REGULATORY MECHANISMS IN CELL-MEDIATED IMMUNE RESPONSES

VII. Presence of *I-C* Subregion Determinants on Mixed Leukocyte Reaction Suppressor Factor*

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Suppressor factors generated by activated murine T lymphocytes constitute a population heterogeneous in a variety of structural and functional characteristics. Common expression of certain features has defined families of suppressor molecules and facilitated study of their integration into a broad regulatory network. Expression of major histocompatibility complex (MHC)[1]-encoded determinants or lack thereof allows structural definition of two categories of suppressor factors. An alternative classification based on properties of antigen-specificity identifies essentially coincident populations. Factors which possess a binding site for the antigen used in their generation or exhibit antigen-specific suppression also generally express determinants encoded by genes of the major histocompatibility complex (1–9). The MHC product associated with each of these suppressor factors, where specifically identified, has been exclusively a product of the *I-J* subregion (3, 4, 10), suggesting a suppressive functional commitment of that subregion as it is currently defined. In contrast, another group of factors, stimulated to suppressor function by antigens or mitogens, function in an antigen-nonspecific fashion or do not specifically bind antigen (11–13). Although less well characterized, these factors appear to lack MHC-encoded determinants (11, 12).

Previous reports from our laboratory have described a soluble factor generated by alloantigen stimulation which suppresses mixed leukocyte reaction (MLR) responses (14–16). MLR suppressor factor (MLR-SF) suppresses proliferative responses in a nonspecific fashion with regard to stimulator cell antigens (14, 16), and does not bind to cells bearing alloantigens used to stimulate its generation (15). Therefore MLR-SF appears to lack antigen specificity. However, in contrast to other antigen-nonspecific factors a role of the MHC has become evident, since MLR-SF exhibits *H-2* encoded genetic restriction in its capacity to interact with MLR responder cells and thereby effect suppression (16, 17). Therefore, we investigated the expression of *H-2* determinants by MLR-SF and subsequently, the relationship of these determinants to those expressed on other MHC-governed suppressor factors. Data contained in this report

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[1] *Abbreviations used in this paper*: B6, C57BL/6; FCS, fetal calf serum; *Lad*, lymphocyte activating determinant; GAT, terpolymer of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}; MEM, minimal essential medium; MHC, major histocompatability complex; MLR, mixed leukocyte reaction; MLR-SF, mixed leukocyte reaction suppressor factor; PBS, phosphate-buffered saline.

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establish that *H-2* gene products are requisite components of the MLR-SF complex. However, unlike other MHC-encoded suppressor factors, *I-J* determinants are not associated with MLR suppressor activity. Rather, determinants encoded by the *I-C* subregion are integral to MLR-SF inhibition of MLR proliferative responses.

Materials and Methods

Mice. BALB/c mice were obtained from the Department of Cell Biology, Baylor College of Medicine, Houston, Tex. The hybrid strain (BALB/c × CBA)F_1 was bred in our animal facility. Inbred and congenic strains C57BL/6J(B6), CBA, DBA/1, B10.BR, B10.D2, and B10.A were purchased from The Jackson Laboratory, Bar Harbor, Maine.

Alloantisera. The antisera (B10.A(4R) × B10.AM)F_1 anti-B10.A(2R), B10.S(9R) anti-B10.HTT, (B10.K × A.TL)F_1 anti-A, (C3H.OL × B10)F_1 anti-C3H and B10.A(3R) anti-B10.A(5R) were produced in the Department of Genetics, Washington University, St. Louis, Mo. Alloantisera (B10 × LP.RIII)F_1 anti-B10.A(2R) (serum designation: D-23); (B10.D2 × SJL)F_1 anti-B10.A(D-23b); (C3H.SW × B10.HTG)F_1 anti-C3H.OH(D-1b); (B10.D2 × A)F_1 anti-B10.A(5R) (D-33); (B10.A(2R) × C3H.SW)F_1 anti-C3H(D-32); (C3H.JK × HTG)F_1 anti-C3H.OH(D-5b); (B10 × A)F_1 anti-B10.D2(D-31); (B10.AKM × 129)F_1 anti-B10.A(D-4); (B10.BR × LP.RIII)F_1 anti-B10.A(2R) (D-28b); and (B10.A(5R) × LP.RIII)F_1 anti-B10(D-2) were obtained from The Jackson Laboratory through the courtesy of the Transplantation Immunology Branch, National Institutes of Allergy and Infectious Diseases, Bethesda, Md. Serum A.TH anti-A.TL was the gift of Dr. Donal Murphy of the Division of Immunology, Stanford University, Stanford, Calif.

