REGULATORY MECHANISMS IN CELL-MEDIATED IMMUNE RESPONSES

II. A Genetically Restricted Suppressor of Mixed Lymphocyte Reactions Released by Alloantigen-Activated Spleen Cells*

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We have previously demonstrated that spleen cells from mice injected with allogeneic cells suppress the response of syngeneic responder cells to allogeneic cells in mixed lymphocyte reactions (MLR).[1] These alloantigen-activated suppressor cells are thymus-derived (T) and act in a nonantigen-specific fashion, i.e., suppressor cells inhibit MLR responsiveness to stimulator cells both syngeneic and allogeneic to cells from the strain used to activate suppressor cell populations (1).

In certain examples of collaborative T- and bone marrow-derived (B)-cell interactions, the requisite cellular activity of T-cells may be substituted by soluble factors isolated from activated T lymphocytes or thymocytes (2, 3). Certain of these soluble mediators contain molecules encoded within the major histocompatibility complex (MHC) (4–6). In addition, several recent studies have demonstrated that optimal cooperative cellular interactions occur only when genetic restrictions determined by the MHC are fulfilled (7–11). Consequently, we examined the alloantigen-activated suppressor T-cell system for production of a soluble suppressor of MLR responses. In addition, genetic constraints imposed on regulatory T-cell factor interactions with responder cells in the development of a T-cell-mediated MLR responses were investigated.

Materials and Methods

Mice. BALB/c and DBA/2 mice were obtained from the Department of Cell Biology, Baylor College of Medicine. C57BL/6, C3H/He, A/Tex, and AKR mice were obtained from the Texas Inbred Mice Company, Houston, Texas. The *H-2* haplotypes of the strains used in these studies are detailed in Table I. Experiments were performed with 6–14-wk-old male animals.

[1] *Abbreviations used in this paper:* Con A, concanavalin A; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; MEM, minimal essential medium; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction.

TABLE I
*H-2 Haplotypes of Strains Utilized in These Studies**

Strain	Haplo-type	H-2 Complex					
			I Region				
		K	I-A‡	I-B‡	I-C	S	D
BALB/c	d	d	d	d	d	d	d
DBA/2	d	d	d	d	d	d	d
A / Tex	a	k	k	k	d	d	d
C3H/He	k	k	k	k	k	k	k
AKR	k	k	k	k	k	k	k
C57BL/6	b	b	b	b	b	b	b

* Modified from Shreffler and David (20).
‡ Previously known as *Ir-1A* and *Ir-1B*.

MLR Culture. MLR were prepared as previously described (1). Briefly, responder and stimulator cell populations were cultured in equal numbers, 1×10^6 cells of each in 0.2-ml cultures in modified Eagle's minimal essential medium (MEM) with 10% fetal calf serum (FCS) (Reheis Chemical Co., Kankakee, Ill.). Supernates were added in 0.1-ml volumes. Stimulator cells (designated throughout by subscript m) were treated before addition to MLR with mitomycin C (Sigma Chemical Company, St. Louis, Mo.). DNA synthesis in MLR was assayed by adding 1.0 μCi of tritiated thymidine ([^{3}H]TdR, sp act 2.0 Ci/mM, New England Nuclear, Boston, Mass.) to cultures for the final 18 h of a 72-h incubation period. Exceptions to this protocol are subsequently detailed.

Data from separate experiments are expressed as mean counts per minute of three to six replicate cultures with the standard error of the mean. The stimulation index (E/C) was calculated by dividing counts per minute from cultures containing stimulating cells allogeneic to the responder population by counts per minute from cultures containing syngeneic stimulating cells. Stimulation indices from grouped replicate experiments represent mean stimulation indices from 3 to 10 separate experiments. Percent MLR suppression was calculated according to the following formula:

$$\frac{[(E/C) \text{ of MLR with supernate}] - 1}{[E/C \text{ of MLR with no supernate}] - 1} \times 100 = \% \text{ control MLR response}$$

