ANTIGEN RECOGNITION BY A T CELL CLONE OUTSIDE THE CONTEXT OF THE MAJOR HISTOCOMPATIBILITY COMPLEX

A Role for Mls in Antigen Presentation

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Functional T cell clones have been useful in defining the specificity of the T cell recognition repertoire at multiple levels (1). Their use has confirmed the importance of the major histocompatibility complex (MHC)[1] in cellular interactions involving the recognition of foreign antigens (2, 3). A number of gene products that are encoded for by loci outside the MHC region may also play a role in lymphocyte interactions. The Mls locus encodes for determinants that induce T lymphocyte proliferation and is a candidate for such involvement in the recognition of antigen.

Recently, individual T cell clones have been isolated that proliferate to both syngeneic MHC and allogeneic Mls determinants (4, 5). The nature of this Mls recognition and its function in the immune response have not been determined. The Mls locus has been mapped to chromosome 1 and is linked to the dipeptidase-1 gene (6). Gene products of two Mls alleles, Mls^a and Mls^d, generate a primary mixed lymphocyte response (MLR) of equal magnitude to that generated by MHC determinants. The two other alleles, Mls^b and Mls^c, can usually be detected only in a secondary MLR. Recognition of allogeneic Mls determinants in the MLR has been shown to occur in association with "self" MHC determinants (7, 8). The high frequency of virgin T cell-bearing receptors for Mls suggests that these determinants may play a role in T cell responsiveness.

The present study was undertaken to characterize a T cell clone selected for its ability to proliferate in the presence of syngeneic Mls^b determinants. The results presented suggest that syngeneic Mls gene products expressed on B lymphocytes and antigen-presenting cells may act in a manner similar to H-2 gene products in antigen presentation to T lymphocytes.

Materials and Methods

Mice. BALB/c, C57BL/6, CB6/F_1, CBA/J, B10.D2, and RIII, 6–8-wk-old, were purchased from The Jackson Laboratory, Bar Harbor, ME. BXD recombinant inbred

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[1] *Abbreviations used in the paper*: BUdR, bromodeoxyuridine; IL-2, interleukin 2; KLH, keyhole limpet hemocyanin; MHC, major histocompatibility complex; MLR, mixed lymphocyte response; OVA, ovalbumin; PFC, plaque-forming cell; RI, recombinant inbred; TNP, trinitrophenyl.

strains were derived from the cross between C57BL/6 (H-2^b, Mls^b) and DBA/2 (H-2^d, Mls^a). All BXD strains were supplied by Dr. Benajamin Taylor of The Jackson Laboratory. AB.Y mice were obtained from the colony of Dr. David Meruelo of the New York University School of Medicine. BALB.B and BALB.K were provided by Dr. Maurice Zuaderer, Columbia University, New York.

Antigens. Keyhole limpet hemocyanin (KLH) (Calbiochem-Behring Corp., San Diego, CA) and ovalbumin (OVA) (Sigma Chemical Co., St. Louis, MO) were conjugated separately with 2,4,6-trinitobenzene sulfonic acid (Eastman Kodak Co., Rochester, NY) in 0.83 M sodium bicarbonate buffer and then dialyzed against 0.1 M potassium bicarbonate buffer. Horse cytochrome *c* (Sigma Chemical Co.) and an isolate of type A influenza virus, PR8, were used as control antigens.

Monoclonal Antibodies. Hybridoma cell lines with defined specificities for I-A^b (25-9-3s) and I-A^d (MK-D6) were purchased from the American Type Culture Collection, Rockville, MD. Ascites of anti-Lyt-1.2 and anti-Lyt-2.2 were purchased from New England Nuclear, Boston, MA. Ascites of hybridoma 30-H12 (anti-Thy-1.2) and 10.2-16 (anti-I-A^k) were generously provided by Dr. Benvenuto Pernis, Columbia University. All anti-I-A ascites used in T cell proliferative blocking experiments were precipitated with 50% saturated ammonium sulfates and purified by ion exchange chromatography.

Preparation of T Cell Clones. Lymph node T lymphocytes were obtained from CB6/F_1 mice primed with 100 μg KLH in Freund's complete adjuvant (Difco Laboratories, Detroit, MI). The mice were injected subcutaneously in the base of the tail and in the hind foot pads. 7 d later, T cells from the periaortic, popliteal, and inguinal lymph nodes were purified on a nylon wool column (9).