Preparation and Use of Insoluble Immunoadsorbents. All sera were heat inactivated at 56°C for 30 min, dialyzed overnight against coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.0) and centrifuged to remove aggregate material. The dialyzed sera were coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N. J.) with the ratio of 20 mg protein to 1 ml Sepharose bed volume, by gentle rotation overnight at 4°C. Unbound material was removed by washing with coupling buffer, and remaining active groups were inactivated by reaction with 1 M ethanolamine (pH 8.0) for 2 h at room temperature. Noncovalently bound protein was removed by three washing cycles of 0.1 M acetate buffer containing 1 M NaCl (pH 4.0) followed by 0.1 M borate buffer containing 1 M NaCl (pH 8.0). Immunoadsorbent columns were prepared by packing 2.0 ml antiserum-conjugated Sepharose in 0.7 × 10 cm columns (Bio-Rad Laboratories, Richmond, Calif.). The immunoadsorbents were stored at 4°C in phosphate-buffer saline (PBS) containing 0.1% sodium azide.

Before use the columns were washed extensively with PBS, followed by a 1 h incubation at 4°C with 3 ml 3% bovine serum albumin in 0.05 M Tris-HCl buffer, pH 7.6, to block nonspecific binding sites. Excess protein was removed by washing with a minimum of 30 ml PBS. The columns were equilibrated with 10 ml supplemented MLR culture medium containing no fetal calf serum (FCS). Control or suppressor culture supernate, diluted 1:1 with serum-free culture medium, was applied to the columns in 1-ml vol and incubated at 4°C for 3 h. The supernates were eluted with serum-free culture medium and four to six fractions of 1.0–1.5 ml each were collected. Each fraction was reconstituted to 2% FCS and assayed in MLR without further dilution, equivalent to 25% final concentration in MLR. Unbound suppressor activity was routinely found in the second fraction; therefore, MLR responses with adsorbed supernates refer to the effect of fraction 2 eluates.

To recover bound suppressor material for assay, the columns were washed with 30 ml PBS to remove any remaining loosely-bound material. Glycine-HCl buffer, pH 2.8, was applied to the column and five to six 1.5-ml fractions were collected and dialyzed overnight against serum-free culture medium. Acid eluates were reconstituted to 2% FCS and assayed in MLR as above.

MLR. MLR were prepared as previously described (17). Briefly, responder and stimulator cell populations were cultured in equal numbers, 1×10^6 cells of each. Cultures were 0.2 ml in supplemented Eagle's minimal essential medium (MEM) with 10% FCS (Grand Island Biological Co., Grand Island, N. Y.) and 50 μg/ml gentamicin (Schering Corp., Kenilworth, N. J.). This culture medium is not supplemented with 2-mercaptoethanol. Stimulator cells (designated throughout by subscript m) were treated before addition of MLR with mitomycin C (Sigma Chemical Co., St. Louis, Mo.). MLR cultures were incubated in an atmosphere of 10% CO_2,

7% O_2, and 83% N_2 at 37°C. DNA synthesis in MLR was assayed by adding 1.0 μCi of tritiated thymidine (sp act 2.0 Ci/mmol; New England Nuclear, Boston, Mass.) to cultures for the final 18 h of a 72 h incubation period.

Data from individual experiments are expressed as mean counts per minute of three to four replicate cultures with the standard error of the mean. Net counts per minute (E-C) were calculated by subtracting counts per minute of cultures with syngeneic stimulating cells (C) from counts per minute of cultures with allogeneic stimulating cells (E). Percent control MLR response was calculated according to the following formula:

$$\frac{\text{(E-C) of MLR with supernate of alloantigen-stimulated cells}}{\text{(E-C) of MLR with supernate of control cells}} \times 100 = \%\ \text{control MLR response.}$$

Preparation of Suppressor and Control Supernates. Suppressor supernates were produced as previously described (17). Normal mice were injected with 2×10^7 allogeneic spleen cells into hind footpads. 4 d later alloantigen-activated spleen cells were cocultured in supplemented Eagle's MEM with 2% FCS with equal numbers of mitomycin C-treated allogeneic spleen cells of the strain used for in vivo sensitization. Supernates were harvested 24 h later. Control supernates were similarly prepared from cocultures of normal spleen cells with equal numbers of mitomycin C-treated syngeneic cells. The shorthand convention designating alloantigen-activated suppressor cells or factor consists of the name of the strain activated to suppressor activity, with the sensitizing strain following in superscript, i.e., $BALB^{B6}$ indicates BALB/c cells sensitized and restimulated with B6 cells.

