REGULATORY MECHANISMS IN CELL-MEDIATED IMMUNE RESPONSES

I. Regulation of Mixed Lymphocyte Reactions by Alloantigen-Activated Thymus-Derived Lymphocytes*

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Functionally distinct subpopulations of thymus-derived lymphocytes (T cells) cooperate in the development of certain cell-mediated immune responses. Initially, Cantor, Tigelaar, and Asofsky (1–3) demonstrated that two distinct populations of T cells, termed effector and amplifier T cells, interacted synergistically in the development of graft-vs.-host (GVH)[1] reactions in the mouse. More recently, cooperative interaction between thymocytes and peripheral T cells has been described for mixed lymphocyte reactions (MLR) (4) and for development of cytotoxic allograft responses in vitro (5).

Evidence is now accumulating that a third T-cell subpopulation, suppressor T cells, may be important in the regulation of cell-mediated immune responses. Hardin, Chused, and Steinberg (6) described a population of splenic suppressor cells in young (6-wk old) (NZB/NZW)F_1 mice which inhibited GVH activity of 25-wk old spleen cells when injected into newborn C3H mice. In addition, Zembala and Asherson (7) reported suppression of contact sensitivity to picryl chloride when T cells from mice unresponsive to picryl sulfonic acid were transferred to normal recipients. Suppression of cytotoxic killer cell generation by concanavalin A (Con A)-activated murine spleen cells has been reported by Peavy and Pierce (8). Folch and Waksman (9, 10) have observed enhanced responses to T-cell mitogens and alloantigens in rat spleen cell cultures depleted of glass-adherent T cells.

We here report the effects of alloantigen activation on generation in vivo of a subpopulation of murine thymus-derived lymphocytes which regulate MLR. The data indicate that regulatory cells with suppressor and amplifier activities in MLR are anatomically segregated after alloantigen sensitization. The generation

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[1] *Abbreviations used in this paper:* Con A, concanavalin A; FCS, fetal calf serum; GVH, graft-vs.-host; HBSS, Hanks' balanced salt solution; MLR, mixed lymphocyte reaction; PFC, plaque-forming cell(s); PHA, phytohemagglutinin.

in vivo and the kinetics and specificity of activated suppressor splenic lymphocytes are characterized. These studies provide additional evidence to support a general concept of active, physiologic regulation of both cell-mediated and humoral immune responses by activated T cells.

Materials and Methods

Mice. BALB/c ($H\text{-}2^d$) mice (Cell Biology Department, Baylor College of Medicine, Houston, Texas) and C57BL/6 ($H\text{-}2^b$) and A/Tex ($H\text{-}2^a$) mice (Texas Inbred Mice Company, Houston) were used in these studies. Experiments were performed with 8- to 14-wk old male animals.

MLR. Single cell suspensions of mouse spleen were prepared by gentle teasing and sedimentation of fragments and debris. Lymph node cell suspensions were similarly prepared from inguinal nodes or pooled cervical, brachial, axillary, and inguinal nodes.

Responder, stimulator, and regulator cell populations were cultured in equal numbers, 1×10^6 cells of each, in a final vol of 0.2 ml in microculture plates (Linbro 1S-FB-96-TC, International Scientific Instruments, Cary, Ill.). Cultures were prepared in RPMI-1640 medium supplemented with 5% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, and penicillin-streptomycin mixture (50 U each per ml) (Grand Island Biological Company, Grand Island, N. Y.), and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. To block appreciable DNA replication by either regulator or stimulator cells, these populations were treated before addition to MLR with mitomycin C (Sigma Chemical Company, St. Louis, Mo.), 50 μg/ml for 30 min at 37°C and washed three times with Hanks' balanced salt solution (HBSS).

Regulator cells, syngeneic to the responding population, were obtained from mice injected with syngeneic or allogeneic spleen cells. Spleen cell suspensions in various doses were injected intravenously, intraperitoneally, or into the hind foot pads. The usual protocol for suppressor cell generation, illustrated in Fig. 1, utilized 2×10^7 cells injected into the hind foot pads. 4 days after injection, single cell suspensions of spleen or lymph node were prepared, treated with mitomycin C and added to MLR.

DNA synthesis in MLR was assayed by adding 1.0 μCi of tritiated thymidine ([^{3}H]TdR, spec act 2.0 Ci/mM, New England Nuclear Corp., Boston, Mass.) to cultures for the final 18 h of a 72-h incubation period. Exceptions to this protocol for certain experiments are subsequently detailed. Cultures were harvested by aspiration of cells onto glass-fiber filters with a saline wash utilizing a multiple automated sample harvester (MASH-II, Microbiological Associates, Inc., Bethesda, Md.). Dry filters were placed in a scintillation cocktail containing toluene, Permaflour, and Soluene (Packard Instrument Co., Inc., Downers Grove, Ill.) and radioactivity was measured in a liquid scintillation spectrometer (Mark II, Nuclear-Chicago, Des Plaines, Ill.).

Data are expressed as mean counts per minute (cpm) of three to six replicate cultures with the standard error of the mean. The stimulation index (E/C) was calculated by dividing cpm from cultures containing stimulating cells allogeneic to the responder population by cpm from cultures containing syngeneic stimulating cells.