Preparations of Suppressor and Control Supernate. Suppressor supernates were produced as follows: normal BALB/c mice were injected into hind footpads with 2×10^7 C57BL/6 spleen cells. 4 days later spleens of these animals were removed, and single cell suspensions were prepared. 1×10^7 alloantigen-activated BALB/c spleen cells were cocultured with 1×10^7 mitomycin C-treated C57BL/6 spleen cells in 1-ml modified Eagle's MEM with 2% FCS. Supernates were harvested 24 h later. These supernates are hereafter referred to as suppressor supernates, with inhibitory activity attributed to suppressor factor(s). Supernates from replicate cultures were pooled, centrifuged for 10 min at 400 g, filtered through a washed 0.45 m Millipore filter (Millipore Corp., Bedford, Mass.), and stored at $-70°$C. Supernates prepared in this fashion were maximally suppressive at final dilution in MLR of 1:2 or 1:5; studies showed some supernatant preparations to retain activity to a final dilution of 1:100. Control supernates were similarly prepared from cocultures of normal BALB/c spleen cells with equal numbers of mitomycin C-treated BALB/c cells.

Treatment of Spleen Cells with Anti-Thy-1.2 Serum and Complement. AKR ascites anti-Thy-1.2 antiserum was obtained from Bionetics Laboratory Products, Kensington, Md. (Lot no. 231-20-1). A 1:10 dilution of this antiserum killed greater than 90% of C3H thymocytes; all cytotoxic activity was removed by absorption with suspensions of C3H brain cells. Pretreatment of BALB/c spleen cells with a 1:2 dilution of this anti-Thy-1.2 and complement reduced mitogenic responses to

concanavalin A (Con A) by 95%, but did not affect responses to *Escherichia coli* lipopolysaccharide. Spleen cells were treated with anti-Thy-1.2 serum and complement as previously described (1).

Results

MLR Suppression by Soluble Factor from Alloantigen-Activated Suppressor Spleen Cells. Cells from mice sensitized in vivo were cocultured with mitomycin C-treated cells of the sensitizing strain, and supernates of these cultures were tested for suppressor activity in MLR. In parallel with supernatant preparation, the direct suppressive capacity of the alloantigen-activated cells was tested by incorporating a mitomycin C-treated portion of the sensitized cells into MLR as a regulator population, as previously described (1). In all studies reported here alloantigen-activated spleen cells used for supernatant production suppressed MLR responses 60–95%. Supernates from C57BL/6-activated BALB/c cells (suppressor) reduced MLR responses by 95% compared with MLR that received no supernatant addition (Table II). In contrast, supernates from unsensitized cell cultures (control) did not affect MLR responses. Neither control nor suppressor supernates significantly affected [^{3}H]TdR incorporation in unmixed cultures with syngeneic stimulator cells. Comparison of cell viability by trypan blue exclusion in the mixed and unmixed cultures containing control or suppressor supernates showed no appreciable cytotoxic effect of the suppressor supernate. Furthermore, the absolute number of viable cells in MLR cultures containing suppressor supernate was equivalent to or greater than that in controls.

Table II (Experiment 2) illustrates in addition the absence of antigen specificity in the suppressor effect. The response of BALB/c cells to C3H stimulator cells was suppressed as effectively as was the response to C57BL/6 stimulator cells (the strain used to stimulate the production of suppressor substance). Responses of BALB/c cells to the alloantigens of A/Tex and AKR strains were similarly inhibited by suppressor supernates generated by BALB/c cells stimulated by C57BL/6 antigens (not shown).