Results

Expression of MHC Determinants by MLR Suppressor Factor. To determine whether MLR-SF displayed determinants encoded by the *H-2* complex, suppressor factors were produced by alloantigen stimulation of $H\text{-}2^d$ (BALB/c) or $H\text{-}2^k$ (CBA) spleen cells and passed over immunoadsorbent columns prepared with antisera against $H\text{-}2^d$ or $H\text{-}2^k$ subregion products. Residual suppressor activity after adsorption was assessed by adding column effluents to MLR cultures prepared with responder cells syngeneic to the suppressor strain. Adsorption of $H\text{-}2^d$ MLR-SF by antibody against determinants of the left hand segment of the $H\text{-}2^d$ complex, including K^d and $I\text{-}A^d$ through $I\text{-}E^d$ subregions, was ineffective (Table I). In contrast, several antisera which share specificity against $I\text{-}C^d$, S^d, and G^d subregion determinants adsorbed suppressive activity of $H\text{-}2^d$ factor. Since suppressor activity was adsorbed regardless of antiserum specificity for D^d region determinants, the essential *H-2* determinant of MLR-SF is coded within the segment containing *I-C, S,* and *G.* CBA ($H\text{-}2^k$) MLR-SF adsorption with alloantisera directed against selected subregion determinants of the $H\text{-}2^k$ complex confirmed the above results. Antisera with specificity for products of all or certain of the I^k subregions removed or significantly decreased CBA suppressor activity; adsorption was not consistent with K^k or D^k specificity. Among the adsorbing sera, (C3H.OL × B10)F_1 anti-C3H antiserum potentially included specificity against $I\text{-}C^k$ but not against *S* and *G* products. Thus, the single subregion specificity expressed by all adsorbing preparations was the *I-C* subregion.

Since suppressor factor with specificity for the terpolymer L-glutamic $acid^{60}$-L-$alanine^{30}$-L-$tyrosine^{10}$ (GAT) possesses bound antigen (2), and because another regulatory factor produced in response to alloantigen stimulation, allogeneic effect factor, has activity associated with stimulator as well as responder *I* region antigens (18), we wanted to determine whether MLR-SF also expressed stimulator cell alloantigens. BALB/c and CBA factors were passed over columns specific for $H\text{-}2^b$ antigens of the C57BL/6 strain used in factor generation (Table I). Similarly, B10.BR factor,

TABLE I

Adsorption of MLR-SF with Anti-H-2 Alloantisera

Suppressor strain‡	Sensitizing strain	Adsorption	Potential *H-2* subregion detected§	MLR Response (E-C)*					
				Control factor	Suppressor factor				
					Unadsorbed	Control MLR	Mean control MLR‖	Adsorbed	Control MLR	Mean control MLR‖
						%	%		%	%
BALB/c ($H\text{-}2^d$)	C57BL/6 ($H\text{-}2^b$)	(B10 × A)F_1 Anti-B10.D2	$K^dA^dB^dJ^dE^d$	4,884	1,204	25	39 ± 6	1,174	24	34 ± 8
		(B10.AKM × 129)F_1 Anti-B10.A	$C^dS^dG^dD^d$	5,026	1,997	40	39 ± 5	4,249	85	85 ± 6
		(B10.BR × LP.RIII)F_1 Anti-B10.A(2R)	$C^dS^dG^dD^b$	12,833	6,338	49	36 ± 6	10,892	85	87 ± 4
		(B10 × LP.RIII)F_1 Anti-B10.A(2R)	$(K^kA^kB^kJ^kE^k)C^dS^dG^d$	11,405	3,654	32	47 ± 3	9,595	84	95 ± 14
		(B10.D2 × A)F_1 Anti-B10.A(5R)	$K^bA^bB^b$	9,392	5,173	55	35 ± 5	3,593	38	21 ± 6
		(B10.A(5R) × LP.RIII)F_1 Anti-B10	$J^bE^bC^bS^bG^bD^b$	2,595	808	31	33 ± 2	1,063	41	44 ± 3
CBA ($H\text{-}2^k$)	C57BL/6 ($H\text{-}2^b$)	(B10.D2 × SJL)F_1 Anti-B10.A	$K^kA^kB^kJ^kE^k$	8,845	4,853	55	42 ± 14	4,976	56	42 ± 13
		(C3H.OL × B10)F_1 Anti-C3H	$K^kA^kB^kJ^kE^kC^k$	9,878	4,506	46	44 ± 2	8,000	81	83 ± 7
		A.TH Anti-A.TL	$A^kB^kJ^kE^kC^kS^kG^k$	7,023	3,549	51	47 ± 3	6,250	89	92 ± 11
		(B10.A(2R) × C3H.SW)F_1 Anti-C3H	$C^kS^kG^kD^k$	4,735	2,239	47	52 ± 4	3,906	82	86 ± 9
		(C3H.SW × B10.HTG)F_1 Anti-C3H.OH	D^k	9,878	4,506	46	42 ± 3	4,280	43	48 ± 4
		(C3H.JK × B10.HTG)F_1 Anti-C3H.OH	D^k	12,105	6,376	53	49 ± 8	7,305	60	50 ± 6
		(B10.D2 × A)F_1 Anti-B10.A(5R)	$K^bA^bB^b$	9,356	3,549	38	43 ± 3	4,055	43	38 ± 6
		(B10.A(5R) × LP.RIII)F_1 Anti-B10	$J^bE^bC^bS^bG^bD^b$	2,581	591	23	27 ± 4	935	36	35 ± 1
B10.BR ($H\text{-}2^k$)	BALB/c ($H\text{-}2^d$)	(B10 × A)F_1 Anti-B10.D2	$K^dA^dB^dJ^dE^d$	1,293	566	44	48 ± 4	453	35	42 ± 5
		(B10.AKM × 129)F_1 Anti-B10.A	$C^dS^dG^dD^d$	1,293	566	44	44 ± 7	331	26	29 ± 3

