SPLEEN CELLS FROM ADULT MICE GIVEN TOTAL LYMPHOID IRRADIATION OR FROM NEWBORN MICE HAVE SIMILAR REGULATORY EFFECTS IN THE MIXED LEUKOCYTE REACTION

I. Generation of Antigen-specific Suppressor Cells in the Mixed Leukocyte Reaction after the Addition of Spleen Cells from Adult Mice Given Total Lymphoid Irradiation*

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Our previous studies have shown that adult BALB/c mice given total lymphoid irradiation (TLI)[1] (17 fractions of 200 rad each) will accept H-2-incompatible allogeneic bone marrow grafts without the development of graft-vs.-host disease (1–8). The resultant chimeras are specifically tolerant of tissues derived from the marrow donor strain, but promptly reject tissues from third-party strains (1, 3–7). Similarly, newborn mice will accept allogeneic bone marrow grafts without graft-vs.-host disease, and become specifically tolerant to the tissues of the marrow donor (9, 10). Spleen cells from both adult mice given TLI (11) and newborn mice (12, 13) contain nonspecific suppressor cells of the mixed leukocyte reaction (MLR) before marrow transplantation. Recent evidence suggests that after transplantation of allogeneic or semiallogeneic lymphoid cells, both TLI-treated adult mice (14, 15) and newborn mice (16) or rats (17–22) develop suppressor cells of transplantation immunity which are specific for the tolerizing tissue antigens.

The object of the present investigation was to examine in detail the regulatory activity of spleen cells from both TLI-treated and newborn mice in the MLR as judged by DNA synthesis, and the generation of cytolytic and suppressor cells. The experimental results suggest that spleen cells from both sources inhibit DNA synthesis and the generation of cytolytic cells regardless of the strain of origin of the responder and stimulator cells. However, the spleen cells do not inhibit the generation of antigen-specific suppressor cells. Thus, blocking of the cytolytic arm of the allogeneic response without blocking of the suppressor arm in vitro may provide a model system for tolerance induction and maintenance in vivo in TLI-treated or newborn mice given allogeneic bone marrow cells.

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[1] Abbreviations used in this paper: CML, cell-mediated lympholysis; FCS, fetal calf serum; MLR, mixed leukocyte reaction; NK, natural killer; TLI, total lymphoid irradiation.

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Materials and Methods

Mice. Male BALB/c (H-2^d), C57BL/Ka (H-2^b), and C3H/Km (H-2^k) mice were obtained from the specific pathogen-free colony of Dr. Robert Kallman, Department of Radiology, Stanford University School of Medicine. Adult mice used for experimentation were between the ages of 2 and 6 mo, and newborn mice were between the ages of 2 and 3 d.

Radiotherapy Procedure (TLI). 4–6-mo-old BALB/c mice were anesthetized daily with pentobarbitol and positioned in an apparatus designed to irradiate the major lymphoid organs, including all major lymph nodes, the spleen, and the thymus, as described by Slavin et al. (23). The skull, lungs, tail, and hind legs were shielded with lead. The mice were given 200 rad/d, five times/wk, to a total dose of 3,400 rad. Irradiation was delivered from a single 250 kV (15 A) source (Phillips Medical System Inc., Shelton, CT). The dose rate was 93 rad/min using an 0.35-mm Cu filter and a 52-cm source-axis distance. Tetracycline was added to the drinking water during TLI and 1 wk after completion of irradiation. TLI-treated mice were killed between 5 and 15 d after completion of TLI in all experiments.

Preparation of Spleen Cells. Spleens were removed aseptically, and single-cell suspensions were prepared by gently pressing the spleen fragments through a nylon fiber mesh (Tetko, Inc., Elmsford, NY). The cells were washed three times and counted in 2% acetic acid before use. Cell viability was determined by trypan blue dye exclusion.

Primary MLR-Suppressor Assay. Responder, stimulator, and co-cultured (putative suppressor) cells were cultured at a concentration of 5×10^5 cells each, in a final volume of 0.3 ml/well, using 96-well flat-bottomed microculture plates (3596; Costar, Data Packaging, Cambridge, MA). The cells were cultured in RPMI 1640 medium supplemented with 25 mM Hepes, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Grand Island Biological Co., Grand Island, NY), 5×10^{-5} M 2-mercaptoethanol, and 5% pooled human serum (MLR medium). Human serum was a kind gift of Dr. Edgar Engleman, Department of Pathology, Stanford University School of Medicine. Stimulator and co-cultured cells were treated with 3,000 and 1,500 rad, respectively, from a ^{137}Ce source (Mark 1 model 25 irradiator; J. L. Shepherd and Associates, Glendale, CA) and washed once just before culture.

Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After 96 h, 1 μCi of [^{3}H]thymidine (sp act 6.7 Ci/mM; New England Nuclear, Boston, MA) was added to each well. 18 h later, cells were harvested onto glass fiber filter paper (Whatman Inc., Chemical Separation Div., Clifton, NJ) using a multiple automated sample harvester (Bio-Plastics, Redwood City, CA). The dried paper disks were placed into mini-vials containing 2.5 ml scintillation cocktail (Betafluor, National Diagnostics, Somerville, NJ) and the incorporated radioactivity was measured in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

Data shown for all MLR cultures are the mean counts per minute of triplicate assays with the standard deviation of the mean. The following formula was used to calculate suppression: percent suppression = (1 − [(cpm with co-cultured cells from TLI-treated mice)/(cpm with co-cultured cells from normal mice)]) × 100.