In Vitro Production of Suppressor Factor: Requirement for Initial In Vivo Sensitization with Subsequent Specific In Vitro Restimulation. Elaboration of

TABLE II

Suppression of MLR by Soluble Factor Produced In Vitro by Alloantigen-Activated Spleen Cells

Exp.	Supernate*	Final dilution in MLR	Allogeneic stimulator cell strain	Syngeneic	Allogeneic	Stimulation index (E/C)	Control MLR response
				cpm ± SEM‡	*cpm ± SEM*		%
1	None	—	C57BL/6	946 ± 179	6,335 ± 1,310	6.70	—
	Control	1:5		808 ± 226	6,397 ± 465	7.92	121
	Suppressor	1:5		1,237 ± 408	1,771 ± 250	1.43	8
2	None	—	C3H/He	484 ± 158	1,680 ± 203	3.37	—
	Control	1:5		413 ± 84	1,402 ± 493	3.39	101
	Suppressor	1:5		382 ± 13	428 ± 85	1.12	5

* Control supernate derived from 24 h culture of BALB/c and BALB/c$_m$ spleen cells. Suppressor supernate derived from 24-h culture of BALB/c* (BALB/c mice sensitized 4 days previously by footpad injection of 2 × 10^7 C57BL/6 spleen cells) and C57BL/6$_m$ spleen cells. Supernate present at final dilution in MLR culture of 1:5.
‡ Mean cpm ± SEM for four replicate cultures.

MLR
RESPONDER: BALB/c
STIMULATOR: C57BL/6

PERCENT CONTROL MLR RESPONSE

160
140
120
100
80
60
40
20

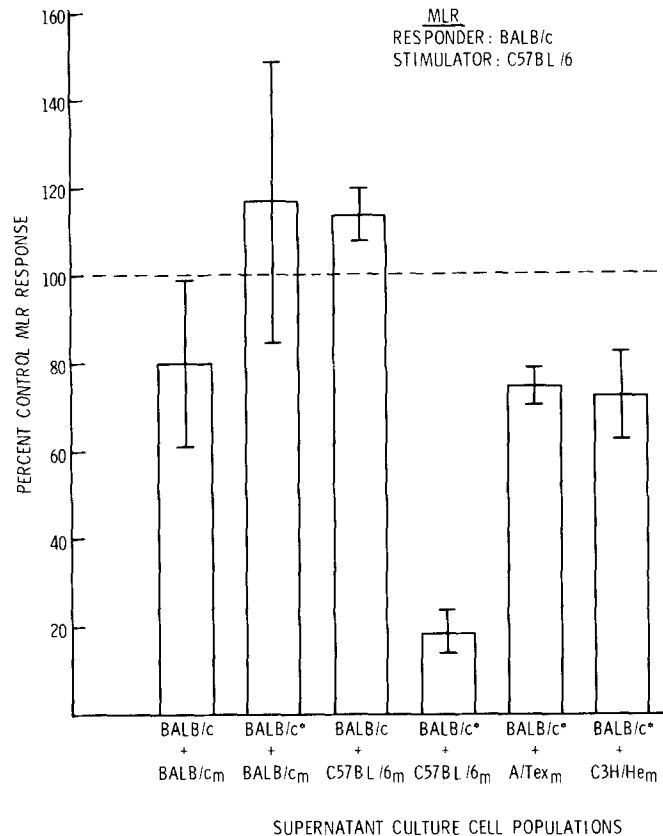| BALB/c | BALB/c* | BALB/c | BALB/c* | BALB/c* | BALB/c* |
| + | + | + | + | + | + |
| BALB/c$_m$ | BALB/c$_m$ | C57BL/6$_m$ | C57BL/6$_m$ | A/Tex$_m$ | C3H/He$_m$ |

SUPERNATANT CULTURE CELL POPULATIONS

FIG. 1. Requirement for in vivo plus in vitro alloantigen stimulation for suppressor supernatant production. Supernatant culture cell populations obtained from spleens of BALB/c mice sensitized to C57BL/6 alloantigens (designated hereafter BALB/c*) or of nonsensitized mice. Supernates obtained from cultures of various cell combinations were present in final dilution of 1:5 in MLR. Data represent mean responses ± SEM of four experiments.

