This suggested a role for I-J\(^k\) suppressor cells in the lack of a primary Tc response observed in primary bulk cultures. Based on these facts, we decided to

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1 Abbreviations used in this paper: C, complement; CD, cytotoxic determinant; Con A, concanavalin A; CML, cell-mediated lympholysis; EHAA, extra high amino acids (abbreviation used in ref. 17 for the enriched medium used for mouse serum cultures); FCS, fetal calf serum; H-199, Hepes-buffered medium 199; HBSS, Hanks' balanced salt solution; IL-2, interleukin 2; LDA, limiting dilution assay; LPS, lipopolysaccharide; MLR, mixed lymphocyte reaction; Mls, M locus; MS, mouse serum; Tc, cytotoxic T lymphocyte; Ts, suppressor T effector cell; Tsp, suppressor T cell precursor.

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test more directly for suppressor activity in cells recovered from primary bulk mixed
lymphocyte reaction (MLR) cultures against H-2-compatible, non-H-2- (including Mls)
incompatible cells.

In the present report, we show that despite the fact that we cannot generate a
primary Tc response to non-H-2 alloantigens in conventional bulk culture, a good
secondary H-2-restricted response is observed in mouse serum (MS) medium using
BALB/c responder and DBA/2 stimulator cells. This has been shown previously in
fetal calf serum (FCS) medium (4–6). Cells obtained from a BALB/c (H-2d Mlsb)
anti-DBA/2 (H-2d Mlsa) MLR were able to totally suppress an in vitro anti-H-2
response. The precursor of the suppressor cell (Tsp) in normal spleen was found to be
Thy-1+, Lyt-1+, 2−, I-J−. The suppressor effector cell (Ts) was shown to express exactly
the same phenotype and to be highly radiosensitive.

Materials and Methods

Mice. BALB/cByJ, DBA/2J, B10.D2SnJ, C3H/HeJ, and AKR/J mice were purchased
from The Jackson Laboratory, Bar Harbor, ME. B10.A, B10.A(1R), B10.HTG, B10.RIII,
B10.S, and B10.S(7R) were obtained from the animal facility of the Immunobiology Research
Center at the University of Wisconsin, Madison, WI. C57BL/6ByJ mice were bred in our
mouse colony at the Sloan-Kettering Institute. Mice between 6 and 12 wk were used and were
sex-matched in each individual experiment.

Serological Reagents. The anti-Lyt-1.2 reagent was a monoclonal antibody prepared and
provided by Dr. F.-W. Shen, and the anti-Thy-1.2 and anti-Lyt-2.2 were monoclonal antibodies
prepared and provided by Dr. U. Hämmerling, both of the Sloan-Kettering Institute. The
anti-I-Jd reagent was an antiserum prepared in the mouse strain combination (D2.GD X
B10.A)F1 anti-B10.D2 by and provided by Dr. C. S. David of the Mayo Clinic Foundation,
Rochester, MN. Rabbit sera selected for low toxicity for mouse cells were used as a source of
lytic C.

Media Components. All media components were supplied by Flow Laboratories, Rockville,
MD, unless otherwise stated.

In Vitro Generation of Cytotoxic Cells. Single-cell suspensions were prepared by gentle teasing
of mouse spleens on sterile steel screens in Hanks' balanced salt solution buffered with 20 mM
Hepes (HBSS). Where secondary responses were measured, the BALB/c mice had been
immunized 2–4 weeks previously by intraperitoneal injection of 2 × 10^7 DBA/2 spleen cells.
Responding cells (2.5 × 10^6/well) were dispensed together with X-irradiated (2,000 rad)
stimulator cells (5 × 10^6/well) in a final volume of 2.5 ml into the wells of 24-well culture plates
(76-033-050; Linbro Chemical Co., Hamden, CT). The culture medium was EHAA (extra-
high amino acids) (17) supplemented with 0.6% mouse serum from mice syngeneic to the
responder strain, 0.135% sodium bicarbonate and 5 × 10^-5 M 2-mercaptoethanol. The cultures
were incubated for 5 d (3 d for a secondary response) in a humidified atmosphere of 5% CO2
in air.

Cell-mediated Lympholysis (CML) Assay and MLR. Concanavalin A (Con A) -induced blast
cells used as target cells were prepared 2–3 d before the assay by culturing 3 × 10^7 mouse spleen
cells in a final volume of 10 ml of medium (EHAA supplemented with 5% FCS and 0.135%
sodium bicarbonate) with Con A (3 μg/ml, Pharmacia Fine Chemicals, Uppsala, Sweden). EL
4 lymphoma cells of C57BL/6 origin and P815 mastocytoma cells of DBA/2 origin were also
used for targets and were maintained by continuous passage in RPMI medium supplemented
with 10% FCS, 1% glutamine (200 mM), and 1% nonessential amino acids (100 X). After
harvesting the effector cells, 0.2 ml aliquots were dispensed into the wells of flat-bottomed
microtiter plates (76-002-03; Linbro Chemical Co.) to assay for cell proliferation by culturing
for a further 6 h in the presence of [3H]thymidine (1 μCi/well) and measuring the level of
incorporation of [3H]thymidine into the cells precipitated onto glass fiber discs with a Skatron
automatic cell harvester (Flow Laboratories). The effector cells were tested for Tc activity in a
^51Cr-release assay as previously described (15). Six control wells to obtain the spontaneous ^51Cr
release (i.e., background values) contained target cells and medium only, and another six wells
containing target cells and acid (1 N HCl) gave the values of maximal $^{51}$Cr release. Percent cytotoxicity was calculated according to the formula: \[
\text{Percent cytotoxicity} = \left( \frac{\text{experimental release (cpm) - spontaneous release (cpm)}}{\text{maximum release (cpm) - spontaneous release (cpm)}} \right) \times 100
\]
All values shown are the means of four replicate samples. Standard deviations are indicated.