Primary Cell-mediated Lympholysis (CML)-Suppressor Assay

PREPARATION OF EFFECTOR CELLS. Responder cells (5×10^5) were cultured with equal numbers of stimulator and co-cultured cells as described in the previous section. On day 5, effector cells were harvested and washed once. Cells were resuspended to desired concentrations in CML medium after counting in 0.04% trypan blue. CML medium was similar to MLR medium except that 10% fetal calf serum (FCS) (Grand Island Biological Co.) was used in place of 5% pooled human serum.

In control cultures (co-cultured cells obtained from normal BALB/c donors), cell recovery at the end of 5 d was 80–140% of the number of responder cells added initially, and cell viability was 60–90%. There were no consistent differences in the recovery and viability of cells in the six different allogeneic combinations studied.

PREPARATION OF TARGET CELLS. P815 (DBA/2, H-2^d) mastocytoma, EL-4 (C57BL/6, H-2^b) lymphoma, and BW5147 (AKR, H-2^k) thymoma lines were a gift of Dr. Irving Weissman, Department of Pathology, Stanford University School of Medicine. Cells were maintained at 37°C in a 5% CO_2 incubator in RPMI 1640 containing 10% FCS. Upon reaching confluence,

the cells were passaged and fresh medium added. 2×10^6 tumor cells were washed and resuspended in 0.4 ml RPMI 1640 with 5% FCS and 100 μCi (^{51}Cr) sodium chromate (New England Nuclear). Cells were incubated at 37°C for 90 min in a CO_2 incubator with occasional agitation. After incubation, target cells were washed four times and adjusted to a concentration of 1×10^5 cells/ml in CML medium. 1×10^4 P815, EL-4, and BW5147 cells incorporated 0.6–0.9 $\times 10^4$ cpm, 0.7–1.1 $\times 10^4$ cpm, and 0.5–0.7 $\times 10^4$ cpm, respectively.

^{51}Cr RELEASE ASSAY. Effector cells and target cells were combined in 96-well V-bottomed microtiter plates (001-010-2701; Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, VA) in a final volume of 0.2 ml. Before incubation for 6 h at 37°C, the plates were lightly centrifuged (100 *g*, 1 min). At the end of the incubation period, the plates were centrifuged (400 *g*, 5 min) again. An aliquot of the supernatant (100 μl) was removed, and radioactivity was measured in a gamma counter (Biogamma II, Beckman Instruments, Inc.). Spontaneous release of ^{51}Cr was obtained by monitoring radioactivity released during the incubation of 1×10^4 target cells alone. Maximum release of ^{51}Cr was determined by monitoring radioactivity released after three rapid cycles of freezing and thawing of labeled target cells suspended in water. This generally released 90% of total radioactivity incorporated in all cells. The range of spontaneous release of the ^{51}Cr-labeled P815, EL-4, and BW5147 after a 6-h incubation was 9–17%, 7–13%, and 5–8% of maximum release in all experiments, respectively. Percent specific lysis was calculated according to the formula: percent specific lysis = ([cpm experimental − cpm spontaneous release]/[cpm maximum release − cpm spontaneous release]) × 100.

The standard deviation of the mean cpm of triplicate assays used to calculate the percent specific lysis was usually <5% of the mean value. The data are expressed as lytic units per culture (24). One lytic unit is defined as the number of effector cells required to achieve 30% specific lysis. The number of lytic units per culture was determined by adding graded volumes obtained from the MLR cultures to the target cells. A curve was constructed to determine the volume of cells producing 30% specific lysis, and calculations were made by setting that volume equal to one lytic unit. The following formula was used to calculate suppression: percent suppression = (1 − [(lytic units/culture with co-cultured cells from TLI-treated mice)/(lytic units/culture with co-cultured cells from normal mice)]) × 100.

Secondary MLR-Suppressor Assay. Primary bulk MLR cultures (2 ml total volume, with 3×10^6 responder, stimulator, and co-cultured cells each) were set up in 24-well flat-bottomed culture plates (3524; Costar). A fixed aliquot (133 μl) of cells was harvested after 6 d of incubation and washed once in medium. These were used as co-cultured cells in the secondary MLR after irradiation in vitro (1,500 rad). Fresh responder cells (5×10^5) and an equal number of fresh stimulator cells were cultured with the aliquot of cells from the primary culture in 0.3 ml of MLR medium in microculture plates. Secondary MLR cultures without co-cultured cells were also set up as controls. Thereafter, [^{3}H]thymidine incorporation was measured as described in the primary MLR-suppressor assay. Percent suppression was calculated by the formula: percent suppression = (1 − [(cpm with co-cultured cells)/(cpm without co-cultured cells)]) × 100.

Secondary CML-Suppressor Assay. Cultures were set up in the identical manner to the secondary MLR-suppressor assay. Effector cells were harvested after 5 d of culture and the number of lytic units per culture was measured as described in the primary CML-suppressor assay. Percent suppression was calculated by the formula: percent suppression = (1 − [(lytic units/culture with co-cultured cells)/(lytic units/culture without co-cultured cells)]) × 100.