* MLR response was measured by determining net alloantigen-stimulated cpm (E-C) from mixed and control cultures which both contained either control or suppressor factors at 25% final concentration. Stimulation indices (E/C) in cultures containing control factor ranged from 2.38 to 7.17. Data from a representative experiment is shown for each antiserum adsorption.

‡ Suppressor factors were assayed with or without adsorption in MLR with responder cells syngeneic to suppressor strain.

§ *H-2* subregion incompatabilities in donor-recipient pairs used in antiserum production. Parentheses enclose subregion specificities not possessed by suppressor strain.

‖ Mean percent control MLR represents the mean ± SEM of all experiments performed with each antiserum (two to five experiments each).

produced in response to BALB/c stimulation, was exposed to immunoadsorbents with anti-$H\text{-}2^d$ specificity. In no instance was suppression reduced after passage over columns specific for alloantigens of the cell stimulating suppressor activity.

Absence of I-J Determinant Association with MLR-SF Activity. In several antigen-specific systems, suppressor factor activity has been uniquely associated with the *I-J* subregion (3, 4, 10). Although the preceding evidence in this report supports an important role of *I-C* encoded determinants in MLR suppression, it was, therefore, necessary to establish any additional participation of *I-J* determinants. For this purpose an immunoadsorbent column was prepared with antiserum B10.A(3R) anti-B10.A(5R), which is putatively monospecific for products of the $I\text{-}J^k$ subregion. Specificity and activity of the B10.A(3R) anti-B10.A(5R) antiserum preparation used in these experiments have been demonstrated in other studies reported by Okuda et al. (38), by its ability to specifically prevent MLR stimulation induced by T-cell-specific $I\text{-}J^k$ determinants. In addition, this particular antiserum pool was shown by Tada and coworkers to adsorb keyhole limpet hemocyanin suppressor factor activity from extracts of primed $H\text{-}2^k$ suppressor T cells (10). MLR-SF was prepared from $H\text{-}2^k$ strains CBA and B10.BR, and $H\text{-}2^{k/d}$ hybrid strain (CBA × BALB/c)F_1, and passed over the B10.A(3R) anti-B10.A(5R) immunoadsorbent. $H\text{-}2^k$ specific suppressor activity contained in each of these supernate preparations was unaffected by exposure to anti-$I\text{-}J^k$ antiserum in multiple experiments (Table II). Anti-Ia sera inhibit a variety of in vitro assays of immune function (19). Conceivably, leaching of anti-$I\text{-}J^k$ antibodies into the column effluent might induce a distinct form of suppression, thereby concealing concomitant binding of suppressor molecules to the adsorbent. To examine this possibility, control rather than suppressor factor was passed over the anti-$I\text{-}J^k$ column before addition to MLR (Table II, exp. III). Control factors thus treated did not suppress MLR responses. As an alternate approach, an immunoadsorbent was prepared with antiserum directed against a recombinant haplotype, (B10 × LP.RIII)F_1 anti-B10.A(2R), which potentially includes specificity against both $I\text{-}J^k$ determinants and $I\text{-}C^d$ determinants. MLR-SF from suppressor cells of $H\text{-}2^k$ (CBA) or $H\text{-}2^d$ (BALB/c) haplotypes were passed over the column, and residual suppression was determined in MLR with responder cells syngeneic to the respective suppressor haplotype (Table II). As previously demonstrated in Table I, suppressor activity of BALB/c MLR-SF was effectively removed, presumably via anti-$I\text{-}C^d$ specific binding. In contrast, the $H\text{-}2^k$ factor retained suppressor activity, despite exposure to anti-$I\text{-}J^k$ antibodies; binding of $H\text{-}2^d$ suppressor factor by the same immunoadsorbent suggests that the antiserum is functionally presented on the Sepharose bed and capable of binding molecules of appropriate haplotype specificity. Collectively, these results are inconsistent with functional association of *I-J* encoded determinants and MLR suppressor activity.