**Generation and Testing of Suppressor Cells.** The experimental procedure used is diagrammatically represented in Fig. 1. Putative suppressor cells were generated by culturing BALB/c responder spleen cells with DBA/2, B10.D2, or control BALB/c stimulator cells under exactly the same conditions described for the generation of Tc. They were assayed for suppressive activity by transfer into cultures containing fresh BALB/c responder cells and third-party H-2 incompatible (usually C57BL/6) irradiated stimulator cells. CML activity against target cells of this third party stimulator strain was tested for on day 5 of culture.

**Cytotoxic Eliminations.** Thy-1.2+, Lyt-1.2+, or Lyt-2.2+ unstimulated BALB/c spleen cells were eliminated by a single-step, two-cycle serological procedure. 20 million spleen cells were treated with the relevant monoclonal antibody (at a final dilution of 1:500) and C (at a final dilution of 1:25) in medium 199, buffered to pH 7.2 with 20 mM Hepes (H-199) in a final volume of 1 ml at 37°C for 40 min. The cells were washed once with H-199, resuspended in 1 ml of the same dilution of antibody and C and again incubated at 37°C for 40 min. They were then washed three times and viable cells were counted by trypan blue exclusion. Controls were treated with C only at the same final dilution and also given two cycles of treatment.

I-J+ cells were eliminated in a two-step, two-cycle procedure in which 2 X 10^7 spleen cells were treated with 1 ml of a 1:10 dilution of anti-I-Jd antiserum in H-199 at 4°C for 30 min. The cells were washed once, resuspended in 1 ml of C at a dilution of 1:25 in H-199, and incubated at 37°C for 40 min. After a single wash, the same procedure was repeated. The cells were then washed three times, and viable cells were counted. Controls were treated with H-199 only in the first step and C only in the second step and were also given two cycles of treatment.

Suppressor effector cells recovered from the first-step culture were treated with the reagents in exactly the same way, except that the monoclonal antibodies (anti-Thy-1.2, anti-Lyt-1.2,

\[\text{BALB/c (H-2d Mls}^b\text{) mice}\]
\[\downarrow\]
\[\text{Responder spleen cell suspension}\]
\[\downarrow\]
\[\text{(Cell treatment [antibodies and C])}\]

**First-step MLR**

\[\text{Suppressor generating MLR: Treated or untreated BALB/c responder cells and irradiated stimulator cells [DBA/2 (H-2d Mls}^b\text{), B10.D2 (H-2a, Mls}^h\text{), or BALB/c]}\]
\[\downarrow\]
\[\text{5-d culture}\]

\[\text{Cultures harvested}\]

\[\text{Aliquots removed for proliferation assay}\]

\[\downarrow\]

\[\text{Suppressor cells washed once and counted}\]

\[\downarrow\]

\[\text{(Cell treatment [antibodies and C/irradiation])}\]

**Second-step MLR**

\[\text{Suppressor assay MLR: treated or untreated suppressor cells with fresh BALB/c responder cells and fresh third-party H-2-incompatible [C57BL/6 (H-2b Mls}^b\text{) in most experiments] irradiated stimulator cells}\]
\[\downarrow\]
\[\text{5-d culture}\]

\[\text{Cultures harvested}\]

\[\text{Aliquots removed for proliferation assay}\]

\[\downarrow\]

\[\text{Cells washed once, counted, and tested for Tc activity against target cells of third-party H-2-incompatible strain used for stimulator cells in second-step MLR}\]

**Fig. 1.** Experimental design for suppressor cell generation and assay for suppression.
and anti-Lyt-2.2) were used at a final dilution of 1:50.

Preparation of Lectin-activated Blasts. Lectin-activated blasts were prepared to test whether blast cells nonspecifically exerted suppressive activity. BALB/c Con A blasts were prepared as described above in the section on the CML assay, except that the EHAA medium used was supplemented with 0.6% MS and not FCS. Lipopolysaccharide (LPS) blast cells were also prepared from BALB/c spleen cells in the same medium at the same cell concentration using the LPS *Salmonella typhosa* (Difco Laboratories, Detroit, MI) at a concentration of 50 μg/ml. The blast cells were harvested on day 2, the height of the blastogenic response, and were tested for suppressor activity in the same way as the MLR-generated suppressor cells.

Results

Lack of a Primary Tc Response to Non-MHC Alloantigens. Cells recovered from a bulk MLR of BALB/c (H-2<sup>d</sup> Mls<sup>b</sup>) responder cells and DBA/2 (H-2<sup>d</sup> Mls<sup>a</sup>) stimulator cells, in MS medium, show no cytotoxic activity against target cells of the stimulator cell strain (Table I), even though a high level of activity was seen if responder cells were obtained from mice injected 2-4 wk previously with DBA/2 spleen cells. This was true whether P815 tumor or DBA/2 Con A blast target cells were used. The cytotoxic activity of these secondarily stimulated Tc was cross-reactive on the B10 background and H-2 restricted (Table II) as shown by Bevan (4-6), who initially described the phenomenon using FCS-supplemented media. Considering the nonspecific effects and polyclonal-activating properties of FCS (18-20), we feel that it is important to carry out these experiments in MS-supplemented media. Secondary proliferative responses to Mls have also been shown to be specific for Mls and not H-2 restricted when the culture medium used for the primary response is supplemented with syngeneic MS (21). However, if FCS medium was used, non-Mls-specific but H-2-restricted prolif-