To standardize results and permit evaluation of regulator cell effects in replicate experiments with differing positive and negative control responses, a ratio was calculated of the stimulation index of cultures incubated with activated regulatory cells to the stimulation index of cultures incubated with nonactivated cells. Each E/C value was first adjusted to express the increment of stimulation over 1.0 (i.e., 1.0 equals no stimulation). The following formula was used:

$$\frac{[(E/C) \text{ of MLR with activated regulators}] - 1}{[(E/C) \text{ of MLR with nonactivated regulators}] - 1} \times 100 = \% \text{ control MLR response.}$$

Thus, reduction of E/C in cultures with suppressor cells to 1.0 was regarded as complete suppression of the MLR (0% control MLR). Data were analyzed statistically by the two-tailed Student's t test, the two-tailed Wilcoxon rank-sum test or the Wilcoxon signed rank test.

Cell Enumeration and Viability Determination. Nucleated cells were enumerated with a model ZBI Coulter Counter (Coulter Electronics, Hialeah, Fla.). Cell viability was determined by trypan

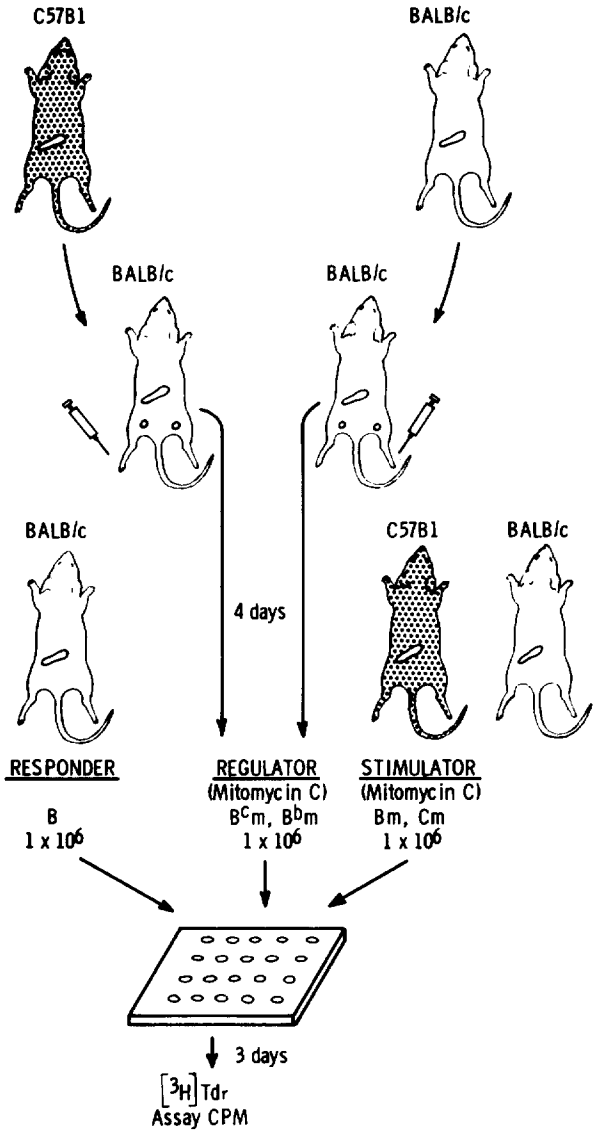

FIG. 1. Diagrammatic representation of preparation of alloantigen-activated spleen and lymph node cells for incorporation into MLR. 2×10^7 syngeneic or allogeneic spleen cells are injected into hind foot pads of normal mice; spleen or lymph node cells or both are obtained 4 days after injection, treated with mitomycin C and added to MLR as regulator populations.

blue exclusion; cells were stained for 30 sec in 0.04% trypan blue, fixed with an equal vol of 4% acetic acid, and counted in a hemocytometer.

Treatment of Spleen Cells with Anti-θ Serum and Complement. AKR anti-θ C3H/HeJ serum, a gift of Dr. Patricia Bealmear Baylor College of Medicine, was prepared by the method of Reif and Allen (11). A 1:20 dilution of this antiserum killed 30-35% of C3H spleen cells and 95-98% of C3H thymus cells.

Spleen cells were incubated in anti-θ serum for 30 min at 4°C (1×10^8 cells in 2.5 ml of a 1:20 dilution of anti-θ serum), 2.5 ml of a 1:2 dilution of mouse liver-absorbed guinea pig complement

(Baltimore Biological Laboratories, Baltimore, Md.) containing 10 μg/ml deoxyribonuclease (Worthington Biochemical Corp., Freehold, N. J.) was added and incubation continued for 45 min at 37°C. Control cells were similarly treated with normal AKR serum, complement and deoxyribonuclease; all cells were washed once in HBSS before subsequent mitomycin C treatment.

^{51}Cr-Release Studies. Cell-mediated cytotoxicity of spleen cells sensitized to alloantigens in vivo was assessed by ^{51}Cr release. Target cells were prepared by culturing 1×10^7 spleen cells with 50 μl PHA-M (Difco Laboratories, Detroit, Mich.) in 1-ml cultures for 72 h. The cultures were harvested, washed with HBSS, and contaminating erythrocytes were lysed by brief exposure to 0.14 M NH_4Cl-Tris. 1-2 $\times 10^8$ cells were resuspended in 1 ml RPMI-1640 containing 1% FCS. 200 μCi ^{51}Cr (as $Na_2{}^{51}CrO_4$, spec act 350 mCi/mg, New England Nuclear Corp.) were added and the suspensions were incubated with frequent agitation for 30 min at 37°C. Cell suspensions were then washed three times in HBSS and resuspended at a concentration of 1×10^5 viable cells per ml in RPMI-1640 with 5% FCS.