Expression of $H\text{-}2^d$- and $H\text{-}2^k$-Specific I-C Subregion Determinants on MLR-SF. Recent difficulties in genetic resolution of the *I-C* subregion have involved both genetic and molecular issues (20–22). Among these have been questions of the haplotype of origin of the *I-C* subregion of the $H\text{-}2^a$ haplotype, originally thought to be $H\text{-}2^d$ (23). Although recent serologic analyses have suggested derivation of this subregion from $H\text{-}2^k$ rather than $H\text{-}2^d$ (20–22), various functional analyses are consistent with expression of activities specific to $I\text{-}C^d$ rather than $I\text{-}C^k$ in immune responses of $H\text{-}2^a$ haplotype cells (16, 17, 24, 25). Therefore, we tested antisera made in reciprocal $I\text{-}C^k$/

TABLE II

Absence of I-J^k Determinants on H-2^k MLR-SF

Adsorption‡	Suppressor strain§	Responder strain	Experiment	MLR response (E-C)*						
				Control factor			Suppressor factor			
				Unadsorbed	Adsorbed	Control MLR	Unadsorbed	Control MLR	Adsorbed	Control MLR
						%		%		%
B10.A(3R) Anti-B10.A(5R) J^k	CBA^{B6}	CBA	I	4,406	—		1,822	41	1,849	42
			II	8,775	—		4,984	57	3,966	45
			III	4,045	3,842	95	2,203	54	—	
	(BALB/c × CBA)F$_1^{B6}$	CBA	I	7,784	—		4,501	58	533	7
			II	1,592	—		582	37	653	41
	B10.BR$^{BALB/c}$	B10.BR	I	1,048	—		256	24	−15	−1
			II	1,293	—		566	45	140	11
(B10 × LP.RIII)F$_1$ Anti-B10.A(2R)	CBA^{B6}	CBA		8,775	—		4,984	57	4,260	49
K^k A^k B^k J^k E^k C^d S^d G^d	BALB/c^{B6}	BALB/c		11,409	—		3,654	32	9,595	84

* MLR response was measured by determining net alloantigen-stimulated cpm (E-C) from mixed and control cultures which both contain either control or suppressor factors at 25% final concentration. Stimulation indices (E/C) in cultures containing control factors ranged from 3.74 to 5.83. Percent control MLR response calculated as described in Materials and Methods.

‡ Donor-recipient strain combinations used in antiserum production and potential *H-2* subregion specificity.

§ Allogeneic strain used to stimulate suppressor activity is denoted by superscript.

TABLE III

Expression of H-2^d and H-2^k Specific I-C Subregion Determinants on MLR-SF

Suppressor strain‡	Responder strain	Adsorption	Potential *H-2* subregion detected§	MLR Response (E-C)*				
				Control factor	Suppressor Factor			
				Unadsorbed	Unadsorbed	Control MLR	Adsorbed	Control MLR
						%		%
BALB/c^{B6}	BALB/c	(B10.A(4R) × B10.AM)F$_1$ Anti-B10.A(2R)	C^{d}S^{d}G^d	9,291	4,130	44	8,263	89
CBA^{B6}	CBA	B10.S(9R) Anti-B10.HTT	(J^s)C^{k}S^{k}G^k	10,753	5,392	50	9,505	88
B10.A$^{DBA/1}$	B10.A	(B10.A(4R) × B10.AM)F$_1$ Anti-B10.A(2R)	C^{d}S^{d}G^d	4,032	1,934	48	3,109	77
		B10.S(9R) Anti-B10.HTT	(J^s)C^{k}S^{k}G^k				1,249	31
(BALB/c × CBA)F$_1^{B6}$	BALB/c	(B10.A(4R) × B10.AM)F$_1$ Anti-B10.A(2R)	C^{d}S^{d}G^d	6,436	2,614	41	5,973	93
		B10.S(9R) Anti-B10.HTT	(J^s)C^{k}S^{k}G^k				3,603	56
	CBA	(B10.A(4R) × B10.AM)F$_1$ Anti-B10.A(2R)	C^{d}S^{d}G^d	7,992	3,115	39	3,976	50
		B10.S(9R) Anti-B10.HTT	(J^s)C^{k}S^{k}G^k				9,787	122
	BALB/c	(B10.K × A.TL)F$_1$ Anti-A	C^{d}S^{d}G^d	1,805	482	27	1,479	82
	CBA			1,592	582	37	636	40
	BALB/c	(B10.K × A.TL)F$_1$ Anti-A (Acid eluate)		2,780	659	24	(993)	(36)
	CBA			2,496	1,229	49	(2,229)	(89)

* MLR response was measured by determining net alloantigen-stimulated cpm (E-C) from mixed and control cultures which both contain either control or suppressor factors or acid eluates from adsorbent columns, at 25% final concentration. Stimulation indices (E/C) in cultures containing control factor ranged from 2.29 to 7.93. Percent control MLR is calculated as described in Materials and Methods. Shown is data from an experiment representative of 2–3 experiments performed with each suppressor factor.