| Table I |
| --- |
| Lack of a Cytotoxic T Cell Response (Tc) in a Primary MLR to Non-H-2 Alloantigens* |
| Type of response | Responders | Stimulators | Effector/target ratio | Percent CML |
| | | | | P815 | DBA/2 Con A blasts |
| Primary‡ | BALB/c | DBA/2<sup>c</sup> | 50:1 | -2.3 ± 1.25 | -0.4 ± 0.8 |
| | | | 10:1 | -2.0 ± 1.20 | 1.0 ± 1.5 |
| | | | 1:1 | -1.8 ± 1.0 | 1.8 ± 1.9 |
| | | | 50:1 | 73.6 ± 7.3 | 26.7 ± 3.9 |
| | | | 10:1 | 38.9 ± 3.3 | 18.8 ± 4.6 |
| | | | 1:1 | 8.9 ± 3.0 | 6.1 ± 4.4 |
| Secondary§ | BALB/c | DBA/2<sup>c</sup> | 50:1 | 1.4 ± 2.9 | -2.0 ± 2.5 |
| | | | 10:1 | 1.0 ± 2.6 | -0.3 ± 4.0 |
| | | | 1:1 | 1.8 ± 2.1 | 0.6 ± 3.5 |

* The responder cells were stimulated with either DBA/2 or BALB/c stimulators for 5 d and then tested for cytotoxic activity against P815 or DBA/2 Con A blast target cells as described in Materials and Methods. Primary responses were tested for with responder spleen cells from normal BALB/c mice, and secondary responses were assayed with responder spleen cells from BALB/c mice injected 2-4 wk previously with 2 × 10<sup>6</sup> DBA/2 spleen cells.

‡ Primary BALB/c, normal BALB/c spleen cells.

§ Secondary BALB/c spleen cells obtained from BALB/c mice injected 2-4 wk previously with 2 × 10<sup>7</sup> DBA/2 spleen cells.
NON-H-2-ACTIVATED T SUPPRESSOR CELLS

Cytotoxic Activity of Secondaryly Stimulated Tc was Restricted by the K-I and D Subregions of the H-2 Complex

* Tc generated by culturing responder spleen cells from BALB/c mice (injected 2-4 wk previously with DBA/2 spleen cells) with irradiated DBA/2 stimulator cells for 3 d were tested for cytotoxic activity against the various Con A blast target cells. Effector/target cell ratio for all values was 50:1. The genetic information shown in this table was taken from ref. 22.

| Target cell | H-2 | Mls | Percent cytotoxicity |
|-------------|-----|-----|----------------------|
| DBA/2       | d   | d   | d                    | d | d | d | d | d | d | d | a | 41.0 ± 3.3 |
| C3H         | k   | k   | k                    | k | k | k | k | k | k | b | -1.5 ± 1.5 |
| AKR         | k   | k   | k                    | k | k | k | k | k | k | a | 3.4 ± 2.6  |
| C57BL/6     | b   | b   | b                    | b | b | b | b | b | b | b | b | 3.9 ± 1.2  |
| B10.D2      | d   | d   | d                    | d | d | d | d | a | d | d | b | 25.7 ± 2.8 |
| B10.HTG     | d   | d   | d                    | d | d | d | d | ? | d | b | b | 15.7 ± 2.9 |
| B10.S(7R)   | s   | s   | s                    | s | s | s | s | s | s | b | 39.7 ± 1.5 |
| B10.HTG     | s   | s   | s                    | s | s | s | s | s | s | d | b | 29.7 ± 4.2 |
| B10.A(1R)   | k   | k   | k                    | k | k | k | k | d | d | d | b | 19.1 ± 2.0 |

Suppressor Cells are Generated in an Anti-Non-H-2 MLR. Because we suspected that the suppressor cells were involved in the lack of a primary Tc response to non-H-2 alloantigens, we decided to look directly for the presence of suppressor cells in cells recovered from a primary MLR against non-H-2 alloantigens. BALB/c spleen cells were stimulated in vitro with irradiated DBA/2 spleen cells for 5 d, harvested, and then transferred into cultures of BALB/c responders and third-party H-2 incompatible stimulator cells. On day 5, the cytotoxic activity of effector cells recovered from these second-step cultures was assayed against target cells of the third-party stimulator cell strain. The proliferative (MLR) response of cells in these cultures was also measured by [3H]thymidine incorporation. Two experiments are shown in Table III. In experiment 1, suppression of the BALB/c anti-C57BL/6 MLR and Tc responses was observed by the cells recovered from the BALB/c anti-DBA/2 MLR, but not by cells from the control BALB/c anti-BALB/c cultures. In some experiments (4 out of 30) the unstimulated cells were slightly suppressive (see Fig. 2, for example) but never by >20% of the response. Sometimes (13 out of 30 experiments) they even exerted an augmenting effect on both the proliferative and Tc responses in the second step MLR (Table III, experiment 2, for example).
Suppressor Cells that Suppress Anti-H-2 Allogeneic MLR and Tc Responses Are Generated in a Primary MLR to Non-H-2 Alloantigens if There Is an Mls Incompatibility