^{51}Cr release from labeled target cells was assayed by culturing untreated or mitomycin-treated effector cells from normal or alloantigen-activated spleens with target cells at an effector cell to target cell ratio of 100:1. Triplicate cultures were incubated 6-8 h at 37°C. Supernates were harvested by centrifugation at 400 *g* for 10 min and ^{51}Cr released from cells was measured in a well-type gamma spectrometer (Model 1085, Nuclear-Chicago Corp.). The release of ^{51}Cr from target cells incubated without effector cells was used as a measure of spontaneous release. Maximal release was achieved by repeated freezing and thawing of target cells in H_2O. The percentage of specific lysis by cytotoxic lymphocytes was calculated according to the formula (12):

$$\frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximal release} - \text{spontaneous release}} \times 100 = \% \text{ specific cytolysis.}$$

Results

Alloantigen-Induced Generation of Regulator Cell Population for MLR. Subcutaneous injection of cells bearing foreign histocompatibility antigens is a potent stimulus of cell-mediated immunity (13). Furthermore, following such sensitization there occurs a time-dependent difference in cell-mediated immune functions between spleen and nondraining lymph node cells and draining lymph node cells (14). These observations were utilized in the design of initial experiments to induce alloantigen activation of populations of lymphocytes with diverse regulatory effects on the development of an MLR.

BALB/c mice were injected with 2×10^7 C57BL/6 spleen cells into the hind foot pads. 4 days later separate dissociated cell suspensions were prepared from spleens and draining (inguinal) lymph nodes. These cell suspensions were treated with mitomycin C, and incorporated into MLR as regulator cells. Lymph node or spleen cells from unsensitized mice or from mice injected with syngeneic spleen cells were utilized as control regulator cells. In most experiments spleen cells from uninjected mice were used, since no differences were detected when these cultures were compared with those containing spleen cells from animals injected with syngeneic cells. Mitomycin C-treated spleen cells from mice sensitized in vivo with allogeneic cells markedly suppressed the mitogenic response of BALB/c responding cells to C57BL/6 stimulating cells, reducing the response by 89% in the experiments illustrated (Table I, group 4). Conversely, cells from the draining lymph nodes of the same sensitized animals (group 8) enhanced stimulation 33% over that observed in stimulated cultures with normal lymph node regulator cells (equal to enhancement of 56% over responses in

TABLE I

Effects of Alloantigen-Activated Spleen and Lymph Node Cells on MLR Responses

Culture group	Responder cells	Stimulator cells	Regulator cells*	Regulator cell source	[^{3}H]TdR incorporation (cpm)‡	Stimulation index (E/C)	% Control MLR response§
1	BALB	$BALB_m$	$BALB_m$	Spleen	596 ± 140	—	
2	BALB	$C57_m$	$BALB_m$	Spleen	2,493 ± 182	4.18	—
3	BALB	$BALB_m$	$BALB^c_m$	Spleen	914 ± 159	—	
4	BALB	$C57_m$	$BALB^c_m$	Spleen	1,269 ± 219	1.34	11
5	BALB	$BALB_m$	$BALB_m$	Lymph Node	653 ± 67	—	
6	BALB	$C57_m$	$BALB_m$	Lymph Node	3,091 ± 262	4.73	—
7	BALB	$BALB_m$	$BALB^c_m$	Lymph Node	668 ± 65	—	
8	BALB	$C57_m$	$BALB^c_m$	Lymph Node	3,981 ± 734	5.96	133

* Regulator cells from spleens and lymph nodes of BALB/c mice sensitized 4 days previously by foot pad injection of 2×10^7 C57BL/6 spleen cells (superscript "c") or spleen cells from control BALB/c mice. Subscript "m" in stimulator and regulator cell columns designates mitomycin C-treated cells.

‡ Mean cpm ± SE of the mean for 12 replicate cultures from three experiments.

§ Formula for calculation in Materials and Methods.

cultures with nonactivated splenic regulator cells [group 2]). Use of normal lymph node cells as the control regulator population (group 6) produced a slight (15–17%) but consistent enhancement of MLR stimulation.

Anatomic segregation of suppressor and amplifier cell activities is further apparent when data from all studies performed utilizing both activated spleen and activated lymph node populations are compared as percent control MLR response (Fig. 2). Basic experimental protocols were identical with that described in the preceeding paragraph. Control MLR responses for these calculations utilized cells from the same organs, i.e., MLR responses in cultures containing activated spleen cells were compared with those in cultures containing nonactivated spleen cells. Similarly, responses in cultures containing nonactivated lymph node cells were utilized as controls for cultures with activated lymph node cells. These data consequently reflect entirely the effects of alloantigen activation on regulatory cell populations from these organs, rather than possible intrinsic suppressor or amplifier activities of spleen or lymph node cells from nonsensitized animals. Activated splenic regulator cells produced consistent marked suppression of MLR. In contrast, activated lymph node cells, usually, but not invariably, enhanced MLR responses (median significantly greater than 100% by the Wilcoxon signed rank test, $P < 0.01$). Subsequent studies were directed toward characterization of the splenic suppressor cell population.