The KLH-reactive T cells were further enriched by culturing the nonadherent population from the nylon wool column with 50 μg KLH/ml for 4 d in T cell medium (RPMI 1640, containing 10% fetal calf serum [Reheis Chemical Co., AZ], 12 mm Hepes, 100 μl penicillin, 100 mg/ml streptomycin, 2×10^{-3} M glutamine, and 3×10^{-5} M 2-mercaptoethanol). After 4 d of incubation, at 37°C and 7% CO_2, lymphoblasts were isolated and established as long-term T cell lines (10, 11).

Proliferation Assays. All T cell clones and subclones were tested for proliferation to a panel of soluble protein antigens and in mixed lymphocyte cultures. 7 d after subculture without antigen, 10^4 cloned T cells and 5×10^5 irradiated syngeneic or allogeneic spleen cells were cultured in 0.2 ml microtiter wells. After 3 d, the cultures were pulsed with [^{3}H]thymidine (1 μCi/well) and harvested 18 h later. No exogenous interleukin 2 (IL-2) was added to the cultures. All responses were measured in triplicate cultures. T cell clones were proven to be monoclonal by the performance of proliferation studies in the presence of bromodeoxyuridine (BUdR) and subsequent exposure to light.

Results

Several T cell clones were derived from lymph nodes of CB6/F_1 mice primed with KLH. After subcloning by limiting dilution, one subclone, A9.4, exhibited the unique property of responding to KLH in the presence of irradiated syngeneic CB6/F_1 spleen cells, as well as spleen cells from either parental strain (BALB/c and C57BL/6). This subclone has been maintained in culture for a period of 18 mo and proliferates in response to KLH, but not to control antigens cytochrome *c* or PR8 (data not shown). Subclone A9.4 was shown to express the phenotype, Thy 1.2^+, Lyt 1^+, and Lyt 2^-.

Subclone A9.4 was maintained in RPMI 1640 medium supplemented with fetal calf serum. Therefore, the proliferative response of this clone was studied to assess whether it could result from proteins contained in fetal calf serum that had become associated with MHC determinants on antigen-presenting cells. Experiments designed to examine this possibility showed that A9.4 cells proliferate in vitro upon culture with KLH and irradiated syngeneic or parental spleen

TABLE I

Proliferation of A9.4 Subclone in Culture Medium Supplemented with Serum from Different Origin

Origin of serum added to culture*	[^{3}H]Thymidine incorporation (cpm)						
	Medium	CB6F$_1$ cells‡		BALB/c cells		C57BL/6 cells	
		Nil	KLH	Nil	KLH	Nil	KLH
Fetal calf (5%)	75 ± 16§	4,073 ± 22	20,644 ± 846	3,970 ± 410	19,826 ± 589	3,944 ± 66	19,714 ± 68
Horse (5%)	66 ± 2	2,891 ± 32	14,070 ± 42	2,819 ± 140	14,086 ± 662	2,884 ± 75	14,276 ± 270
Mouse (2%)	44 ± 6	2,747 ± 33	12,975 ± 219	2,515 ± 41	12,277 ± 345	2,830 ± 48	13,348 ± 84
Human (5%)	71 ± 10	2,316 ± 24	14,560 ± 56	2,233 ± 140	12,033 ± 1058	2,189 ± 28	13,254 ± 517

* RPMI 1640 supplemented with heat-inactivated pooled serum from various sources.

‡ Subclone A9.4 (1 × 10^5 cells/culture) was stimulated with syngeneic CB6/F$_1$ or parental BALB/c, or C57BL/6 (1 × 10^6 cells/culture). 50 μg/ml KLH was added to cultures.

§ Mean ± SD of triplicate cultures.

TABLE II

Absorption of Anti-IA Antibodies with Subclone A9.4 Cultured with Syngeneic Filler Cells

Monoclonal antibody	Complement	Absorption*	Percent viable cells‡
Anti-IA^b	−	−	3 ± 1
	+	−	87 ± 5
	+	+	10 ± 5
Anti-IA^d	−	−	2 ± 1
	+	−	83 ± 5
	+	+	12 ± 5
Nil	+	−	2 ± 1

* Absorption experiments were carried out by incubation for 45 min at 37°C of 0.2 ml of antibody with 10^6 A9.4 T cell clones cultured on CB6/F$_1$ filler cells. Cb6/F$_1$ T-depleted splenic lymphocytes were incubated for 45 min at 37°C with ascitic fluid of monoclonal antibodies specific for I-A^b (1:12,000) and I-A^d (1:20,000), and rabbit complement.