soluble suppressor factor into culture supernates required both in vivo alloantigen sensitization and specific in vitro restimulation (Fig. 1). Supernates of BALB/c spleen cells sensitized only in vitro to C57BL/6 alloantigens showed no suppressive effect, but instead slightly enhanced MLR responses. Neither normal BALB/c spleen cells nor C57BL/6-activated BALB/c spleen cells, cultured with syngeneic BALB/c cells, generated supernates with suppressor activity. Furthermore, when BALB/c spleen cells sensitized to C57BL/6 alloantigens were restimulated in vitro with cells of strains other than C57BL/6, i.e. A/Tex or C3H/He, significant suppressor activity was not elicited. In addition, these latter supernates, which did not suppress MLR of BALB/c responder cells and C57BL/6 stimulator cells, also did not suppress MLR that contained A/Tex or C3H/He stimulator cells (not shown).

Characterization of MLR Inhibited by Suppressor Factor. The effect of suppressor supernate added at culture initiation on the kinetics of MLR response was determined by daily harvesting MLR cultures, containing control or

suppressor supernates, after a 6 h pulse with [³H]TdR. Cultures containing either control or suppressor supernates exhibited peak alloantigen-induced [³H]TdR incorporation on day 3, at the same time as cultures containing no supernate. However, addition of suppressor supernate reduced the peak response by more than 60%. Therefore, suppressor reflected a quantitative inhibition of MLR response rather than altered response kinetics.

It was further of interest to determine whether the suppressor supernate primarily affected initial cell interactions in the MLR or if the suppressed response represented an effect on subsequent proliferative events. Control or suppressor supernates were added to MLR cultures at varying times after culture initiation, and cultures were then assayed for alloantigen-induced [³H]TdR incorporation at 72, 96, or 120 h of incubation (Fig. 2). Maximal suppression resulted with addition of suppressor supernate at 0 h, and the degree of suppression decreased with addition of supernate at later times after culture initiation. When assayed at 96 or 120 h, cultures with suppressor supernate added at 24 or 48 h showed increasingly reduced responses. Under no conditions of suppressor supernate addition or culture assay was an enhanced proliferative response observed.

T-cell Dependence of Suppressor Factor Production In Vitro. Spleen cells from BALB/c mice sensitized to C57BL/6 alloantigens or from normal unsensitized BALB/c mice were treated with AKR anti-Thy-1.2 serum or normal AKR serum and complement before coculturing with C57BL/6 or BALB/c stimulator cells for supernatant production. Supernates were then added to MLR to assess production of suppressor factor in cultures depleted of Thy-1.2 positive cells (Table III). Characteristic suppression of MLR response was observed in cultures containing suppressor supernate produced by alloantigen-activated spleen cells pretreated with normal AKR serum and complement (Group 2). In contrast, activated cells treated with anti-Thy-1.2 serum and complement produced super-
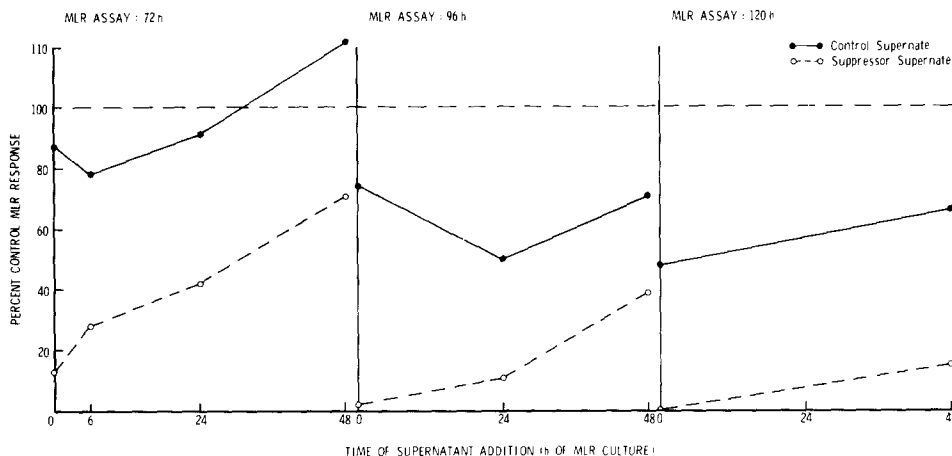

Fig. 2. Effect of suppressor supernate added after MLR culture initiation. Control (●—●) or suppressor (O– – –O) supernate was added to MLR at culture initiation or at intervals thereafter (final dilution in MLR 1:2); MLR cultures were harvested at 72, 96, or 120 h of incubation. Data represent mean responses ± SEM of two experiments.