‡ Allogeneic strain used to stimulate suppressor activity is designated by superscript.

§ *H-2* subregion incompatabilities in donor-recipient pairs used in antiserum production. Parentheses enclose subregion specificities not possessed by suppressor strain.

I-C^d presumed disparate combinations for their ability to specifically remove *H-2^d* or *H-2^k* MLR-SF activity (Table III).

According to previous definition of the strains involved, antiserum (B10.A(4R) × B10.AM)F$_1$ anti-B10.A(2R) potentially possesses specificity for determinants of the *I-C* subregion of B10.A(2R) which are distinct from *I-C^k* determinants found in the antiserum producer. Suppressor activity of BALB/c factor (*H-2^d*), as well as of B10.A factor, was removed by exposure to this immunoadsorbent. Antiserum detecting specificities of (*J^s*), *C^k*, *S^k*, and *G^k* subregions of B10.HTT was produced in strain B10.S(9R), which bears an *I-C^d* haplotype. CBA suppressor activity was abolished by this adsorbent. In contrast, B10.A factor retained full suppression. Thus, antiserum prepared against *I-C* determinants of *H-2^a* consistently removed suppressor factor activity associated with the *I-C^d* subregion. Since previous studies as well as observations in this report have shown that the *S* and *G* regions, as currently defined, do not appear to play a role in MLR suppressor interactions (26), the relevant determinant defined by these sera appears to be an *I-C* product.

To verify *H-2^d* and *H-2^k* specificity of the suppressor factor-antiserum interactions, suppressor factor was produced by (BALB/c × CBA)F$_1$ cells, passed over each of the antiserum preparations and residual suppressor activity tested in MLR responses of parental BALB/c and CBA responder cells (Table III). We have previously shown that such F$_1$ suppressor factors contain two distinct populations of suppressor molecules, one specific for each of the parental haplotypes (27). Unadsorbed F$_1$ factor suppressed responses of both parental strains, while not affecting response of *H-2*

unrelated B6 cells. Anti-$C^dS^dG^d$ adsorption of F_1 factor removed suppressor activity for the $H\text{-}2^d$ (BALB/c) response while suppression of the $H\text{-}2^k$ (CBA) response remained in the column effluent. Conversely, the same F_1 factor exposed to the anti-$C^kS^kG^k$ preparation lost its suppressive capacity for CBA MLR; residual F_1 suppression of BALB/c responses was unaffected. An additional antiserum with specificity for C^d, S^d, and G^d subregion products, detected by differences from the k haplotype in those regions, was produced in strain combination (B10.K × A.TL)F_1 anti-A. F_1 MLR-SF was exposed to this antiserum and residual suppressor activity determined in BALB/c and CBA MLR. In addition, haplotype specificity of the column-adsorbed suppressor product was confirmed by assay of glycine HCl (pH 2.8) column eluates in MLR of the respective parental strains. This anti-$C^dS^dG^d$ antiserum also reduced or removed suppressor activity of the adsorbed F_1 factor for BALB/c MLR responses, while failing to affect suppressor activity for CBA responses. Acid eluates, which were dialyzed before addition to fresh MLR, produced only $H\text{-}2^d$ haplotype-specific suppression, when tested against both parental strains. These data indicate that $H\text{-}2^d$ and $H\text{-}2^k$ haplotypes encode serologically as well as functionally distinct suppressor products controlled by the *I-C* subregion.

Discussion

Evidence in this report supports the conclusion that alloantigen-induced MLR suppressor factor expresses a product of the *I-C* subregion as a requisite structural component. In contrast to the class of MHC-controlled suppressor factors which are distinguished by expression of *I-J* determinants, as well as by antigen-specificity, no evidence was found for functional participation of determinants encoded by the *I-J* subregion. Thus, MLR-SF represents an immunoregulatory molecule with properties closely related to, yet genetically distinct from, the family of antigen-specific T-cell suppressive factors.

Analyses of immunoadsorption studies carried out with a variety of anti-*H-2* region and subregion sera, tested with suppressive factors derived from multiple strains of mice, are consistent with expression of an *I-C* encoded determinant. This conclusion is supported by removal of suppressor activity only by antisera produced in donor-recipient pairs in which potential incompatability existed for *I-C* region products of the haplotype relevant to the suppressor strain. $H\text{-}2^k$ suppressor activity was unaffected by antiserum (B10.D2 × SJL)F_1 × anti-B10.A, specific for the left-hand segment of the k haplotype through *I-E*, but adsorbed by (C3H.OL × B10)F_1 anti-C3H; the latter preparation differs only by the additional specificity for $I\text{-}C^k$ gene products. Thus, determinants specified by the left-hand portion of the *H-2* complex are inactive or not expressed in MLR-SF preparations. In addition, since the antiserum (C3H.OL × B10)F_1 anti-C3H lacks specificity against *S* and *G* region products, these regions are similarly unnecessary to suppression. The single anti-*H-2* subregion specificity consistently associated with effective adsorption of MLR suppressor factor activity is directed against products of the *I-C* subregion. It should be noted that certain of the antisera used in these studies also potentially contain activity against products of the *Tla* or adjacent *Qa-1* and *Qa-2* loci. However, there was no association of anti-*Tla* region specificity and adsorptive capacity.