‡ Cytotoxic activity of anti-Ia antibodies was measured by trypan blue dye exclusion on duplicate wells.

cells independently of the species difference in serum used to supplement the culture (Table I).

Although proliferation to KLH is a measure of the immunological activation and specificity of subclone A9.4, it does not demonstrate a relationship between the helper phenotype and helper function. The helper capacity tested in an in vitro antibody synthesis assay by incubation of KLH-primed lymph node cells, subclone A9.4, and clone F6, with highly purified B cells from trinitrophenyl (TNP)-OVA-primed CB6/F$_1$ mice in the presence of TNP-KLH.

The magnitude of direct and indirect anti-TNP plaque-forming cell (PFC) responses of B cells incubated with clone F6 was consistently higher in comparison with lymph node cells derived from KLH-primed CB6/F$_1$ mice. However, no significant helper effect was detected with subclone A9.4, tested four times during an 18 mo period (unpublished results). These results indicate that subclone A9.4, despite its ability to proliferate to KLH, is unable to exhibit helper function.

An interesting observation is the ability of this subclone to proliferate to KLH in the presence of irradiated spleen cells from either parent. To exclude the possibility that hybrid I-A determinants were involved in the genetic restriction

TABLE III

Inhibition of Subclone A9.4 Proliferative Response to Stimulator Cells with Monoclonal Anti-Ia Antibodies

Monoclonal antibodies added during the culture	Percent inhibition with A9.4 subclone cultured 6 wk on:								
	$CB6F_1$ filler cells			BALB/c filler cells			C57BL6 filler cells		
	$CB6F_1$*	BALB/c*	C57BL6*	$CB6F_1$*	BALB/c*	C57BL6*	$CB6F_1$*	BALB/c*	C57BL6*
Anti-Ia^d (MK-D6)	69.4	58.9	65.3	55.9	52.8	32.6	6.6	−6.7	12.1
	71.4	69.9	60.8						
	69.5	69.9	60.8						
Anti-Ia^b	49.7	57.7	56.4	6.8	6.6	1.1	68.8	71.1	77.1
	83.8	73.4	70.1						
	82.7	76.9	70.1						
Anti-Ia^k (10.2-16)	−0.4	6.8	−3.7	6.8	−4.2	−7.3	6.5	−2.2	−16.3
	16	21.0	−0.9						
	9.9	2.4	−0.9						

Subclone A9.4 was repeatedly cultured on syngeneic or parental filler cells for 6 wk before assaying for inhibition of proliferation to stimulator splenic cells with monoclonal anti-Ia antibodies. All cultures contained Iscove's serum-free medium supplemented with 1% affinity-purified monoclonal antibodies. Results are presented as percent inhibition of proliferation. Three separate experiments were performed on cells grown as syngeneic $CB6/F_1$ filler cells.

* Stimulating cells

to either parent, the effect of anti-Ia monoclonal antibodies on the proliferative response of this clone was studied. The results, presented in Table II, show that anti-I-A^b and anti-I-A^d monoclonal antibodies inhibited the proliferative response of subclone A9.4 to both irradiated parental spleen cells. To determine whether these results were due to Ia molecules passively absorbed by A9.4 T cells from the irradiated filler cells, the following experiment was performed. A9.4 cells were cultured for 6 wk with either $CB6/F_1$, BALB/c, or C57BL/6 irradiated spleen cells. The cells harvested from each of these cultures were used to test the effect of anti-IA monoclonal antibodies on the proliferative response to $CB6/F_1$, BALB/c, and C57BL/6 irradiated spleen cells. The results presented in Table III show that the response of subclone A9.4 cultured on BALB/c filler cells was inhibited by anti-I-A^d antibodies, but not by anti-I-A^b antibodies. Conversely, the proliferative response of this clone cultured on C57BL/6 was inhibited only by anti-I-A^b and not by anti-I-A^d antibodies. These results suggest that the inhibition of proliferation of subclone A9.4 by monoclonal anti-I-A antibodies is due to the passive absorption of Ia molecules from the filler cells onto the T cell clone.

The presence of Ia molecules bound to subclone A9.4 was further substantiated in absorption experiments. The results illustrated in Table II show that the complement-mediated cytotoxic activity of monoclonal anti-I-A^b and anti-I-A^d antibodies was drastically reduced after incubation with subclone A9.4 cultured on $CB6/F_1$. The cytotoxic activity of both monoclonal antibodies could not be absorbed by the T cell clone after it had been cultured for 7 d in IL-2 without any irradiated filler cells. These data further support the suggestion that anti-I-A inhibition is due to the passive absorption of class II molecules onto this clone. Indeed, there are recent reports that demonstrate that highly purified, antigen-specific T blasts or T cell clones (13) can passively bind Ia determinants from allogeneic or syngeneic feeder cells.