TABLE III
T-Cell Dependence of Suppressor Factor Production in Vitro

Culture group	Supernate*	Final dilution in MLR‡	Treatment of control or suppressor cells before culture for supernate production§	Stimulation index (E/C) ± SEM	Control MLR response
					%
1	Control	1:2	NMS + C	4.13	
		1:5		5.03	—
2	Suppressor	1:2	NMS + C	1.60	19
		1:5		2.54	38
3	Control	1:2	Anti-Thy-1.2 + C	3.77	—
		1:5		5.88	—
4	Suppressor	1:2	Anti-Thy-1.2 + C	2.69	61
		1:5		6.31	109

* Control supernate derived from 24-h culture of BALB/c and BALB/c$_m$ spleen cells. Suppressor supernate derived from 24-h culture of BALB/c* (BALB/c mice sensitized four days previously by footpad injection of 2×10^7 C57BL/6 spleen cells) and C57BL/6$_m$ spleen cells.

‡ MLR consists of BALB/c responder cells and BALB/c$_m$ stimulator cells.

§ Control and suppressor cells treated with AKR anti-Thy-1.2 or normal AKR serum (NMS) plus complement, as described in Materials and Methods.

nates with little or no suppressor activity (Group 4). Treatment of control cells with anti-Thy-1.2 serum and complement had no demonstrable effect.

Activity of Soluble Suppressor Factor in MLR Responses of Cells from Various Mouse Strains. The biological activity of a soluble factor with regulatory capacities presumably requires that it interact with the antigen, with one or more of the cells participating in the induction or expression of an immune response, or with both antigen and responding cells. Such interactions might thus require that the soluble factor express some degree of specificity for the cell or antigen involved. Since our observations indicated that the suppressor supernate did not exhibit alloantigenic specificity for stimulator cells in MLR, it was important to determine whether the suppressor factor did exhibit specificity with respect to alloantigens on responder cells in the MLR. Suppressor supernates prepared by culturing activated BALB/c spleen cells with C57BL/6 cells were tested for suppressor activity in MLR with responder cells from strains BALB/c, DBA/2, A/Tex, C3H/He, AKR, and C57BL/6. A variety of stimulating cell strains were used throughout. As shown in Fig. 3, the suppressor supernate produced by activated BALB/c cells not only inhibited the response of BALB/c responder cells, but also suppressed the responses of DBA/2 and A/Tex responder cells. In contrast, however, responses of cells of strains C3H/He, AKR, and C57BL/6 were unaffected by the addition of the same supernate. The stimulator cell type was irrelevant to demonstration of suppressor activity, since C57BL/6 stimulator cell populations were participants in MLR which either were sup-
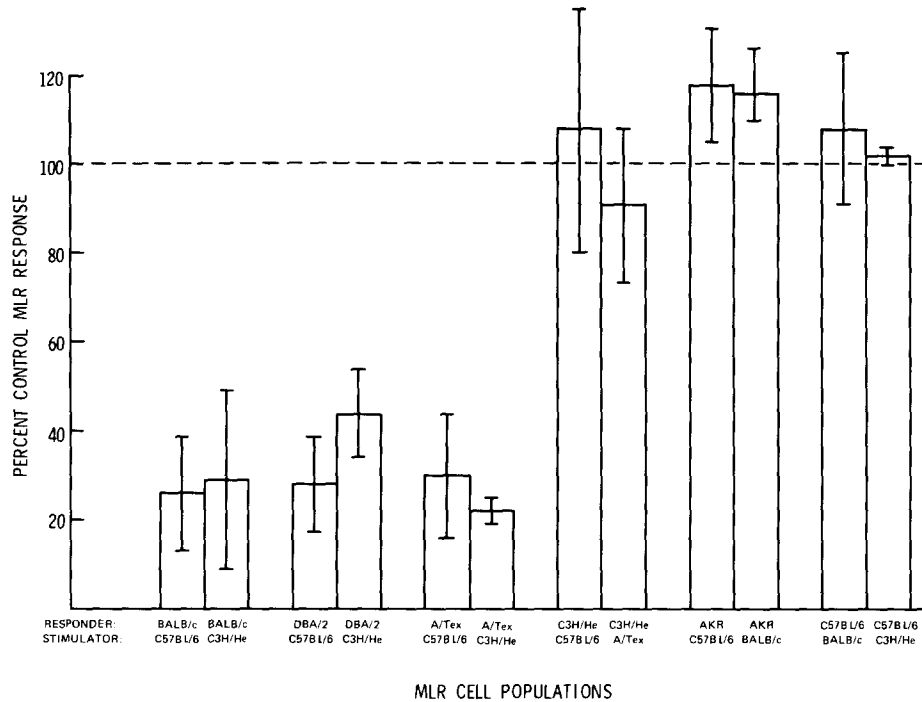
FIG. 3. Activity of suppressor supernate from activated BALB/c cells on MLR of various strains. Suppressor supernates from C57BL/6-activated BALB/c spleen cells were added to MLR of responder and stimulator cell combinations of various mouse strains (final dilution 1:5). Data represent mean responses ± SEM of five to seven experiments.