Genetic definition of the *I-C* subregion has recently become somewhat ambiguous. Originally, Ia.7 defined the *I-C* subregion, occurring as a public specificity determined

by both $H\text{-}2^k$ and $H\text{-}2^d$ haplotypes (28), and Ia.6 was a private *I-C* specificity determined uniquely by $H\text{-}2^d$ (23). Original assignment of the *I-C* subregion in the $H\text{-}2^a$ recombinant haplotype to $H\text{-}2^d$ origin rested on demonstration of Ia.6 (23). Recent serological identification of this specificity has been problematic, allowing the possibility that the *I-C* subregion of $H\text{-}2^a$ is of $H\text{-}2^k$ origin (20–22). However, sequential immunoprecipitation studies have suggested that Ia specificity 7 be reassigned to *I-E*, and that the *I-C* subregion of $H\text{-}2^a$ is, in fact, of $H\text{-}2^d$ origin (39). In addition, the present studies provide clear serological evidence of $H\text{-}2^d$ specificity of *I-C* products of the $H\text{-}2^a$ haplotype, supporting previous functional assignment of $H\text{-}2^d$-associated MLR suppressor activity to the $H\text{-}2^a$ *I-C* subregion (16, 17). Two sera, (B10.A(4R) × B10.AM)F_1 anti-B10.A(2R) and (B10.K × A.TL)F_1 anti-A were specific for *I-C*, *S*, and *G* region determinants from an $H\text{-}2^a$ haplotype, recognized as distinct from the *k* haplotype of the recipients used in antiserum production. Both sera removed suppressor activity from $H\text{-}2^d$ as well as $H\text{-}2^a$ suppressor factors. $H\text{-}2^k$-specific suppressor activity associated with (BALB/c × CBA)F_1 suppressor factor was unaffected by these adsorbents, which simultaneously removed $H\text{-}2^d$ specific suppression of the same F_1 factor. Reciprocal analysis using a serum with $I\text{-}C^d$ anti-$I\text{-}C^k$ gene product reactivity demonstrated no adsorptive capacity for $H\text{-}2^a$ factors, while abolishing suppression of $H\text{-}2^k$ factor. These results indicate that the *I-C* subregion of $H\text{-}2^a$ is syngeneic with $H\text{-}2^d$ for genes encoding determinants possessed by MLR-SF. Mixed leukocyte reaction studies of Okuda and David (29) with purified T cells have identified a lymphocyte activating determinant (*Lad*) locus controlled by the *I-C* subregion; allelic products of this locus stimulate reciprocal MLR reactivity between $I\text{-}C^k$ and $I\text{-}C^d$ disparate cells. The antisera just discussed, which identify *I-C* determinants on MLR-SF, also inhibit the *I-C* specific MLR responses. Adsorption by purified T cell, B cell, and macrophage populations revealed that the relevant *I-C* specificities are expressed solely on T cells (29). Moreover, since these antisera also contain activity against products of *S* and *G* regions, removal of these specificities through appropriate cell adsorptions confirmed that the T cell *Lad* was indeed an *I-C* subregion product (29). Thus, the *I-C* subregion controls Lad determinants with T-cell-restricted expression, as well as determinants requisite to MLR suppressor function. There is no direct evidence that the MLR stimulating determinant is the same *I-C* gene product involved in suppression. However, both kinds of molecules are determined by the *I-C* subregion, they react with the same panel of antisera, and both represent T cell-specific activities. Further characterization will be required to determine the molecular relationship of these activities.