Results

Nonspecific Inhibition of [^{3}H]Thymidine Incorporation in the Primary MLR by TLI-treated BALB/c Spleen Cells. In several experiments, spleen cells from TLI-treated BALB/c mice were co-cultured with equal numbers of responder and stimulator cells from a variety of strains. Co-cultured cells were given 1,500 rad in vitro just before incubation. Similarly treated spleen cells from normal (unirradiated) BALB/c mice were used as co-cultured cell controls. One representative experiment (four experiments performed) is shown in Table I. Spleen cells obtained from mice between 5 and 15 d after

TABLE I

Nonspecific Inhibition of the MLR by Spleen Cells from Donors Given TLI

Responder cells*	Stimulator cells‡	Co-cultured cells§	[^{3}H]Thymidine incorporation (mean cpm ± SD)	Percent‖ suppression
BALB	BALB	BALB	6,637 ± 581	
BALB	C57	BALB	119,165 ± 4,951	
BALB	C57	TLI¶	27,787 ± 2,835	76.7
C57	C57	C57	2,132 ± 229	
C57	BALB	BALB	79,170 ± 5,529	
C57	BALB	TLI	14,581 ± 3,015	81.6
C3H	C3H	C3H	3,540 ± 1,447	
C3H	C57	BALB	133,372 ± 6,482	
C3H	C57	TLI	12,298 ± 2,676	90.8
BALB	BALB	BALB	6,673 ± 581	
BALB	C3H	BALB	45,782 ± 687	
BALB	C3H	TLI	12,070 ± 3,560	73.6
C57	C57	C57	2,132 ± 229	
C57	C3H	BALB	62,863 ± 2,825	
C57	C3H	TLI	9,056 ± 1,388	85.6
C3H	C3H	C3H	3,540 ± 1,447	
C3H	BALB	BALB	103,678 ± 8,145	
C3H	BALB	TLI	24,243 ± 1,366	76.6

* 5×10^5 responder cells per well were used. BALB, C3H, C57 indicate spleen cells from normal BALB/c, C3H/Km, and C57BL/Ka donor strains, respectively.

‡ 5×10^5 stimulator cells per well were given 3,000 rad in vitro.

§ 5×10^5 co-cultured cells per well were given 1,500 rad in vitro.

‖ Percent suppression = (1 − [(cpm with TLI co-cultured cells)/(cpm with BALB co-cultured cells)]) × 100.

¶ TLI-treated BALB/c donors; other donors were untreated adult mice.

completion of TLI suppressed the MLR by 55–95% with a mean of 77% as compared with control cultures with cells from normal mice. There was no specificity for responder or stimulator cells in any strain combination used in these experiments.

Nonspecific Inhibition of the Generation of Cytolytic Cells in the Primary MLR by TLI-treated BALB/c Spleen Cells. As shown in Table II, TLI-treated spleen cells suppressed the generation of alloantigen-specific cytolytic cells as measured by lytic units per culture. The suppressor activity was also nonspecific and varied from 50 to 90% with a mean of 73% in four different experiments. In simultaneous MLR and CML experiments using the same surce of TLI-treated spleen cells, the degree of suppression was generally within 10–15% agreement.

Generation of Antigen-specific Suppressor Cells in the Primary Bulk MLR (BALB Responder Cells) After the Addition of TLI-treated Spleen Cells. Putative suppressor cells were generated in the primary bulk MLR consisting of BALB/c responder cells, C57BL/Ka stimulator cells (3,000 rad), and co-cultured cells (1,500 rad) from either normal or TLI-treated BALB/c donors at a concentration of 3×10^6 cells each per well.

TABLE II

Nonspecific Inhibition of the Generation of Cytolytic Cells in the MLR by Spleen Cells from Donors Given TLI

MLR*			CML assay target cell	Lytic units/culture‡	Percent suppression§
Responder cells	Stimulator cells	Co-cultured cells			
BALB	C57	BALB	EL-4	444.4	
BALB	C57	TLI	EL-4	178.6	59.8
C57	BALB	BALB	P815	227.3	
C57	BALB	TLI	P815	26.7	88.3
C57	C3H	BALB	BW5147	55.6	
C57	C3H	TLI	BW5147	<12.5	>77.5
BALB	C3H	BALB	BW5147	149.3	
BALB	C3H	TLI	BW5147	66.7	55.3
C3H	BALB	BALB	P815	294.1	
C3H	BALB	TLI	P815	106.4	63.8
C3H	C57	BALB	EL-4	135.1	
C3H	C57	TLI	EL-4	<25.0	>81.5

* 5×10^5 responder, stimulator, and co-cultured cells per well were used in each MLR culture.

‡ Calculation for lytic units per culture is explained in Materials and Methods.

§ Percent suppression = (1 − [(lytic units/culture with TLI co-cultured cells)/(lytic units/culture with BALB co-cultured cells)]) × 100.

Additional control MLR cultures containing BALB/c responders, BALB/c stimulator, and either normal or TLI-treated BALB/c co-cultured cells were simultaneously set up. On day 6, cells from a fixed volume (133 μl) of each primary bulk MLR culture were added to a fresh micro MLR (secondary MLR) as co-cultured cells. Cells from primary cultures were irradiated in vitro (1,500 rad) before transfer. Suppressor activity generated in the primary bulk MLR was always expressed in comparison to [^{3}H]thymidine incorporation in a secondary MLR culture without co-cultured cells. Results of these experiments are shown in Table III. Experiments 1 and 2 are representative of nine and three experiments, respectively. TLI-treated BALB/c spleen cells used in these experiments had shown >50% suppression in the primary MLR and CML-suppressor assays.