Table III

| Suppressor | MLR<sub>a</sub> | Suppressor/ | MLR<sub>b</sub> | Percent cytotoxicity |
|------------|----------------|-------------|----------------|---------------------|
|            | [C57BL/6(H-2<sup>b</sup>)EL4 cells] |
|            | MLR<sub>a</sub> responder MLR<sub>b</sub> ratio |               | [C57BL/6(H-2<sup>b</sup>)EL4 cells] |
| Experiment 1 |                |               |               |                     |
| + BALB/c (H-2<sup>d</sup>Mls<sup>a</sup>) | 6443 ± 385 | 0.5:1 | 4153 ± 907 | 7.8 ± 1.2 |
| + DBA/2 (H-2<sup>d</sup>, Mls<sup>a</sup>) | 0.25:1 | 4539 ± 413 | 4.7 ± 1.2 |
| + BALB/c | 726 ± 256 | 0.5:1 | 15946 ± 1163 | 78.9 ± 2.6 |
| + BALB/c, None | 726 ± 256 | 0.5:1 | 16325 ± 4153 | 81.4 ± 5.2 |
| + BALB/c, None | 726 ± 256 | 0.5:1 | 15946 ± 1163 | 78.9 ± 2.6 |
| + B10.D2(H-2<sup>d</sup>Mls<sup>b</sup>) | 0.125:1 | 50136 ± 1740 | 22.2 ± 3.0 |
| + BALB/c | 592 ± 31 | 0.5:1 | 54247 ± 2910 | 46.7 ± 3.1 |
| + BALB/c, None | 592 ± 31 | 0.5:1 | 41284 ± 907 | 22.6 ± 4.3 |

*The suppressor cells were generated in primary MLR cultures as described in Materials and Methods. The MLR<sub>a</sub> column shows the degree of cell proliferation in cpm of [H<sup>3</sup>]-thymidine incorporated into the cells in the first step suppressor generating cultures. The suppressor/responder ratio indicates the relative number of suppressors added to a constant number of BALB/c responder cells (2.5 × 10<sup>5</sup> cells/well) in 2.5 ml complete EAA medium. 2.5 × 10<sup>5</sup> irradiated stimulator cells (C57BL/6 in experiment 1 and B10.RIII in experiment 2) were added per well. The MLR<sub>b</sub> column shows the degree of cell proliferation in cpm of [H<sup>3</sup>]-thymidine incorporated into the cells in the second step suppressor assay cultures. The level of Tc activity in cells recovered from the cultures, when tested against EL4 (experiment 1) and B10.RIII Con A blast (experiment 2) target cells at an effector to target cell ratio of 40:1, is given in the last column.

Part of experiment 2 (in Table III) is a titration of BALB/c suppressor cells generated by stimulation with DBA/2 spleen cells and tested in a BALB/c anti-B10.RIII (H-2<sup>d</sup> Mls<sup>b</sup>) Tc response. When BALB/c cells were cultured with B10.D2 (H-2<sup>d</sup> Mls<sup>b</sup>) stimulators (an H-2<sup>d</sup>- and Mls-compatible combination) and then tested for suppressive activity, at the suppressor/responder ratios used, no suppression was detected. In this combination (i.e., BALB/c anti-B10.D2), no proliferative response is observed, whereas a strong MLR is induced across the Mls incompatibility (i.e., BALB/c anti-DBA/2) (Table III). Both DBA/2 and B10.D2 were shown to differ from responder BALB/c mice with respect to alleles at seven out of eight different non-H-2 loci (23). Thus, it is probable that it is the proliferation inducing incompatibility at the Mls locus that facilitates the generation of the suppressor cells. However, to confirm this, a segregation analysis of backcross animals should be carried out. These experiments are in progress.

The suppression is not selective, as both a BALB/c anti-B57BL/6 (experiment 1) and a BALB/c anti-B10.RIII (experiment 2) response were suppressed. It appears from the two experiments shown that the suppression was far more potent in the anti-C57BL/6 response than that observed in the anti-B10.RIII combination used in the second-step assay cultures. However, this effect is not a consistent one, and reflects variability in the strength of the suppressors generated rather than higher susceptibility of one anti-H-2 MLR combination to suppression than another. Because of this...
Suppressor cell activity is inversely proportional to the proliferation observed in suppressor generating cultures. BALB/c anti-DBA/2 suppressor cells were generated in MLR cultures at different responder/stimulator cell ratios. The degree of cell proliferation was measured at the time of harvesting (day 5), and the suppressor activity of the cells obtained was assayed in terms of their ability to suppress a BALB/c anti-C57BL/6 Tc response in second-step suppressor assay cultures. Suppressor cells were added at a suppressor/responder ratio of 0.25:1, and Tc activity was tested for against C57BL/6 Con A blast target cells at an effector target cell ratios of 40:1. Counts per minute of $[^{3}H]$thymidine incorporated into cells in the suppressor generating cultures are indicated on the left-hand ordinate. Tc activity of the cells recovered from the suppressor assay cultures (in percent cytotoxicity) is shown on the right-hand ordinate. The ratios of responder/stimulator cells at which the different suppressors were generated are shown on the abscissa. $\Box$, cpm of incorporated $[^{3}H]$thymidine in suppressor-generating cultures; $\blacksquare$, percent cytotoxicity in cells recovered from suppressor assay cultures. Standard deviations are indicated, and the percent suppression is the figure in parenthesis at the head of columns showing percent cytotoxicity. Percent suppression = ($x - y$)/$x$ X 100, where $x$ = percent cytotoxicity of cells recovered from cultures without suppressors, $y$ = percent cytotoxicity of cells recovered from suppressed culture.

Variability, in all further experiments, at least three suppressor cell concentrations were tested, although, to keep the tables and figures as uncluttered as possible, not all are shown. Suppression was observed when stimulator cells expressing H-2 haplotypes other than those shown here (i.e., H-2s, H-2k, and H-2k) were used in the suppressor assay cultures (data not shown).