Cytotoxicity Studies of Alloantigen-Activated Suppressor Cells. Two approaches were used to investigate the possibility of a direct or indirect cytotoxic action of activated spleen cells either specifically, on allogeneic stimulator cell populations, or nonspecifically, on other cells in cultures (Table II). First, trypan blue exclusion studies of cell viability in control and experimental cultures were

performed at the time of MLR harvesting; no decreased viability was observed in cultures containing allogen-activated spleen cells when compared to those containing control regulator populations. Second, ^{51}Cr-labeled, PHA-stimulated target cells syngeneic either to the regulator or stimulator population were cultured with normal or alloantigen-sensitized spleen cells. The potentially cytotoxic population was either untreated or mitomycin C-treated to provide a population of cells comparable to that tested in MLR. ^{51}Cr release was not increased in cultures containing alloantigen-sensitized spleen cells as potential killers. No effector population induced ^{51}Cr release greater than spontaneous ^{51}Cr release, thereby resulting in negative values. However, a portion of the same activated cells used in the ^{51}Cr release test demonstrated marked suppression when added as regulator cells in MLR.

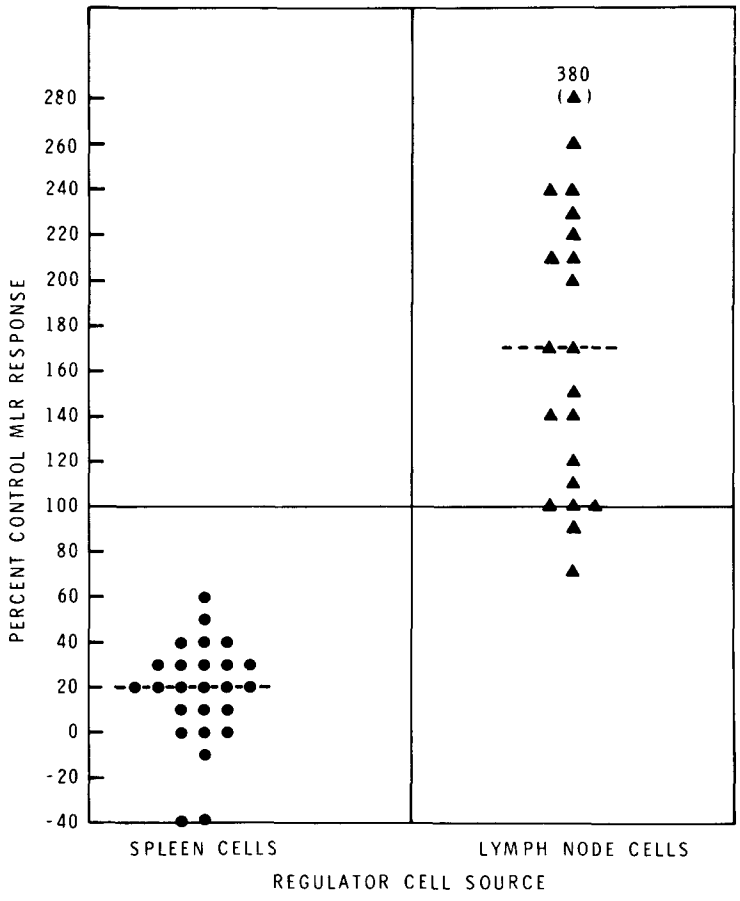

FIG. 2. Anatomic segregation of regulatory cell populations for MLR responses. Spleen or lymph node cells were obtained from BALB/c mice which had been injected into foot pads with 2×10^7 C57BL/6 spleen cells or control BALB/c spleen cells 4 days previously. Percent control MLR responses are calculated as described in Materials and Methods. Data points represent mean responses of triplicate cultures from separate experiments. Median responses for experiments with regulator cells from each source are designated by the dashed line.

TABLE II

Cytotoxic Activity of Alloantigen-Activated Spleen Cells

Culture group	^{51}Cr release assay*			MLR assay		
	Effector	Target	% ^{51}Cr release‡	Constituents	% Viability§	Stimulation index (E/C)
1	B	B	0	—		
2	B	C	−4	—		
3	B_m	B	−6	B B_m B_m	49 ± 2	—
4	B_m	C	−26	B B_m C_m	53 ± 6	3.88
5	B^c	B	−13	—		
6	B^c	C	−9	—		
7	B^c_m	B	−17	B B^c_m B_m	45 ± 2	—
8	B^c_m	C	−6	B B^c_m C_m	50 ± 3	1.43

* Effector/target cell ratio = 100:1. Effector cells incubated with target cells for 6 h before harvest. Subscript "m" indicates mitomycin C-treated spleen cells. Superscript "c" designates spleen cells from BALB/c mice sensitized 4 days previously by foot pad injection of 2×10^7 C57 spleen cells.
‡ Percent specific ^{51}Cr release represents average of three replicate cultures for each group.
§ Determined by trypan blue exclusion following 72 h of culture.

Kinetics of Suppressor Cell Activity in MLR. Kinetics of MLR responses were studied to determine whether the apparent suppressive effect of activated spleen cells reflected instead a shift in peak response to an earlier or later time. Mixed leukocyte cultures containing regulator populations of normal or alloantigen-sensitized spleen cells were harvested daily after a 4 h pulse with [^{3}H]TdR, and stimulation indices were calculated for each day of culture (Fig. 3). Cultures containing either normal or activated regulator populations both exhibited peak alloantigen-induced [^{3}H]TdR incorporation on day 2. MLR responses were reduced in cultures containing activated regulator spleen cells on each day of assay. Numerous similar kinetic studies utilizing 4 h [^{3}H]TdR pulses demonstrated maximum MLR responses to occur between day 2 and day 3, and subsequent studies were performed with an 18-h labeling period to encompass this peak of stimulation. High levels of incorporation at the initiation of culture are characteristic of mouse spleen cell cultures in vitro under these conditions and have been described by others (15).