When cells from syngeneic primary cultures (BALB × BALB × BALB, or BALB × BALB × TLI) were added to the secondary MLR, slight suppression (<18%) or enhancement was observed in seven out of nine experiments. There was little difference in the percent suppression observed with the two syngeneic combinations (TLI vs. BALB co-cultured cells). Therefore, nonspecific suppressor activity of TLI-treated BALB/c spleen cells (Table I) did not persist in culture with syngeneic cells. When cells from BALB × C57 × BALB primary cultures were added to the secondary MLR (experiments 1 and 2), marked suppression (mean, 85.6%) was observed regardless of the strain of origin of the secondary responder or stimulator cells (range, 43–97% suppression) (Table III). On the other hand, when cells from BALB × C57 × TLI primary cultures were added to the secondary MLR, marked suppression was observed

TABLE III

Generation of Antigen-specific Suppressor Cells in the Bulk MLR (BALB × C57) after Addition of Spleen Cells from Donors Given TLI: Secondary MLR Assay System

Secondary MLR			[^{3}H]Thymidine incorporation (mean cpm ± SD)	Percent suppression‖
Responder cells*	Stimulator cells‡	Co-cultured cells (primary culture)§		
Experiment 1				
BALB	BALB		7,114 ± 566	
BALB	C57		114,829 ± 7,263	
BALB	C57	BALB × BALB × BALB	100,526 ± 14,204	12.5
BALB	C57	BALB × BALB × TLI	105,054 ± 6,662	8.5
BALB	C57	BALB × C57 × BALB	7,842 ± 1,284	93.2
BALB	C57	BALB × C57 × TLI	26,580 ± 3,361	76.9
BALB	BALB		7,114 ± 566	
BALB	C3H		126,303 ± 14,563	
BALB	C3H	BALB × BALB × BALB	155,176 ± 5,746	−22.9
BALB	C3H	BALB × BALB × TLI	104,394 ± 16,273	17.3
BALB	C3H	BALB × C57 × BALB	18,203 ± 2,838	85.6
BALB	C3H	BALB × C57 × TLI	97,340 ± 11,517	22.9
Experiment 2				
C3H	C3H		5,315 ± 811	
C3H	C57		72,450 ± 5,629	
C3H	C57	BALB × BALB × BALB	85,366 ± 8,640	−17.8
C3H	C57	BALB × BALB × TLI	74,255 ± 10,159	−2.5
C3H	C57	BALB × C57 × BALB	10,256 ± 1,664	85.8
C3H	C57	BALB × C57 × TLI	14,204 ± 1,070	80.4
C3H	C3H		5,315 ± 511	
C3H	BALB		65,101 ± 7,259	
C3H	BALB	BALB × BALB × BALB	100,737 ± 6,491	−54.7
C3H	BALB	BALB × BALB × TLI	83,362 ± 10,225	−28.1
C3H	BALB	BALB × C57 × BALB	10,332 ± 1,982	84.1
C3H	BALB	BALB × C57 × TLI	51,760 ± 2,358	20.5

* 5×10^5 responder cells per well were used in the secondary MLR.

‡ 5×10^5 stimulator cells per well were given 3,000 rad in vitro.

§ Co-cultured cells obtained from the bulk MLR culture at day 6 were given 1,500 rad. 3×10^6 responder, stimulator, and co-cultured cells used in the bulk MLR are shown.

‖ Percent suppression = (1 − [(cpm with co-cultured cells)/(cpm without co-cultured cells)] × 100.

with C57, but not with C3H or BALB stimulator cells. Similar suppression was observed with BALB or C3H responder cells. Thus, suppressor activity was antigen-specific, but was not restricted by the H-2 type of the secondary responder cells as has been reported in several experimental systems (25–28). The magnitude of the suppressor activity using C57 stimulators was 81.4% on the average (range, 48–95%). However, the percent suppression using BALB or C3H stimulators was 15.5% on the average (range, −22–38%).

Putative suppressor cells from primary MLR cultures were also assayed for their ability to inhibit the generation of cytolytic cells in secondary MLR cultures. The results of these experiments are shown in Table IV. Experiments 1 and 2 are representative of seven and three experiments, respectively. TLI-treated BALB/c

TABLE IV

Generation of Antigen-specific Suppressor Cells in the Bulk MLR (BALB × C57) after Addition of Spleen Cells from Donors Given TLI: Secondary CML Assay System

Secondary MLR			CML assay	Lytic units/	Percent
Responder cells*	Stimulator cells‡	Co-cultured cells (primary culture)§	target cell	culture	suppression‖
Experiment 1					
BALB	C57		EL-4	357.1	
BALB	C57	BALB × BALB × BALB	EL-4	322.6	9.7
BALB	C57	BALB × BALB × TLI	EL-4	327.9	8.2
BALB	C57	BALB × C57 × BALB	EL-4	<50.0	>86.0
BALB	C57	BALB × C57 × TLI	EL-4	<50.0	>86.0
BALB	C3H		BW5147	66.7	
BALB	C3H	BALB × BALB × BALB	BW5147	>100.0	−49.9
BALB	C3H	BALB × BALB × TLI	BW5147	90.9	−36.3
BALB	C3H	BALB × C57 × BALB	BW5147	<25.0	>62.5
BALB	C3H	BALB × C57 × TLI	BW5147	56.8	14.8
Experiment 2					
C3H	C57		EL-4	185.2	
C3H	C57	BALB × BALB × BALB	EL-4	ND¶	
C3H	C57	BALB × BALB × TLI	EL-4	ND	
C3H	C57	BALB × C57 × BALB	EL-4	<50.0	>73.0
C3H	C57	BALB × C57 × TLI	EL-4	<50.0	>73.0
C3H	BALB		P815	277.8	
C3H	BALB	BALB × BALB × BALB	P815	ND	
C3H	BALB	BALB × BALB × TLI	P815	ND	
C3H	BALB	BALB × C57 × BALB	P815	125.0	55.0
C3H	BALB	BALB × C57 × TLI	P815	208.3	25.0

* As for Table III.
‡ As for Table III.
§ As for Table III.
‖ Percent suppression = (1 − [(lytic units/culture with co-cultured cells)/(lytic units/culture without co-cultured cells)]) × 100.
¶ Not done.