An experiment was carried out to determine the ratio of responder to stimulator cells that would give optimum suppression. Constant numbers of BALB/c responder cells (2.5 X $10^{6}$/well) were cultured for 5 d with various numbers of X-irradiated DBA/2 stimulator cells, harvested, and then tested for suppressive activity. As the stimulator cell number was increased, a proportionate increase in the proliferative response in the suppressor generating cultures was found and a proportional decrease in the suppressor activity of the cells was observed (Fig. 2). It appears that some degree of stimulation, and perhaps cell proliferation, is required for the activation of these suppressors, as control unstimulated cells (Table III and Fig. 2) or cells
stimulated with H-2-compatible, Mls-compatible stimulators (Table III, experiment 2) were not significantly suppressive. However, "strong" stimulation results in a decreased suppressor cell response (Fig. 2). The finding that the cells recovered from cultures, in which strong proliferation was observed, showed decreased suppressive activity, suggests that the observed suppression is not a totally nonspecific effect mediated by any blast cells. If this were the case, one would predict that the cells obtained from the cultures with the higher stimulator cell numbers, in which a far greater number of blast cells were observed, would be more suppressive. That the suppression is not a nonspecific property of any blast cell is also shown by the data in Table IV, in which the suppressive activity of cells generated in an Mls MLR is compared with those of Con A and LPS blast cells. Con A is a T cell mitogen (24) and LPS is a B cell antigen (25). Strong suppression was observed with the MLR generated cells but none was seen with Con A and LPS blasts when tested at the same concentrations.

**The Phenotype of the Suppressor Precursor Cells.** The Thy-1, Lyt-1, and Lyt-2 and I-J phenotypes of the suppressor cell precursor were investigated by pretreating normal BALB/c spleen cells with the relevant antibodies and C, as described in Materials and Methods, before stimulation in bulk MLR with X-irradiated DBA/2 spleen cells. Cells recovered from cultures of responder cells pretreated with C only, anti-Lyt-2.2 and C or anti-I-Jd and C were strongly suppressive when tested in a second suppressor assay culture (Fig. 3). However, the suppressive activity was totally inhibited by pretreatment of the responder cells with anti-Thy-1.2 and C and anti-Lyt-1.2 and C. To test whether the anti-Lyt-2.2 and C treatment had been complete or not, a portion

| Cells added to test culture | Ratio of added cells to responders | Percent cytotoxicity |
|-----------------------------|-----------------------------------|---------------------|
| None                        | None                              | 72.7 ± 3.4          |
| BALB/c + DBA/2, MLR         | 0.25:1                            | 5.5 ± 1.4           |
|                             | 0.125:1                           | 39.9 ± 5.4          |
|                             | 0.06:1                            | 80.1 ± 5.1          |
| BALB/c Con A blasts         | 0.25:1                            | 81.7 ± 3.5          |
|                             | 0.125:1                           | 79.9 ± 3.9          |
|                             | 0.06:1                            | 78.3 ± 2.3          |
| BALB/c LPS blasts           | 0.25:1                            | 82.1 ± 5.7          |
|                             | 0.125:1                           | 77.1 ± 3.4          |
|                             | 0.06:1                            | 75.3 ± 4.7          |
| BALB/c + BALB/c, MLR        | 0.25:1                            | 76.2 ± 2.5          |
|                             | 0.125:1                           | 79.7 ± 5.2          |
|                             | 0.06:1                            | 72.2 ± 4.4          |

* BALB/c anti-DBA/2a and BALB/c anti-BALB/c MLR cells and Con A and LPS blast cells were generated as described in Materials and Methods and tested for suppressor activity in a BALB/c anti-C57BL/6 Tc response. Tc activity was tested against EL4 tumor target cells at an effector/target cell ratio of 40:1.
of the treated cells was tested for their ability to mount a Tc response, as Tc precursors have repeatedly been shown to be Lyt-2⁺ (26, 27). Whereas a high level of activity was seen with responders treated with C alone, none was observed when the responders were treated with anti-Lyt-2 and C (data not shown). Thus, it appears that the cytotoxic elimination with anti-Lyt-2 and C was efficient and the suppressor precursor cell was indeed Lyt-2⁻. There was no internal positive control for the anti-I-J⁺ treatment. However, the experiment shown is a representative one of five and was run using the same C used for the eliminations with anti-Thy-1.2, anti-Lyt-1.2 and anti-Lyt-2.2. Also using the same antiserum and C, it has recently been shown by colleagues in our laboratory that suppressor cells from tumor-bearer animals, which suppress BCG-potentiated anti-tumor responses, are I-J⁺, i.e., the same antiserum at the same dilution and C treatment was effective (28). These tumor inducer suppressor cells were also from BALB/c mice. Thus, we conclude that the suppressor cell generated in the anti-Mls MLR is derived from a precursor T cell that is Thy-1⁺, Lyt-1⁺,2⁻, and I-J⁻. We shall refer to this cell as Tsp.

The Phenotype of the Suppressor Effector Cells. The same phenotypic determinants that were tested for on the Tsp were examined on the suppressor effector cells by treating cells recovered from bulk BALB/c anti-DBA/2 MLR cultures with the four different antibodies and C, as described in Materials and Methods, before testing them in a second culture for their ability to suppress an anti-H-2 Tc response.