pressed (BALB/c × C57BL/6$_m$, DBA/2 × C57BL/6$_m$, A/Tex × C57BL/6$_m$) or were unaffected (C3H/He × C57BL/6$_m$, AKR × C57BL/6$_m$). In addition, cells of strains that were characteristically suppressed as responder cells (BALB/c, A/Tex) were utilized as stimulator cells in MLR which were not suppressed by the supernatant factor. Control supernates did not appreciably affect MLR responses in any of the strain combinations tested (range 83–119% of control MLR responses).

The H-2^a haplotype of strain A/Tex is a recombinant between the H-2^k and H-2^d haplotypes, represented in strains C3H/He and AKR and strains BALB/c and DBA/2, respectively. It was therefore of interest to further exploit the H-2^a haplotype in a reciprocal study of suppressor supernatant production and effect. Suppressor supernate was harvested from A/Tex spleen cells which had been activated and restimulated by C57BL/6 cells. This supernate was tested for suppressor activity in MLR with responder cells from strain BALB/c, which shares the D-end and the I-C subregion of the MHC with A/Tex, and from strain C3H/He, which shares the K-end with the H-2^a haplotype (Fig. 4). In addition to suppressing the response of A/Tex responder cells, the suppressor factor from activated A/Tex cells also inhibited the response of BALB/c responder cells. On the other hand, the proliferative response of MLR with C3H/He responder cells was not affected.
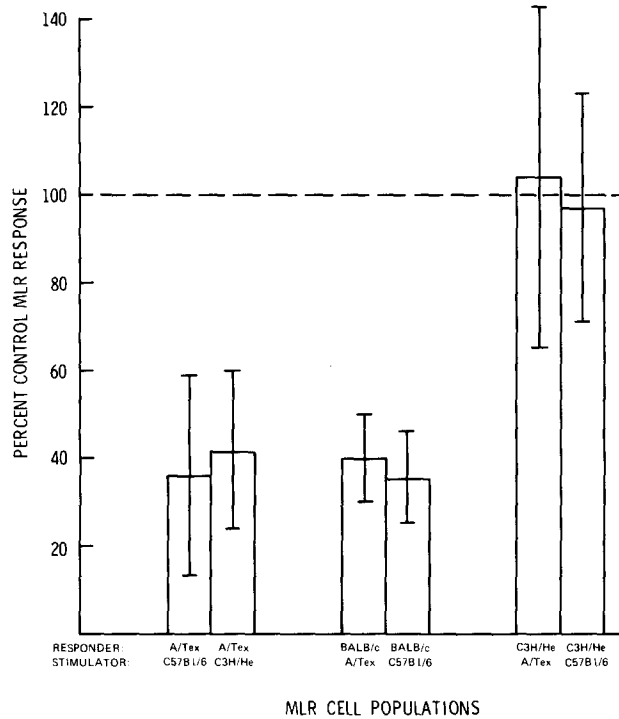
FIG. 4.   Activity of suppressor supernate from activated A/Tex cells on MLR of various strains. Suppressor supernatants from C57BL/6-activated A/Tex spleen cells were added to MLR of responder and stimulator cell combinations of various mouse strains (final dilution 1:2). Data represent mean responses ± SEM of three experiments.