spleen cells used in these experiments had shown >50% suppression in the primary MLR and CML-suppressor assays. The results of the secondary CML-suppressor assays were similar to those of the secondary MLR-suppressor assays. Briefly, neither BALB × BALB × BALB, nor BALB × BALB × TLI primary culture cells showed substantial suppression of the generation of cytolytic cells in secondary cultures. Cells from BALB × C57 × BALB primary cultures inhibited the generation of cytolytic cells by roughly the same magnitude in all secondary responder and stimulator combinations used with an average of >78.2% suppression (range, 55–94%). On the other hand, cells from BALB × C57 × TLI primary cultures showed antigen-specific suppressor activity. The mean percent suppression was >79.7% (range, 72–94%) when C57 stimulator cells were used in the secondary MLR. However, it was 15.2% (range, −46–54%) when C3H or BALB stimulator cells were used in the secondary MLR. Even in the experiment that showed 54% suppression (upper limit of range of values), the number of lytic units per secondary culture was still sixfold higher using BALB

× C57 × TLI primary culture cells as compared with BALB × C57 × BALB primary culture cells.

It is unlikely that the specific suppression of the MLR and CML mediated by the BALB × C57 × TLI primary cells is due to specific killing of C57 stimulator cells in the secondary cultures. In several experiments, ~90% suppression of the generation of cytolytic cells was observed in the primary cultures. At an effector/target cell ratio of 1:4, these primary cells produced no cytolysis above background levels. Nevertheless, aliquots of the same primary cells produced marked specific suppression (~80%) of the secondary cultures despite the fact that the ratio of the primary cells to secondary stimulator cells was considerably less than 1:4.

Generation of Antigen-specific Suppressor Cells in the Primary MLR (C57 Responder Cells) After the Addition of TLI-treated Spleen Cells. In all of the secondary culture experiments described above, responder cells in the primary cultures were syngeneic (BALB) with the primary co-cultured cells (either normal or TLI-treated BALB). In further experiments, putative suppressor cells were also generated in primary bulk MLR cultures consisting of C57BL/Ka responder cells, BALB/c stimulator cells (3,000 rad), and co-cultured cells (1,500 rad) from either normal or TLI-treated BALB/c donors. Suppressor activity was tested again in both secondary MLR and CML-suppressor assays.

Experiments 1 and 2 shown in Table V are representative of four experiments in each category. TLI-treated BALB/c spleen cells used in these experiments had shown >60% suppression in the primary MLR and CML-suppressor assays. When C57 × BALB × BALB primary culture cells were added to the secondary MLR cultures, marked suppression was observed with all combinations of secondary responders and stimulators. The mean percent suppression using C57 responders was 80.4% (range, 75–87%). The mean percent suppression using C3H responders was 66.4%, and there was a wider range of values (59%, 84%, 91%, 93%, with BALB stimulators; −4%, 35%, 80%, 93% with C57 stimulators).

On the other hand, when C57 × BALB × TLI primary culture cells were added to the secondary MLR, marked suppression was only observed with C57 responders and BALB stimulators (mean of 70.5% suppression; range, 54–80%). All other secondary responder and stimulator cell combinations showed considerably less suppression (mean of 11.3%; range, −17–28%). Thus, in this series of experiments, suppressor cells generated in the primary MLR cultures containing TLI-treated spleen cells showed antigen specificity as well as restriction with regard to the strain of origin of the responder cells. Marked suppression was observed only when the secondary responder-stimulator combination was identical to the primary responder-stimulator combination.

A similar pattern of suppression was seen in the secondary CML-suppressor assay (Table VI). Experiments 1 and 2 (Table VI) correspond to experiments 1 and 2 shown in Table V. A total of four experiments were performed in each category. C57 × BALB × BALB primary culture cells nonspecifically suppressed the generation of cytolytic cells with an average of >78.1% (range, 37–>96%) in all secondary responder-stimulator combinations with the [^{3}H]thymidine incorporation assay. More uniform values were obtained with C57 responder cells (mean, >82.9% suppression; range, >65–>96%). The mean percent suppression with C3H responder cells was 73.3% (range, 37–92%). Addition of C57 × BALB × TLI primary culture cells to the

TABLE V

Generation of Antigen-specific Suppressor Cells in the Bulk MLR (C57 × BALB) after Addition of Spleen Cells from Donors Given TLI: Secondary MLR Assay System

Secondary MLR			[^{3}H]Thymidine incorporation (mean cpm ± SD)	Percent suppression‖
Responder cells*	Stimulator cells‡	Co-cultured cells (primary culture)§		
Experiment 1				
C57	C57		893 ± 234	
C57	BALB		27,346 ± 3,689	
C57	BALB	C57 × BALB × BALB	6,032 ± 843	77.9
C57	BALB	C57 × BALB × TLI	7,775 ± 1,523	71.6
C57	C57		893 ± 234	
C57	C3H		21,779 ± 3,517	
C57	C3H	C57 × BALB × BALB	3,541 ± 462	83.7
C57	C3H	C57 × BALB × TLI	15,836 ± 3,293	27.3
Experiment 2				
C3H	C3H		5,296 ± 876	
C3H	BALB		102,679 ± 13,753	
C3H	BALB	C57 × BALB × BALB	7,197 ± 1,185	93.0
C3H	BALB	C57 × BALB × TLI	74,106 ± 1,150	27.8
C3H	C3H		5,296 ± 876	
C3H	C57		126,456 ± 1,465	
C3H	C57	C57 × BALB × BALB	8,593 ± 2,124	93.2
C3H	C57	C57 × BALB × TLI	115,273 ± 2,162	8.8

* 5×10^5 responder cells per well were used.

‡ 5×10^5 stimulator cells per well were given 3,000 rad in vitro.