Treatment with C alone, anti-Lyt-2.2 and C, and anti-I-J⁺ and C had no effect on the suppression, but both anti-Lyt-1.2 and C and anti-Thy-1.2 and C treatment completely eliminated the suppressive activity of these cells (Table V). It is our experience that T cells recovered from culture are often refractory to treatment with
TABLE V

Phenotype of the Suppressor Effector Cell*

| Experiment | Suppressors | Treatment | Suppressor/responder ratio | Percent cytotoxicity |
|------------|-------------|-----------|---------------------------|----------------------|
| 1          | None        | None      | 0.25:1                    | 44.1 ± 3.2           |
|            | BALB/c + DBA/2* | C alone | 0.25:1                    | -0.3 ± 0.8           |
|            | BALB/c + DBA/2* | an-Tly-1.2 + C | 0.25:1                    | 48.9 ± 3.5           |
|            | BALB/c + DBA/2* | an-Lyt-1.2 + C | 0.25:1                    | 48.9 ± 1.0           |
|            |            |           | 0.08:1                    | -1.0 ± 1.4           |
|            |            |           | 0.08:1                    | 52.1 ± 1.7           |
|            |            |           | 0.08:1                    | 39.8 ± 0.5           |
| 2          | None        | None      | 0.25:1                    | 56.6 ± 6.3           |
|            | BALB/c + DBA/2* | C alone | 0.25:1                    | 3.2 ± 1.0            |
|            |            |           | 0.125:1                   | 25.1 ± 7.8           |
|            | BALB/c + DBA/2* | an-Lyt-2.2 + C | 0.25:1                    | 2.7 ± 0.6            |
|            |            |           | 0.125:1                   | 11.3 ± 0.8           |
| 3          | None        | None      | 0.25:1                    | 27.1 ± 5.8           |
|            | BALB/c + DBA/2* | C alone | 0.25:1                    | 0.3 ± 1.4            |
|            |            |           | 0.125:1                   | 4.2 ± 2.0            |
|            | BALB/c + DBA/2* | an-I-Jd + C | 0.25:1                    | -0.8 ± 1.8           |
|            |            |           | 0.125:1                   | 0.3 ± 1.9            |

* Normal BALB/c spleen cells were stimulated in MLR cultures with irradiated DBA/2 stimulator cells for 5 d, harvested, and then treated with C alone (experiments 1, 2, and 3), an-Thy-1.2 and C, an-Lyt-1.2 and C (experiment 1), an-Lyt-2.2 and C (experiment 2), or an-I-Jd and C (experiment 3), as described in Materials and Methods. They were then tested for suppressive activity in a BALB/c anti-C57BL/6 Tc response at two suppressor/responder ratios (0.25:1 and 0.08:1 in experiment 1 and 0.25:1 and 0.125:1 in experiments 2 and 3). Tc activity of cells recovered from these cultures on day 5 was assayed at an effector/target cell ratio of 40:1 against EL4 target cells in experiments 1 and 2 and C57BL/6 Con A blasts in experiment 3.

Some antibodies to Thy-1 and LYT determinants and C, and so we had to ensure that the monoclonal anti-LYT-2.2 we used could eliminate functional LYT-2+ cells recovered from culture. Tc generated in anti-H-2b MLR using BALB/c splenic responder cells were totally eliminated by treatment with anti-LYT-2 and C, whereas C alone had no effect (data not shown). As Tc themselves have also been shown to be LYT-2+ (26, 27), we conclude that our anti-LYT-2 antibody was efficient at eliminating LYT-2+ cells recovered from culture. Again, there was no positive control for the activity of the anti-I-Jd antisem. But, by the same criteria used for Tsp, we would argue that the suppressor effector was I-J-. Thus, we conclude the suppressor effector cell, which we designate Ts, is a Thy-1+, LYT-1-,2-, I-J- T cell.

Radiosensitivity of the Ts. BALB/c spleen cells that had been cultured for 5 d with DBA/2 stimulators were γ-irradiated with 100, 250, 500, 1,000, or 1,500 rad before being assayed for suppressor activity in a BALB/c anti-C57BL/6 Tc response (Table VI). It appears that all five doses of irradiation were sufficient to eliminate the suppressive activity. Thus, the Ts generated in the anti-Mls MLR are highly radio-
Table VI

| Suppressor                  | Dose of $\gamma$ irradiation | Percent cytotoxicity |
|-----------------------------|-------------------------------|----------------------|
| None                        | None                          | 55.4 ± 1.1           |
| BALB/c + DBA/2x             | None                          | 9.3 ± 2.9            |
|                             | 0.25:1                        | 17.0 ± 2.4           |
|                             | 0.125:1                       | 40.4 ± 4.3           |
| BALB/c + DBA/2x             | 1,500                         | 78.3 ± 3.5           |
|                             | 0.25:1                        | 80.4 ± 1.5           |
|                             | 0.125:1                       | 75.4 ± 2.9           |
| BALB/c + DBA/2x             | 1,000                         | 73.7 ± 2.9           |
|                             | 0.25:1                        | 81.6 ± 7.3           |
|                             | 0.125:1                       | 82.0 ± 8.9           |
| BALB/c + DBA/2x             | 500                           | 65.6 ± 2.2           |
|                             | 0.25:1                        | 65.6 ± 2.2           |
|                             | 0.125:1                       | 58.1 ± 1.6           |

* Normal BALB/c spleen cells were stimulated in MLR cultures with irradiated DBA/2 stimulator cells, harvested, and aliquots were exposed to five different doses of $\gamma$ irradiation. Each was then tested for suppressive activity in a BALB/c anti-C57BL/6 Tc response at two suppressor/responder ratios (0.25:1 and 0.125:1). Tc activity of cells recovered from these cultures was assayed against EL4 tumor target cells and an effector/target cell ratio of 40:1.

sensitive. Not only does the irradiation eliminate suppression, but enhanced Tc responses were observed in cultures to which irradiated cells had been added. This finding is consistent with the contention that elimination of the suppressor cells exposes the expression of radioresistant helper cells generated in the first-step MLR and that these helper cells then enhance the anti-H-2 Tc response in the second-step assay MLR.