Discussion

Alloantigen sensitization in vivo activates a population of T-cells in mouse spleen which suppress MLR in a noncytotoxic fashion (1). This paper demonstrates that alloantigen-activated suppressor cells elaborate a soluble noncytotoxic suppressor factor. Furthermore, this suppressor factor appears to function in a genetically restricted fashion with regard to responding cells in the MLR, while expressing no detected specificity toward the alloantigens of the stimulating cells.

Production of suppressor factor was specifically elicited by re-exposure to the original stimulating antigen. Requirement for a secondary specific challenge to permit physiologic release of suppressor factor has been indicated in reports of suppressor factors active in both T-cell (12) and B-cell (13) effector systems. Restimulation of activated or primed cells may serve as the trigger mechanism which most efficiently releases maximal quantities of soluble mediators of immune response regulation. The less physiologic mechanism of mechanical disruption may serve to release suppressor factor from primed cells which are not secondarily challenged (14).

Addition of supernates containing suppressor factor at the initiation of MLR culture resulted in a quantitative inhibition of responsiveness. When added at

later times, suppressor supernate inhibited MLR responses to an extent proportional to the length of time present in culture. However, with extended culture periods the degree of suppression increased to a level comparable to that observed when supernates were added at culture initiation. If suppression in this system were predicated upon interference with initial antigen recognition, it might be assumed that addition of the factor at 24 and 48 h of culture would be less effective. This was not the case; the data support an hypothesis that suppression results from inhibition of proliferation of a responding clone of cells subsequent to initial antigen recognition.

Production of suppressor factor was abolished by anti-Thy-1.2 serum and complement treatment of activated spleen cells before culture for supernate production. Therefore, generation of suppressor factor appears to be a T-cell dependent process. There are many examples of soluble factors released directly by T lymphocytes which influence immune responses or alter the behavior of other cells (15). Consequently, it seems probable that the alloantigen-activated T-cell is the direct source of the suppressor factor. However, it is known that peritoneal exudate macrophages can secrete soluble factors with significant modulatory effects on surrounding cells (16). It is therefore possible that suppressor factor is derived from a secondary source such as a macrophage in response to a specific T-cell signal. Thus depletion of Thy-1.2-bearing cells would remove the necessary primary cell signal with resultant abolition of suppressor factor production or release.

Our data suggest that suppressor factors liberated by activated spleen cells function in a nonantigen-specific fashion. That is, proliferative responses were inhibited to alloantigens both syngeneic and allogeneic to the strains used to activate suppressor cell populations. It is, however, possible that the specificity of the suppressor factor(s) is quite broad within the alloantigen system, acting via those specificities shared by the strains studied, or that it reflects a composite effect of multiple factors with individual specificities.