§ Co-cultured cells were obtained from the bulk MLR at day 6 and given 1,500 rad in vitro. 3×10^6 responder, stimulator, and co-cultured cells used in the bulk MLR are shown.

‖ As for Table III.

secondary MLR cultures again showed marked suppression only with secondary C57 responder cells and BALB stimulator cells (mean, >83.7% suppression; range, 75–>89%). Minimal suppression was seen in other combinations (mean, 12.4% suppression; range, −29–62%). Even in the experiment that showed 62% suppression (upper limit of range of values), the number of lytic units per secondary culture was still ninefold higher using C57 × BALB × TLI primary culture cells as compared with C57 × BALB × BALB primary culture cells. Thus, suppression of the generation of cytolytic cells in the secondary MLR was antigen-specific, and restricted by the strain of origin of the secondary responder cell. This pattern of suppression cannot be explained by specific killing of secondary stimulator cells by cytolytic cells generated in primary cultures, because destruction of stimulator cells should have produced suppression of the secondary MLR and CML assays regardless of the origin of the responder cells.

Correlation between the Degree of Nonspecific Suppression of Primary MLR Cultures and the Degree of Specificity of Suppression in Secondary MLR Cultures. The ability of spleen cells from donors given TLI to suppress the primary MLR with BALB responder and C57 stimulator cells was tested in 27 different experiments. In 24 experiments, nonspecific suppression of [^{3}H]thymidine incorporation or generation of cytolytic cells was at least

TABLE VI

Generation of Antigen-specific Suppressor Cells in the Bulk MLR (C57 × BALB) after Addition of Spleen Cells from Donors Given TLI: Secondary CML Assay System

Secondary MLR			CML assay target cell	Lytic units/ culture	Percent suppression‖
Responder cells*	Stimulator cells‡	Co-cultured cells (primary culture)§			
Experiment 1					
C57	BALB		P815	203.0	
C57	BALB	C57 × BALB × BALB	P815	<25.0	>87.7
C57	BALB	C57 × BALB × TLI	P815	<25.0	>87.7
C57	C3H		BW5147	55.6	
C57	C3H	C57 × BALB × BALB	BW5147	<12.5	>77.5
C57	C3H	C57 × BALB × TLI	BW5147	47.6	14.4
Experiment 2					
C3H	BALB		P815	312.5	
C3H	BALB	C57 × BALB × BALB	P815	<12.5	>96.0
C3H	BALB	C57 × BALB × TLI	P815	119.0	61.9
C3H	C57		EL-4	451.9	
C3H	C57	C57 × BALB × BALB	EL-4	<25.0	>94.5
C3H	C57	C57 × BALB × TLI	EL-4	342.1	24.3

* As for Table III.
‡ As for Table III.
§ As for Table III.
‖ As for Table IV.

50%, however, in three experiments, <50% suppression was observed. In the latter experiments, there appeared to be a correlation between the degree of nonspecific suppression in the primary cultures and the degree of antigen specificity of suppression in the secondary cultures. The degree of antigen specificity can be taken as the difference in the percent suppression using secondary stimulator cells syngeneic to the primary stimulator cells, and that using secondary stimulator cells allogeneic to the primary stimulator cells (i.e., percent suppression with secondary C57 stimulator cells minus the percent suppression with secondary C3H or BALB stimulator cells). When the percent nonspecific suppression of primary cultures was at least 50%, the mean percent difference was 54.9% in the secondary MLR assay and 63.7% in the secondary CML assay. When the percent nonspecific suppression in the primary MLR assay was 28.8%, 33.4%, and 41.3%, the specificity difference in the secondary MLR assay was 10.6%, 6.8%, and 34.1%, respectively. Similarly, when the percent nonspecific suppression of the primary CML assay was 26.5%, 30.2%, and 37.8%, then the specificity difference in the secondary CML assay was 15.8%, 27.7%, and 21.9%, respectively.

Nonspecific Inhibition of [^{3}H]Thymidine Incorporation in the Primary MLR by Newborn BALB/c Spleen Cells. Spleen cells from newborn BALB/c mice were co-cultured with equal numbers of normal adult responder and stimulator cells from a variety of strains. Spleen cells from adult BALB/c mice were used as co-cultured cell controls. Six experiments were performed using 2- or 3-d-old newborn BALB/c mice, and one representative result is shown in Table VII. Newborn (2 or 3 d after birth) spleen cells

TABLE VII

Nonspecific Inhibition of the MLR by Newborn Spleen Cells

Responder cells*	Stimulator cells‡	Co-cultured cells§	[^{3}H]Thymidine incorporation (mean cpm ± SD)	Percent suppression‖
BALB	BALB	BALB	5,186 ± 568	
BALB	C57	BALB	100,998 ± 3,119	
BALB	C57	NB¶	42,101 ± 948	58.3
C57	C57	C57	2,479 ± 467	
C57	BALB	BALB	51,770 ± 1,446	
C57	BALB	NB	16,872 ± 762	67.4
C3H	C3H	C3H	5,803 ± 923	
C3H	C57	BALB	100,764 ± 5,895	
C3H	C57	NB	37,945 ± 1,037	62.3
BALB	BALB	BALB	5,186 ± 568	
BALB	C3H	BALB	97,075 ± 5,993	
BALB	C3H	NB	36,076 ± 2,392	62.8
C57	C57	C57	2,479 ± 467	
C57	C3H	BALB	44,372 ± 7,568	
C57	C3H	NB	12,193 ± 2,307	72.5
C3H	C3H	C3H	5,803 ± 923	
C3H	BALB	BALB	104,571 ± 4,819	
C3H	BALB	NB	45,482 ± 2,657	56.5

* 5×10^5 responder cells per well were used.