Time-Course of Suppressor Cell Generation. The hind footpads of BALB/c mice were injected with 2×10^7 C57BL/6 spleen cells at intervals ranging from 15 days to 1 day before the incorporation of mitomycin C-treated spleen cells from these animals into MLR (Fig. 4). Suppressor activity was detected 48 h after injection of allogeneic cells and was maximal at 4 days. Spleen cells from animals sensitized 7 days or more before culturing did not suppress MLR responses.

Effect of Injection Route on Generation of Suppressor Cells. BALB/c mice received 2×10^7 C57BL/6 spleen cells by intravenous, intraperitoneal, or footpad injection. 4 days later mitomycin C-treated spleen cells from each group of mice were incorporated into MLR as regulator populations; an equal number of normal mitomycin C-treated spleen cells was added to control cultures (Table III). Spleen cells from animals injected with allogeneic cells intravenously (group 6) or into hind footpads (group 8) induced 70–90% suppression of control MLR

responses. However, in evaluation of many experiments, generation of highly active suppressor cells most consistently followed footpad immunization. Spleen cells from animals receiving the same sensitizing dose of cells intraperitoneally had only minimal suppressor effects.

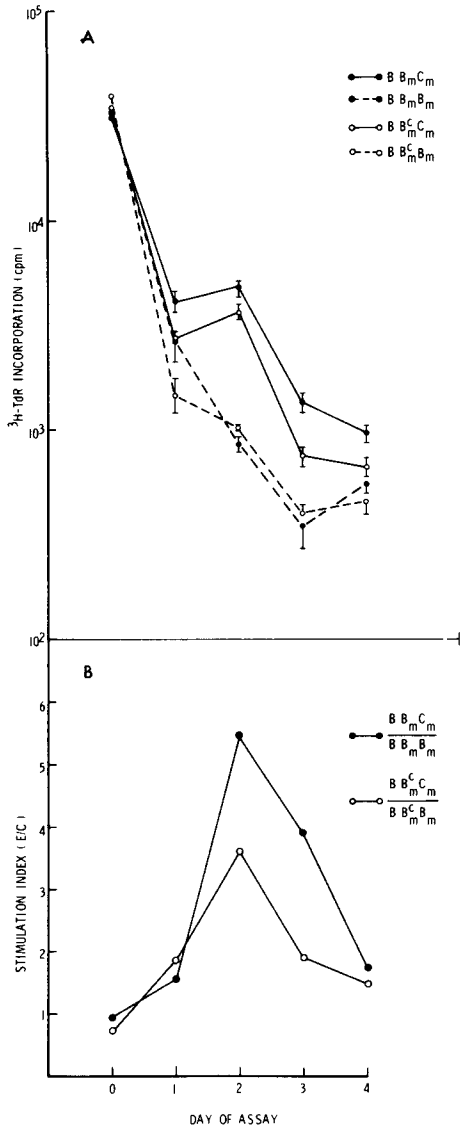

FIG. 3. Kinetics of alloantigen-activated spleen cell suppression in MLR. Cultures containing normal or C57BL/6-sensitized BALB/c spleen regulator cells were harvested daily following a 4 h pulse with tritiated thymidine. Fig. 3 A illustrates daily [^{3}H]TdR incorporation into mixed (●—● B Bm Cm; O—O B $B_m{}^c$ C_m) and unmixed control (●---● B B_m B_m; O---O B $B_m{}^c$ B_m) cultures. Data represent mean cpm ± standard error of the mean of quadruplicate cultures at each point. Fig. 3 B illustrates the stimulation indices (E/C) derived from [^{3}H]TdR incorporation detailed in Fig. 3 A.

Effect of Anti-θ Serum and Complement on Suppressor Cell Activity. Spleen cells from BALB/c mice injected with 2×10^7 C57BL/6 spleen cells by footpad 4 days before culture or from normal BALB/c mice were treated with AKR anti-θC3H serum and complement. They were then treated with mitomycin C and added to MLR as regulator populations (Table IV). Control cells were treated with normal AKR serum, complement, and mitomycin C. Characteristic suppression of MLR response was observed in cultures containing regulator