An alternative explanation of this paradoxical proliferative response is that the

TABLE IV
Mls Reactivity of Subclone A9.4

Stimulator strain	H-2	Mls	Exp. 1		Exp. 2	
			Nil	KLH	Nil	KLH
			cpm			
Nil	—	—	266 ± 91*	266 ± 97	209 ± 19	130 ± 30
$CB6F_1$	b/d	b	1,954 ± 109	4,829 ± 841	3,668 ± 630	13,820 ± 533
C57BL/6	b	b	1,107 ± 77	3,457 ± 137	3,765 ± 805	10,955 ± 777
BALB/c	d	b	2,173 ± 128	4,632 ± 416	4,768 ± 106	12,607 ± 627
BALB/B	b	b	1,770 ± 197	3,457 ± 61	ND	ND
BALB/K	k	b	1,157 ± 119	3,055 ± 364	4,481 ± 272	10,313 ± 980
BXD:1	d	b	2,021 ± 148	4,568 ± 302	4,902 ± 197	11,742 ± 1,564
2	b	a	158 ± 23	209 ± 140	511 ± 116	781 ± 123
6	d	b	2,267 ± 340	5,070 ± 138	4,413 ± 328	7,541 ± 786
8	b	a	108 ± 16	148 ± 50	593 ± 419	378 ± 126
12	d	b	1,757 ± 128	3,990 ± 97	2,941 ± 786	10,521 ± 1,445
24	d	a	127 ± 49	381 ± 106	158 ± 67	150 ± 72
29	b	a	158 ± 12	86 ± 45	248 ± 121	421 ± 55
B10.D2	d	b	925 ± 71	3,736 ± 341	ND	ND
AB.Y	b	c	245 ± 145	218 ± 196	ND	ND
RIII/J	r	a	188 ± 50	145 ± 93	ND	ND
CBA/J	k	d	ND	ND	455 ± 55	530 ± 54

Proliferation of subclone A9.4(10^4 cells/culture) to syngeneic and allogeneic splenic stimulator cells was carried out with 5×10^5 stimulator cells in Exp. 1 and 1×10^6 in Exp. 2. In both experiments, the antigen proliferation was carried out with 50 μg/ml KLH.
* Mean ± SD of triplicate cultures. ND, not done.

genetic restriction is due to molecules encoded outside the MHC. The proliferative response of this clone was therefore tested in the presence of irradiated spleen cells derived from animals of different Mls haplotypes. The rationale of this investigation was based on information indicating that BALB/c and C57BL/6 share the same silent Mls^b determinants, and that Mls gene products induce strong T cell proliferative responses, similar to the I-A gene product. Data in Table IV show that only stimulator strains which express Mls^b determinants were able to induce the proliferative response of subclone A9.4 with KLH. In particular, BALB.K ($H\text{-}2^k$, Mls^b) induced proliferation, whereas AB.Y ($H\text{-}2^b$, Mls^c) did not. This indicates that subclone A9.4 can recognize KLH in association with Mls determinants. In addition to the antigen-specific proliferation, this clone showed significant proliferation in the absence of antigen to syngeneic ($CB6/F_1$) cells, as well as to both parental cells (BALB/c and C57BL/6). This clone did not proliferate, however, in the presence of irradiated spleen cells from mice bearing $H\text{-}2^k$, $H\text{-}2^r$, $H\text{-}2^s$, $H\text{-}2^a$, $H\text{-}2^u$, or $H\text{-}2^q$ haplotypes (data not shown).

The genetic restriction of subclone A9.4 to Mls^b determinants is further supported by the results obtained with recombinant inbred (RI) BXD mice. Only the recombinant strains that possess chromosome 1 from BALB/c mice and bear Mls^b determinants were able to induce a proliferative response with or without KLH. Interestingly, the RI BXD 6 strain, exhibiting a recombination in chromosome 1 and bearing Mls^d determinants, was unable to stimulate the proliferation of A9.4 T cells. Therefore, these results suggest that this T cell clone recognizes autologous determinants encoded by the Mls^b allele as well as the foreign antigen associated with such determinants.