‡ 5×10^5 stimulator cells per well were given 3,000 rad in vitro.

§ 5×10^5 co-cultured cells per well were given 1,500 rad in vitro.

‖ Percent suppression = (1 − [(cpm with NB co-cultured cells)/(cpm with BALB co-cultured cells)]) × 100.

¶ Newborn BALB/c donors (3 d after birth).

suppressed the MLR by 55–90% with a mean of 71% as compared with adult spleen cells. Thus, the pattern of inhibition of the MLR was similar to that observed with cells from TLI-treated donors, in that no specificity for responder or stimulator cells was seen in any of the strain combinations.

Discussion

We have previously shown that potent nonspecific suppressor cells of the MLR and of graft-vs.-host disease are present in the spleens of adult BALB/c mice immediately after TLI, and gradually disappear during the subsequent 3–4 wk (11). The present study confirms and extends this work. Spleen cells obtained from BALB/c mice within the first 1–2 wk after TLI suppressed the MLR by 55–95% regardless of the strain of origin of the responder or stimulator cells. Similarly, the generation of cytolytic cells in MLR cultures were suppressed by 50–90%. It is unlikely that the suppression is mediated by natural killer (NK) cells, as NK activity of spleen cells from mice given TLI was similar to cells from normal donors (A. Oseroff and S. Strober, unpublished observations).

Recently, several investigators have reported that antigen-specific and nonspecific suppressor cells are generated in MLR cultures along with cytolytic cells (29–42). To determine the effect of spleen cells from TLI-treated donors on the generation of suppressor cells in the MLR, primary bulk MLR cultures containing normal responder cells, normal stimulator cells, and co-cultured cells obtained from either TLI-treated or untreated donors were allowed to incubate for 6 d. At the end of the primary culture period, the cells were irradiated (1,500 rad) and added to a secondary MLR culture. The secondary cultures were assayed for both suppression of [^{3}H]thymidine incorporation and generation of cytolytic cells. Antigen specificity of suppression was tested by comparing the effect of cells from primary cultures on secondary cultures in which the secondary stimulator cells were either syngeneic or allogeneic to the primary stimulator cells. Haplotype restriction of suppression was tested by comparing the effect of cells from primary cultures on secondary cultures in which the secondary responder cells were either syngeneic or allogeneic to the primary responder cells.

Cells from primary cultures in which responder, stimulator, and co-cultured cells were all syngeneic (all obtained from normal adult BALB/c donors) generally showed little suppression of [^{3}H]thymidine incorporation or generation of cytolytic cells in the secondary MLR cultures. However, primary cultures containing normal BALB/c responder cells, normal C57BL/Ka stimulator cells, and normal BALB/c co-cultured cells markedly suppressed [^{3}H]thymidine incorporation and the generation of cytolytic cells regardless of the source of responder and stimulator cells in the secondary cultures. Thus, the suppressor cells generated in the primary cultures under the latter conditions were antigen nonspecific, and were not restricted by the major histocompatibility complex haplotype of the responders in the secondary cultures. It is unlikely that the nonspecific suppression of the secondary cultures was due to killing of secondary responder or stimulator cells by cytolytic cells generated in the primary cultures, as cells from primary cultures suppressed secondary cultures in which neither the secondary responder nor stimulator cells were syngeneic to the stimulator cells in the primary cultures.

Although fresh spleen cells from TLI-treated BALB/c mice nonspecifically suppressed primary MLR cultures, their suppressive activity disappeared after several days of in vitro incubation with BALB/c responder and stimulator cells. Cells harvested from such syngeneic primary cultures failed to show suppressive activity after addition to secondary allogeneic MLR cultures. On the other hand, cells from primary cultures containing normal BALB/c responders, normal C57BL/Ka stimulators, and co-cultured cells from primary cultures containing normal BALB/c responders, normal C57BL/Ka stimulators, and co-cultured cells from BALB/c mice given TLI strongly suppressed secondary cultures only when the secondary stimulator cells were syngeneic to the primary stimulator cells. These antigen-specific suppressor cells were not restricted by the haplotype of the secondary responder cell, as suppressive activity was about equal whether or not the secondary responder was syngeneic or allogeneic to the primary responder.

It is unlikely that antigen-specific suppression observed in secondary cultures was caused by specific killing of secondary stimulator cells by cytolytic cells generated in primary cultures containing cells from TLI-treated donors. In several experiments with marked suppression (~90%) of the generation of cytolytic cells in the primary cultures, no cytolysis above background was produced by primary cells at effector/

target ratios of 1:4. However, marked suppression of the secondary MLR and CML assays was observed with the same primary cells at a primary cell/secondary stimulator cell ratio of 1:4. In addition, the primary cells (BALB × C57 × TLI) failed to inhibit the MLR or CML responses of C57BL/Ka cells to BALB/c stimulators at the same concentrations that inhibited secondary cultures with C57BL/Ka stimulators (S. Okada and S. Strober, unpublished observations).

It is of interest that the magnitude of nonspecific suppression of the primary cultures by spleen cells from TLI-treated mice was directly proportional to the degree of antigen specificity of the suppressor cells of the secondary cultures. In those experiments in which primary cultures were minimally suppressed by the addition of spleen cells from TLI-treated mice, there was minimal specificity of suppression in the secondary cultures (considerable suppression with all combinations of responders and stimulators).