**Discussion**

We have shown that whereas a secondary in vitro H-2-restricted response to non-H-2 alloantigens was readily obtainable in bulk cultures, no such primary response was observed (Table I). This is true whether MS- (our data) or FCS- (4–7) supplemented media were used. We mapped the H-2 restriction to the D end and some region to the left of the I-C subregion of the H-2 complex (Table II). It has been postulated that the primary in vitro Tc response to non-H-2 alloantigens of responder cells from autoimmune NZB mice was mediated by hyperactive helper cells (13). However, this seems unlikely in the light of the recent demonstration of defective interleukin 2 (IL-2) production by cells from NZB mice (29). Also, in the non-H-2 system described in this report and in others (1–6), Mls incompatibilities, which have been shown to stimulate powerful help in normal mouse responder cells (30), are
usually included. In fact, a number of cloned helper cell lines with Mls specificity have been derived (31). So the lack of such a Tc primary response in non-autoimmune normal mice does not seem to be caused by the lack of a help-generating stimulus. Indeed, we have shown that, whereas a strong NZB (H-2\(^b\) Mls\(^a\)) anti-BALB/c (H-2\(^d\) Mls\(^b\)) primary Tc response was observed in FCS-supplemented medium, no such response was observed in MS-supplemented medium, even though the latter medium did support the generation of a good anti-H-2 response in the same NZB responder cells (15). Thus, whatever the actual mechanism of such cytotoxic responses in the NZB cells, it is dependent on the presence of xenogeneic serum in the medium.

Among the possible explanations for the failure to generate Tc after primary in vitro sensitization against non-H-2 antigens, one is that suppressors are induced during the reaction that are sufficient to inhibit a primary response. During the course of an allogeneic MLR there is simultaneous generation of Tc, helper, and suppressor T cells. The activity detected in effector cell populations recovered from culture must be an average of the augmenting and suppressive influences generated during the reaction. In a primary anti-H-2 response that includes an I region incompatibility, the help-suppression balance is probably tipped in the favor of help, whereas in the case of a non-H-2 response it is shifted in the favor of suppression. It is unlikely that there is a defect in the presentation of cytotoxic determinants (CD) to Tc precursors, as strong secondary responses are easily inducible. It is possible, however, that a lower density of CD is required to restimulate Tc memory cells than to stimulate Tc precursors in a primary response. However, in support of the role of suppressor cells in the lack of a primary response, it was possible, using a LDA procedure, to generate a primary Tc response to non-H-2 alloantigens in normal mouse responder cells (15). At the higher responder cell concentrations, a markedly decreased response was observed, suggesting the presence of relatively infrequent suppressor cells which, at the lower dilutions, are not sufficient to exert an effect.

For these reasons, we decided to test directly whether suppressor cells were generated in an anti-non-H-2 alloantigen MLR. Clearly, a strong nonspecific suppressor cell response was produced (Table III). This response required, at least, some antigenic stimulation, as cells from unstimulated control cultures either had no effect, minimally suppressed (never by >20% of the response), or sometimes augmented the Tc response in the second step anti-H-2 suppressor assay MLR. This latter finding would appear to be at variance with the previously described (32-34) work on the culture generated suppressor cells, where unstimulated cells were cultured alone for 3–5 d in the generating phase. However, all those experiments were carried out in FCS or horse serum medium, and indeed, if we carry out our suppressor generating MLR in FCS medium, “unstimulated” control cells also exert powerful suppressive activity (data not shown), suggesting that these culture generated suppressor cells are activated by FCS determinants. Cells recovered from an MLR across non-H-2 non-Mls incompatibilities in which no cell proliferation is observed showed no suppressive activity (Table III), suggesting that there is a requirement for a proliferative stimulus for the induction of the suppressor cells. However, increasing the stimulator cell number in the BALB/c anti-DBA/2 suppressor generating MLR, which caused a proportional increase in the proliferative response, actually resulted in a decreased level of suppression (Fig. 2). Thus, it is possible that IL-2 (and/or other lymphokines) levels critically determine whether a response is shifted in the favor of help or suppression.
We are currently investigating the antigenic/lymphokine requirements for the generation of these suppressor cells.

The finding that an increased proliferative response, with the concomitant generation of a greater proportion of blast cells (as observed microscopically), resulted in less suppression, would also suggest that the suppression itself is not a totally nonspecific phenomenon mediated by all blast cells. This contention is also supported by the fact that Con A and LPS blast cells were not suppressive when tested at the same concentrations as those at which anti-Mls MLR-generated suppressors were active (Table IV). We do not, however, exclude the possibility that the suppressor effector is in the blast cell population generated during the MLR.