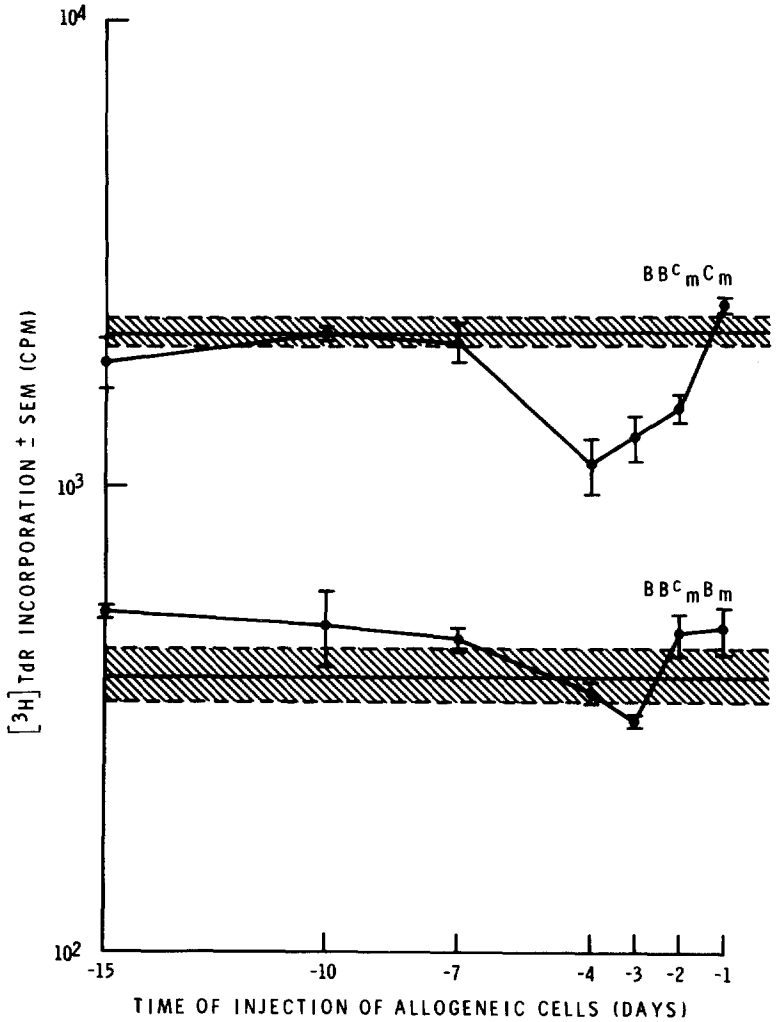

FIG. 4. Time-course of alloantigen-activated suppressor cell generation. BALB/c mice injected by foot pad with 2×10^7 C57BL/6 spleen cells at indicated times before use of spleen cells from these animals as regulator cells in MLR. Data represent mean cpm ± SE of the mean for quadruplicate cultures at each point. Means and SE of control mixed and unmixed cultures containing regulator cells from nonsensitized mice are indicated by lateral solid and dashed lines. Responses of mixed cultures containing activated regulator cells from animals injected 2, 3, or 4 days before culture were significantly different from the control mixed culture ($P < 0.001$ by two-tailed Student's t test). No other responses were statistically different from controls.

TABLE III

Effect of Sensitization Route on Generation of Alloantigen-Activated Suppressor Spleen Cells

Culture group	Responder cells	Stimulator cells*	Regulator cells	Route of sensitization	[^{3}H]TdR incorporation (cpm)‡	Stimulation index (E/C)	% Control MLR response§
1	BALB	$BALB_m$	$BALB_m$	—	2,160 ± 154	—	
2	BALB	$C57_m$	$BALB_m$	—	6,073 ± 246	2.81	—
3	BALB	$BALB_m$	$BALB^c_m$	IP	2,192 ± 401	—	
4	BALB	$C57_m$	$BALB^c_m$	IP	5,391 ± 391	2.46	81
5	BALB	$BALB_m$	$BALB^c_m$	IV	3,046 ± 309	—	
6	BALB	$C57_m$	$BALB^c_m$	IV	3,466 ± 141	1.14	8
7	BALB	$BALB_m$	$BALB^c_m$	FP	1,979 ± 141	—	
8	BALB	$C57_m$	$BALB^c_m$	FP	3,023 ± 171	1.53	29

* Regulator cells derived from spleens of BALB/c mice sensitized 4 days previously by injection of 2×10^7 C57BL/6 spleen cells (superscript "c") or spleen cells from control BALB/c mice. Subscript "m" in stimulator and regulator cell columns designates mitomycin C-treated cells.

‡ Mean cpm ± SE of the mean for quadruplicate cultures.

§ Formula for calculation in Materials and Methods.

TABLE IV

Effect of Treatment with Anti-θ Serum and Complement on Suppressor Activity of Alloantigen-Activated Spleen Cells

Culture group	Responder cells	Stimulator cells	Regulator cells*	Regulator treatment‡	[^{3}H]TdR incorporation (cpm)§	Stimulation index (E/C)	% Control MLR response‖
1	BALB	$BALB_m$	$BALB_m$	NMS + C	699 ± 50	—	
2	BALB	$C57_m$	$BALB_m$	NMS + C	4,439 ± 259	6.35	—
3	BALB	$BALB_m$	$BALB^c_m$	NMS + C	649 ± 43	—	
4	BALB	$C57_m$	$BALB^c_m$	NMS + C	2,079 ± 101	3.20	41
5	BALB	$BALB_m$	$BALB_m$	Anti-θ + C	444 ± 42	—	
6	BALB	$C57_m$	$BALB_m$	Anti-θ + C	2,574 ± 230	5.80	—
7	BALB	$BALB_m$	$BALB^c_m$	Anti-θ + C	492 ± 43	—	
8	BALB	$C57_m$	$BALB^c_m$	Anti-θ + C	2,651 ± 252	5.39	91

* Regulator cells from spleens of BALB/c mice sensitized 4 days previously by foot pad injection of 2×10^7 C57BL spleen cells (superscript "c") or spleen cells from control BALB/c mice. Subscript "m" in stimulator and regulator cell columns designates mitomycin C-treated cells.

‡ Regulator spleen cells treated with AKR anti-θC3H or normal AKR serum (NMS) plus complement as described in Materials and Methods.

§ Mean cpm ± SE of mean of six replicate cultures for each point.

‖ Formula for calculation in Materials and Methods.

populations of activated spleen cells treated with normal AKR serum and complement (group 4). In contrast, treatment of activated spleen cells with anti-θ serum and complement (group 8) abolished the suppressive activity of this regulator population ($P > 0.10$ by the two-tailed Wilcoxon rank-sum test).