A hypothesis derived from previous reports (29–41) that explains the data above is that responder cells in the MLR, in the absence of co-cultured cells from TLI-treated mice, generate at least three types of cells: cytolytic cells, nonspecific suppressor cells, and antigen-specific suppressor cells (see Fig. 1). Spleen cells from TLI-treated mice inhibit the generation of the cytolytic cells and nonspecific suppressor cells, but do not inhibit the generation of antigen-specific suppressor cells. Because nonspecific suppressor cells generated in the primary cultures can "mask" the specific suppressor cells, the latter cells are not ordinarily observed in primary cultures. However, the addition of spleen cells from TLI-treated mice "unmasks" the antigen-specific suppressors. Spleen cells obtained from neonatal mice within 2–3 d of birth show the same pattern of nonspecific inhibition of the MLR as observed with cells from TLI-treated mice (Table VII). In addition, the neonatal cells show a similar pattern of inhibition of the generation of cytolytic and nonspecific suppressor cells without blocking the generation of specific suppressor cells.[2]

In all of the experiments with secondary cultures described above, the primary cultures were made up such that the co-cultured cells were syngeneic with the responder cells. To determine whether spleen cells from TLI-treated mice have similar regulatory effects on allogeneic responder cells in primary cultures, normal C57BL/Ka responder cells and normal BALB/c stimulator cells were co-cultured with cells from TLI-treated BALB/c mice. A similar pattern of nonspecific suppression of the primary cultures and antigen-specific suppression of the secondary cultures was observed. However, optimum suppression of secondary cultures occurred only when the secondary responder and stimulator combination was identical to that in the primary culture. Thus, antigen-specific suppression could not be mediated by specific killing of secondary stimulator cells.

In conclusion, spleen cells from TLI-treated mice can inhibit the generation of cytolytic cells and nonspecific suppressor cells in the MLR when the responder cell is either syngeneic or allogeneic to the co-cultured cells. However, these spleen cells do not inhibit the generation of antigen-specific suppressor cells. The overall effect is that allogeneic stimulator cells in the presence of cells from TLI-treated or neonatal

[2] Okada, S., and S. Strober. Spleen cells from adult mice given total lymphoid irradiation or from newborn mice have similar regulatory effect in the mixed leukocyte reaction. II. Generation of antigen-specific suppressor cells in the MLR after the addition of spleen cells from newborn mice. Manuscript submitted for publication.

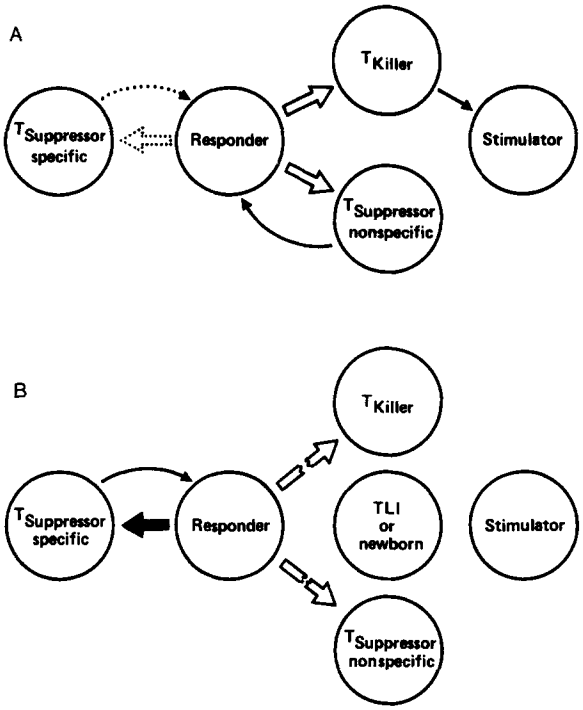

FIG. 1. Regulatory Cell Model. In the two-party MLR culture containing normal responder cells and normal stimulator cells (irradiated in vitro) (A), the responder cells generate cytolytic cells, antigen-nonspecific suppressor cells, and perhaps antigen-specific suppressor cells masked by antigen-nonspecific suppressor cells. After the addition of spleen cells from adult mice given TLI or from newborn mice, the generation of cytolytic cells and nonspecific suppressor cells is blocked. However, the generation of antigen-specific suppressor cells (B) proceeds without impairment. Thus, continued alloantigen stimulation produces a pool of antigen-specific suppressor cells which continue to block the generation of cytolytic cells. The latter pool may play an important role in maintaining tolerance in vivo even after the nonspecific suppressor cells found after TLI or in newborn mice disappear.

mice tend to stimulate the production of large numbers of antigen-specific suppressor cells rather than cytolytic cells. This regulatory phenomenon may render these mice highly susceptible to tolerance induction in vivo for short periods of time after birth or radiotherapy.

Summary

We added spleen cells from adult BALB/c mice treated with total lymphoid irradiation (TLI) to the mixed leukocyte reaction (MLR) using a variety of responder and stimulator cells. The spleen cells nonspecifically suppressed the uptake of [^{3}H]-thymidine and the generation of cytolytic cells regardless of the responder-stimulator combination used. We also examined the effect of the spleen cells on the generation of antigen-nonspecific and antigen-specific suppressor cells in the MLR. The experimental results suggest that the spleen cells from TLI-treated mice inhibit the generation of nonspecific suppressor cells, but do not inhibit the generation of antigen-specific suppressor cells. Thus, alloantigenic stimulation of normal responder cells in vitro in the presence of spleen cells from TLI-treated mice generates large numbers of

antigen-specific suppressor cells, but few cytolytic cells or nonspecific suppressor cells. Similar nonspecific inhibition of the MLR was observed with neonatal spleen cells. This in vitro system provides a regulatory model for the induction and maintenance of tolerance in vivo, in which adult mice given TLI or neonatal mice accept allogeneic bone marrow transplants without graft-vs.-host disease.

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