A separate issue addressed by these studies have been the role of the antigenic determinants stimulating MLR suppressor activity as components of the suppressor molecule. It has been previously suggested that the suppressor molecule is not functionally antigen-specific (14–16) and does not possess an antigen-binding site (15). However, it is conceivable that such a binding site could exist but be occupied by shed stimulator cell alloantigens. T cells activated in MLR bind stimulator cell H-2K and D or I alloantigens to specific cell-surface receptors (30). Therefore, it is possible in the generation of MLR-SF that in vivo alloantigen sensitization, or in vitro restimulation induces binding of stimulator cell alloantigens to suppressor cell receptors. Suppressor factor activity might then reside in shed receptor-stimulator H-2 antigen complexes. Consequently, the antigen-binding site, while present, would be

functionally unavailable to identification by the cell adsorption approach previously reported. Moreover, in another suppressor factor system it has been demonstrated through immunoadsorption studies that antigen (GAT) is a component of active suppressor factor (GAT-TsF) (2). Alternatively, it has been reported that AEF, which is produced in a manner somewhat similar to MLR-SF generation, involving allogeneic activation, derives a genetically-restricted component of its helper effect by virtue of Ia antigens obtained from the sensitizing allogeneic cell (18). It was, therefore, possible that part of MLR suppressor factor activity might be associated with sensitizing cell alloantigens. This was directly investigated by exposure of MLR-SF to antisera with specificity for sensitizing alloantigens. However, suppressor factor activity was not removed by these adsorptions, suggesting that the sensitizing alloantigens are not constituents of MLR suppressor factor, either independently or as part of a complex with alloantigens of the putative suppressor cell.

Expression of *I-C* subregion determinants on MLR-SF, as well as failure to identify a role of *I-J* encoded products, contrasts with MHC control associated with antigen-specific suppressor systems. Several groups have demonstrated that suppressor T cells (31–35) and antigen-specific suppressor factors derived from T cells (3, 4, 10) bear antigens coded by the *I-J* subregion. In vivo potentiating effects of anti-I-J sera have recently been documented which may also reflect indirectly an association of *I-J* products and immune suppression (36, 37). Determinants of the *I-J* subregion are selectively restricted to T-cell expression (31); in addition to a role in suppressor activities, these determinants also have MLR-stimulating properties when tested in a purified T cell MLR (38). Therefore, *I-J* subregion properties of restricted T-cell expression, MLR-stimulating character and participation in immune suppression are analogous to those now described for the *I-C* subregion. Gene duplication has been a hallmark characteristic of the *H-2-Tla* complex, giving rise to multiple gene products with functional as well as structural homologies. Thus, identification of distinctly mapped but functionally similar MHC-directed regulatory activities is not surprising. Differences between antigenic stimuli driving *I-J* and *I-C* associated suppressive mechanisms suggest that these are not redundant functions. The *I-C* subregion may possess unique recognitive or regulatory functions specifically involved in response toward MHC-encoded alloantigens. Quantitative or qualitative alteration of *I-C* determinant expression may be induced by allogeneic MHC stimulation; in turn, these *I-C* molecules may provide an intrinsic antigenic stimulus to a regulatory cell system characterized by receptors for self *I-C* determinants. In contrast, the *I-J* subregion suppressor molecule is distinguished by specificity for extrinsic antigen, in addition to specificity toward self *I* region determinants, suggesting a molecule more complex in its dual specificity. Continuing genetic, functional and biochemical analyses of MHC-encoded products will provide understanding of the relationship of distinct, yet related, activities such as those involved in immune regulation, as well as more broadly define the role of the MHC in immune mechanisms.

Summary

The presence of *H-2* gene products on mixed leukocyte reaction (MLR) suppressor factor was investigated by passage of MLR-suppressor factor (SF) over solid immunoadsorbents prepared with various anti-H-2 subregion sera. Antisera with specificity for all or certain *I* subregion determinants removed or significantly reduced suppressor

activity; adsorption was not consistent with *K* or *D* region specificity. The single *I* subregion specificity common to all adsorbing preparations was *I-C*. Serologic differentiation of *I-C* products of *k* and *d* haplotypes expressed on MLR-SF was established with antisera prepared in $I\text{-}C^d/I\text{-}C^k$ disparate strain combinations. These sera define allelic T cell restricted Lad determinants encoded by *I-C* genes. MLR-SF prepared from (BALB/c × CBA)F_1 mice and exposed to the $I\text{-}C^d$ and $I\text{-}C^k$ specific adsorbents demonstrated *d* and *k* haplotype specific adsorption respectively. F_1 suppressor activity adsorbed on an anti-$I\text{-}C^d$ column was eluted by glycine-HCl buffer and suppressed only BALB/c ($H\text{-}2^d$) responses. B10.A suppressor activity was removed by anti-$I\text{-}C^d$ sera, but was unaffected by anti-$I\text{-}C^k$ sera, indicating that B10.A suppressor activity is encoded by an *I-C* subregion derived from the *d* haplotype. Antisera with anti-$I\text{-}J^k$ specificity did not remove suppressor activity of various $H\text{-}2^k$ factors. Finally, adsorption with antisera directed against *H-2*-associated determinants of the allogeneic cell used to stimulate suppressor factor generation demonstrated that sensitizing alloantigens are not components of MLR suppressor factor. Thus among the major histocompatibility complex (MHC)-controlled suppressor factors, MLR suppressor factor is uniquely determined by the *I-C* subregion.

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