Specificity of Suppressor Activity. Preliminary studies of the antigenic specificity of alloantigen-sensitized suppressor spleen cells were performed. BALB/c mice were injected into footpads with 2×10^7 spleen cells from strains A/Tex or C57BL/6. Regulator populations derived from these mice were incorporated into cultures with stimulating cells either syngeneic or allogeneic to the sensitizing cell strain (Table V). Groups 5 and 6 illustrate the nonspecific nature of suppression expressed by activated cells. BALB/c spleen cells activated by C57BL/6 alloantigens suppressed by 68% the MLR response to A/Tex stimulating cells (group 6); the same regulator cells suppressed the response to C57BL/6 stimulator cells by 65% (group 5). Comparable data were obtained when regulator cells were stimulated by spleen cells from A/Tex mice (groups 7, 8, 9). A similar series of experiments performed with A/Tex as responder and regulator cells and BALB/c and C57BL/6 as sensitizing and stimulator cells similarly demonstrated nonspecificity of MLR suppression. In addition, these

TABLE V

Nonspecificity of Suppressor Activity of Alloantigen-Activated Spleen Cells

Culture group	Responder cells	Stimulator cells	Regulator cells*	[^{3}H]TdR incorporation (cpm)‡	Stimulation index	% Control MLR stimulation§
1	BALB	$BALB_m$	$BALB_m$	693 ± 166	—	
2	BALB	$C57_m$	$BALB_m$	3,004 ± 572	4.33	—
3	BALB	A_m	$BALB_m$	2,523 ± 631	3.64	—
4	BALB	$BALB_m$	$BALB^c_m$	710 ± 142	—	
5	BALB	$C57_m$	$BALB^c_m$	1,530 ± 227	2.15	35
6	BALB	A_m	$BALB^c_m$	1,316 ± 64	1.85	32
7	BALB	$BALB_m$	$BALB^A_m$	1,105 ± 571	—	
8	BALB	$C57_m$	$BALB^A_m$	2,349 ± 1,227	2.13	34
9	BALB	A_m	$BALB^A_m$	1,820 ± 676	1.65	25

* Regulator cells derived from spleens of BALB/c mice sensitized 4 days previously by foot pad injection of 2×10^7 C57BL/6 (superscript "c") or A/Tex (superscript "A") spleen cells or spleen cells from control BALB/c mice. Subscript "m" in stimulator and regulator cell columns designates mitomycin C-treated cells.

‡ Mean cpm ± SE of the mean for 12 replicate cultures from three experiments.

§ Formula for calculation in Materials and Methods.

experiments indicate that alloantigen-induced activation of suppressor T cells for MLR responses was not unique to BALB/c mice.

Discussion

Regulation of immune responses by activated T cells has recently gained increasing experimental support as a general immunological phenomenon. The cooperative interaction between T and B lymphocytes in development of antibody responses to most complex antigens is well established (16–18). Furthermore, numerous investigators utilizing diverse experimental models have

now demonstrated that T-cell subpopulations can also suppress antibody synthesis (18, 19). The regulatory functions of T cells in cell-mediated immune responses are less clear. It appears, however, that effector and amplifier T-cell populations interact synergistically in the development of GVH reactions in vivo (1-3) and in expression of T-cell-mediated cytotoxicity (5) and MLR reactivity (4) in vitro.

Recently suppressor T-cell activities in GVH reactions (6, 20, 21) and contact hypersensitivity (7) have been described. As complex manifestations of the last effector phase of cell-mediated immune responses in vivo, however, these systems are relatively limited in dissection and elucidation of mechanisms of T-cell-mediated regulation of cellular immune responses.

The present investigation begins a comprehensive analysis of the phenomena and mechanisms of regulation of cell-mediated immune responses in vitro by activated T cells. This paper establishes an anatomic segregation of suppressor and amplifier cells for MLR responses in alloantigen-sensitized mice. In addition, effects of alloantigen activation on the in vivo generation, kinetics, and specificity of splenic MLR suppressor T cells are described.

The spleen is comprised of functionally heterogeneous populations of lymphocytes; subpopulations of T cells of varying stages of maturity and function are represented (1–3, 22–24). Spleen or spleen-seeking cells are less reactive in MLR than lymph node or lymph node-seeking cells (23). This difference may reflect anatomic segregation of either responder or regulatory cell populations or both. In studies of differential lymphocyte migration, suppressor thymocytes for GVH reactions have been reported to localize preferentially in the spleen (25). Furthermore, Folch and Waksman (10) have recently described thymus-dependent adherent cells in normal rat spleens which inhibited full MLR reactivity.

In our studies, sensitization by injection of allogeneic lymphocytes into footpads promoted development of a subpopulation of splenic T cells with suppressor activity markedly greater than that of nonsensitized spleen cells. Furthermore, the same sensitizing procedure resulted in enhanced activity of an MLR amplifier population in regional lymph nodes. Events leading to this anatomic segregation of T-cell-mediated regulatory functions are uncertain, however, several alternatives should be considered. Triggering of inherently segregated but nonactivated suppressor and amplifier populations, with resultant enhanced manifestation of each regulatory function is possible. Intrinsic differences in the immunological functions of spleen-seeking and lymph node-seeking T cells (3, 23–25), as well as the limited regulatory activities of nonsensitized spleen vs. lymph node cells in MLR observed in our experiments, are consistent with this hypothesis. Alternatively, differential migration of regulatory subpopulations of T cells may occur following sensitization. Frost and Lance (26) have reported trapping of particulate antigen in the draining lymph nodes following foot pad injection. MLR amplifiers may be recruited from the spleen to a site of local antigen concentration thereby establishing an amplifier cell-rich population in the regional lymph node and leaving a suppressor cell-rich population in the spleen. Finally, it is possible that these diverse effects reflect opposing functions of a single regulatory population present in differing concentrations in spleen and lymph nodes. The experiments of Dutton (27),

however, suggest that different subpopulations of Con A-activated T cells enhance and suppress antibody responses to sheep erythrocytes, and we regard this last explanation as unlikely.