Suppression of MLR by supernates of alloantigen-activated cells was genetically restricted with regard to the responder cells in MLR. Initial studies indicated that suppressor factors produced by activated BALB/c spleen cells not only suppressed responses of BALB/c responder cells, but also responses of DBA/2 and A/Tex strains to a variety of stimulating cell types. Interestingly, however, the same supernate had no suppressive effect on C3H/He responder cells. Major non-*H-2* genetic barriers exist among the strains of mice utilized in this study. Thus it is obvious that the suppressor factor effectively operates across these barriers in selected instances. However, studies of collaborative cell-cell interactions have suggested that products of the MHC, particularly of genes located within the *I* region, play critical roles in the mediation of specific cellular collaboration (7-11). It was therefore of interest to examine the MHC of the strains involved in these studies. The strain BALB/c, in which the factor was produced, and the strain DBA/2, upon which it was effectively suppressive, share the same *H-2* complex but differ in non-*H-2* background, suggesting *H-2* directed restriction of effect. Moreover, homology for only the *D*-end of the *H-2* complex including the *I-C* subregion was sufficient as defined by suppression of strain A/Tex which shares those regions with BALB/c. In contrast, the C3H/He

strain differs from BALB/c by all regions although sharing K-end regions with strain A/Tex and was not suppressed. Additional studies indicated that strains C57BL/6 (H-2^b), which differs for the entire H-2 complex, and AKR (H-2^k), which carries the same H-2 haplotype as C3H/He, were similarly not suppressed. Consequently, in these studies the suppressor factor generated by activated BALB/c cells effectively inhibited MLR responses of responder cells sharing with BALB/c the D-end and/or I-C subregion of the H-2 complex. Responder cells which did not share these regions were not inhibited by the same suppressor factor. These data were extended by the demonstration that activated A/Tex cells, with the recombinant haplotype exhibiting I-C and D-end regions of the H-2^d haplotype, produced a factor that suppressed responder cells sharing the I-C^d, S^d, and D^d regions (BALB/c), but did not suppress C3H/He (H-2^k) responder cells, which differ in these regions but do show K-end homology with the H-2^a haplotype. Therefore, identity at regions other than I-C, S, and D is not sufficient for successful suppressive T-cell regulation.

Polymorphic genes and their products dissociated from the H-2 complex appear not to play a critical role in cooperative T-cell–B-cell interactions (7, 17). Dorf et al. (17) have demonstrated effective carrier-primed T-cell cooperation in vivo with hapten-primed B cells which are identical at the H-2 complex, but dissimilar with respect to background genotypes. In that system, for optimal T-B-cell cooperative interactions there must exist gene or gene product identities in the I region of the murine MHC (18, 19). A parallel situation appears to exist for macrophage-T-lymphocyte interactions (9). Furthermore, thymocyte or T-lymphocyte soluble factors with facilitative or suppressive functions in T-dependent antibody production are adsorbed by antisera specific for gene products coded by the I region (4, 5, 6, 14). The soluble mediators of cooperative T-B-cell interaction described above appear to contain molecules encoded by genes in the I-A and/or I-B subregions of the H-2 complex (4, 6). In addition, effective cell cooperation requires homology for critical genes which also map in the I-A and I-B subregions (17, 19).

Conversely, the suppressor factor described in this report, effective in T-T-cell regulatory interactions, would appear to be coded by the I-C subregion and/or D-end of the MHC, since suppression was demonstrable only with strain combinations homologous for these regions. Studies of suppressor factor generation and effect in congenic resistant strains of mice should further define the mapping of genes relevant to suppressor activity.

Summary

The mechanism of alloantigen-activated spleen cell suppression of mixed lymphocyte reaction (MLR) is explored in this report. Activated murine suppressor spleen cells elaborated a soluble noncytotoxic factor which suppressed MLR responses by 55–95%. Generation of suppressor factor required both in vivo alloantigen sensitization and specific in vitro restimulation. Suppressor factor was not produced by activated spleen cells which had been treated with anti-Thy-1.2 serum and complement. Antigenic specificity toward alloantigens of the stimulator cells was not demonstrable. In contrast, suppressor factor effectively inhibited MLR responses only of responder cells of those strains that shared the

D-end and the *I-C* subregion of the *H-2* complex with the cells producing suppressor factor. Therefore, active suppression appears to require an MHC-directed homology relationship between regulating and responder cells in MLR.

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