We have shown that Tsp in normal spleen are Thy-1+, Lyt-1+,2- by their sensitivity or lack of sensitivity to pretreatment with the various monoclonal antibodies and C (Fig. 3). By the same criteria, the Ts recovered from anti-Mls MLR are also Thy-1+, Lyt-1+,2- (Table V). Although we lack an internal positive control for the activity of the anti-I-J* antiserum we used, it appears that both the Tsp and the Ts are I-J* (Fig. 3 and Table V). The Ts is highly radiosensitive, shown by the fact that it is eliminated by doses of γ-irradiation as low as 100 rad (Table VI). Macrophages or adherent non-T cells have also been shown to exhibit suppressive activity (35, 36), and we do not exclude a role for them in the suppression described here. However, as they are relatively radioresistant (37) and are not sensitive to treatment with anti-Thy-1 and anti-Lyt-1 and C, macrophages alone are not able to mediate the observed phenomenon.

The suppression observed was not antigen specific. Attempts made to detect specific suppression were unsuccessful (data not shown). As no primary Tc response is observable in the system, the specific suppressor cells had to be tested for in a secondary response using responder spleen cells from previously primed animals. A possible reason for the failure to detect suppression is that the secondary response is not as sensitive to suppression as a primary response. It has in fact been shown that a secondary response to non-H-2 alloantigens can be specifically suppressed by cells recovered also from a secondary MLR to non-H-2 alloantigen but not by cells from a primary MLR (38). Immunization of mice with Mls-disparate spleen cells results in the production in the lymph nodes, of suppressive activity which effects MLR, in vitro Tc responses and polyclonal proliferative responses (39-41). The mechanism of this in vivo generated suppression may be the same as the in vitro generated suppression described here.

The mechanism of action of the Ts is not clear. Because it is Lyt-2- and Tc are Lyt-2+ (26, 27), it would seem that in this system, at least, the Ts is not a Tc acting against either the responder or stimulator in the second-step assay cultures, as has been suggested in other systems (42, 43). The conclusion is also supported by the fact that Ts are not detectable in a conventional bulk anti-non-H-2 MLR culture. Most suppressor T effector cells have been shown to be Lyt-1-,2+ (44-46). However, an Lyt-1+,2- culture generated suppressor of an in vitro antibody response has been observed (47) and in an in vivo graft-vs.-host system, an Lyt-1+,2- suppressor of antibody responses was described (48). More recently, a number of groups have described suppressor circuits in which Lyt-1+ suppressor-inducer cells act via soluble factors and an Lyt-1+,2- intermediate cell to induce Lyt-2+ suppressor effectors (49, 50). It is possible that the Lyt-1+ suppressor cell generated in the anti-Mls MLR
induces, by a similar mechanism, a second suppressor effector in the normal responder population of the second step assay cultures. A report has been published (51) in which a factor from a predominantly Lyt-2+ responder cell population, from an anti-H-2 MLR, has been shown to induce a second suppressor of Tc in a normal spleen cell population. The actual suppressor effector cell could be acting directly on the Lyt-2+ Tc precursors themselves or indirectly on Lyt-2- helper cells or the soluble lymphokines they produce (i.e., IL-2). We have preliminary evidence that they act by either inhibiting production of, inactivating or absorbing IL-2 (data not shown). In an LDA designed to estimate the precursor frequencies of IL-2 producing cells responding in both anti-H-2 and anti-Mls MLR, it was shown that at lower responder cell doses higher levels of IL-2 per precursor were observed than both at the higher cell doses or in conventional bulk MLR culture (52). This finding is also suggestive of a relatively infrequent suppressor cell that acts either on the IL-2 producing cell or on the soluble IL-2 itself.

If these I-J- suppressor cells do indeed mask a Tc response to non-H-2 alloantigens, the interpretation of our earlier finding that pretreatment of a responder spleen cell population with an anti-Ia serum, which included a high titer of anti-I-J activity, and C led to significant primary anti-minor locus response was probably incorrect (16). As I-J-encoded determinants have been demonstrated on suppressor cells in a variety of systems (53-55), we interpreted the result as indicating that anti-Ia and C treatment removed a suppressor cell and thus facilitated a non-H-2 alloantigen-specific Tc response. It is possible that the suppressor expresses some Ia determinant(s) other than I-J, because the antiserum which was prepared in the mouse combination (B10.HTT X A.TH)F1 anti-A.TL also contained strong anti-I-AK specificities. It is also possible that some non-C-fixing antibodies in the antiserum exerted a nonspecific or polyclonal activating effect on responder cells expressing the appropriate antigens. The Tc responsiveness seen in primary MLR of NZB spleen cells to non-H-2 alloantigens might reflect a deficiency of suppressor cells of the kind reported here or insensitivity of Tc precursors or helper cells to suppression rather than hyperactive helper cells as previously postulated (13). There is evidence for a decreased level of suppressor activity in (NZB X NZW)F1 (56) and sensitivity to suppression in NZB mice (57).

Thus, it seems that radiosensitive nonspecific Thy-1+, Lyt-1+,2-, I-J- suppressor T cells are generated in a primary MLR to non-H-2 alloantigens, including a stimulatory Mls antigen or group of antigens. These suppressor cells probably play a role, together with other factors, such as low Tc precursor frequency, in the lack of a primary Tc response to non-H-2 alloantigen.

Summary

Normal mouse spleen cells are not capable of mounting a primary cytotoxic T lymphocyte (Tc) response to non-H-2 alloantigens in vitro, although a good secondary H-2-restricted response is observable after in vivo immunization of the responder animals. Suppressor cells are generated in such a primary response provided a Mls incompatibility exists between the responder and stimulator. These suppressors are not antigen specific, are Thy-1+, Lyt-1+,2-, I-J-, and are highly radiosensitive. The suppressor cell precursors in normal spleen express the same phenotype. These suppressor cells are probably implicated in the lack of a primary Tc response in a primary mixed lymphocyte reaction across non-H-2 incompatibilities that include an
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