Spleen cells activated in the manner described are not cytotoxic toward allogeneic lymphocytes bearing the original stimulating histocompatibility markers, nor toward syngeneic target cells as measured by ^{51}Cr release. Viability assays by trypan blue exclusion of control and experimental cultures similarily reflect lack of specific or nonspecific cytotoxic effects. Generation of cytotoxic T lymphocytes subsequent to in vivo sensitization with allogeneic tumor cells has been established by Brunner et al. (28); lack of cytotoxicity in our system probably reflects differences in kinetics of generation of functionally differing subpopulations of T cells. Whereas 10–11 days are required for development of full cytotoxic capacity in a normal unirradiated host (28), maximal MLR suppressor activity was demonstrated at 4 days, and was not detected after 7 days.

Mitomycin C treatment of alloantigen-activated spleen cells prevents appreciable [^{3}H]TdR incorporation by this regulator population into total DNA synthesis in MLR. Consequently, activated suppressor cells can express their inhibitory effects in the absence of detectable proliferation. After irradiation of Con A-activated suppressor T cells, suppressor activity independent of cell proliferation has been reported for antibody synthesis by Rich and Pierce (29). Similar observations of radio-resistant suppressor T-cell activity for GVH reactivity have been reported by Gershon et al. (21). Soluble factors with inhibitory effects on cell proliferation may be secreted by activated lymphocytes (30–33). Synthesis of migration inhibitory factor by activated T cells does not require cell proliferation (34), and production of a soluble regulator of lymphocyte proliferation by activated but nondividing T cells may account for our observations.

Our data suggest that T cells activated to suppress MLR responses function nonspecifically. That is, proliferative responses were inhibited to alloantigens both syngeneic and allogeneic to the cell pools used to activate suppressor populations. Taussig (35) has similarly demonstrated that suppressor T cells for humoral immune responses express their suppressor activities nonspecifically; suppressor cells activated by a synthetic polypeptide antigen also markedly inhibited plaque-forming cell (PFC) responses to sheep erythrocytes. In the present experiments, it is possible that triggering of suppressor T-cell activity is specific, while the subsequent anti-proliferative effect may be largely nonspecific or may act via those specificities shared by the strains studied.

Kinetic analysis of MLR responses in cultures containing suppressor spleen cells indicate that apparent suppression at the peak of an MLR response is not simply the result of a change in kinetics of a proliferative response, but reflects instead a quantitative inhibition of responsiveness. It should be noted that in our microculture system the peak incorporation of [^{3}H]TdR was reached up to 24 h earlier than that observed by investigators of the kinetics of mouse MLR in tube culture systems (15).

These experiments do not identify the point during an MLR response at which suppressor cell activity is manifested. Interference with initial alloantigen

recognition or inhibition of subsequent proliferation of a responding clone of cells are possibilities which must be considered. Rich and Pierce (33) have studied effects of Con A-activated T cells on the kinetics of primary PFC responses to heterologous erythrocytes in vitro. Their data demonstrated that although suppressor T cells inhibited PFC responses only if present during the inductive phase of the response, suppression was not expressed until the later phase of exponential PFC proliferation. The development of primary cytotoxic lymphocyte responses in vitro is thought to require initial proliferation during the sensitizing MLR (35, 36), although it is probable that the T-cell population responding in MLR is different from that which expresses cytotoxicity (37, 38). Therefore, activated suppressor cell effects documented in studies of killer cell generation (8) may reflect suppression either of requisite proliferation in the sensitizing phase or of postproliferative differentiation of cytotoxic lymphocytes.

Investigation of preproliferative events associated with antigen recognition and triggering, in conjunction with further studies of proliferative processes and of the relationship of MLR responder cells to killer cells will help clarify the mechanisms responsible for these phenomena. Demonstration in this report of regulatory functions of antigen-activated T cells should facilitate the development of unifying concepts of physiologic regulation of immune responses. Further dissection of events which promote the anatomic segregation of suppressor and amplifier activities will be critical to this development.

Summary

Regulatory effects of alloantigen-activated thymus-derived lymphocytes in mixed lymphocyte reactions have been demonstrated. Mice were injected into foot pads with allogeneic spleen cells; 4 days following sensitization spleen or regional lymph node cells from these animals were treated with mitomycin C and incorporated into MLR as regulator populations syngeneic to the responder cell type. Activated spleen cells suppressed MLR responses 60–90% whereas activated lymph node cells from the same animals enhanced MLR responses. Suppression by activated spleen cells was not due to cytotoxic effects nor to altered kinetics of the proliferative response. Studies of splenic suppressor cell generation in vivo revealed peak activity four days after alloantigen stimulation with no activity demonstrable at 7 days or at later times. Suppressor cell activity was abrogated by treatment with anti-θC3H serum and complement, and was not alloantigen specific.

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