Discussion

The aim of this communication has been to characterize a T cell clone that recognizes antigen in the context of non-MHC determinants. Antigen recognition by this T cell clone was shown to be restricted to determinants encoded by the Mls^b allele. The frequency of precursor T cells specific for syngeneic Mls^b determinants appears to be a rare event; only one clone out of our panel of KLH-specific clones exhibited this pattern of proliferation. While class I and II MHC determinants play a major role in antigen recognition by T lymphocytes, non-MHC determinants may also be involved in regulating this important mechanism of immunological responsiveness.

Various congeneic and RI strains were used to characterize the autologous histocompatibility determinants recognized by subclone A9.4. The KLH-induced proliferation is independent of classic MHC determinants, as demonstrated by the lack of T cell activation by AB.Y ($H\text{-}2^b$, Mls^c). The results using the RI BXD strains demonstrate that non-MHC determinants were responsible for the proliferation of this clone. Four of the strains tested contain recombinations within chromosome one. Two strains, RI BXD 2 and 8, containing a recombination between the dipeptidase-1 gene of C57BL/6 and the Mls gene of DBA/2, did not induce proliferation of this clone, whereas strain RI BXD 6, which contains a reciprocal recombination between the dipeptidase-1 gene of DNA/2 and the Mls locus of C57BL/6, did induce proliferation of the T cell clone. The implication of these findings is that T cell clone A9.4 recognizes KLH in the context of syngeneic Mls gene products.

Critical differences between our KLH-Mls- and KLH-MHC-restricted T cell clones were observed. First, the clone that recognizes KLH in the context of non-MHC gene products does not provide help to TNP-OVA-primed B cells in the presence of TNP-KLH to the generation of an anti-TNP response. In contrast, T cell clones that demonstrate recognition of KLH- and MHC-encoded determinants exhibited helper activity for both IgM and IgG responses. We cannot rule out the possibility that other Mls-restricted T cell clones will be able to provide such help. Second, the A9.4 T cell subclone passively binds Ia antigens derived from irradiated filler cells, since its proliferative response to $CB6F_1$, BALB/c, and C57BL/6 stimulatory cells was inhibited with anti-Ia antibodies corresponding to filler cell haplotypes. This inhibition may be related to a steric hinderance mechanism by which anti-Ia antibodies bound to passively absorbed Ia alter the ability of the receptor of A9.4 clones to interact with Mls antigens expressed on stimulatory cells.

The mechanisms by which Ia binds to this T cell clone are unclear. Other investigators have shown that both murine and human activated T cells can bind Ia molecules (12–14). There are at least three possible mechanisms for this type of binding: (*a*) The binding of Ia molecules may be due to specific anti-Ia receptors. This is unlikely since a single clone binds to both parental haplotypes as well as the F_1 Ia antigen. (*b*) Ia molecules may bind to a nonspecific receptor for these molecules by mechanisms similar to the binding of the Fc fragments of immunoglobulin to the Fc receptor. (*c*) Ia binding may occur through complimentary binding of these molecules by the Ia molecules that are expressed on activated T cell clones. These hypotheses are currently under investigation.

MHC determinants play a major role in the differentiation and maturation of T lymphocytes. They are also involved in genetically restricted antigen recognition during the effector phases of cell-mediated immune responses. It is possible that class II MHC molecules which bind to activated T cells interact in a unique way with Mls determinants presented on antigen-presenting cells. The existence of a T cell clone restricted to syngeneic Mls determinants suggests that this locus may also play a role during the developmental stages of T lymphocyte differentiation.

Summary

A T cell clone isolated from antigen-primed CB6/F_1 mice was shown to proliferate to keyhole limpet hemocyanin (KLH) in the presence of irradiated syngeneic F_1 spleen cells, as well as spleen cells from either parental strain (BALB/c and C57BL/6). The genetic restriction involved in this antigen-specific proliferation was mapped using BXD (C57BL/6 × DBA/2) recombinant inbred strains of mice to the Mls gene on chromosome one. To exclude the role of Ia antigens as the restricting determinants, monoclonal anti-Ia antibodies were used to block the in vitro proliferative response of this clone. Although anti-Ia^b and anti-Ia^d blocked the proliferation of this clone to KLH in the presence of irradiated spleen cells from either parent, this effect was shown to be dependent on Ia molecules passively absorbed by the T cell clone from the irradiated filler cells.

Since the T clone expressed Thy-1.2 and Lyt-1^+ differentiation markers, its helper activity was compared with other KLH carrier-specific clones in an in vitro antibody synthesis assay. The Mls-KLH-restricted T cell clone, in contrast to other carrier-specific, major histocompatibility complex (MHC)-restricted T cell clones, was unable to cooperate with trinitrophenyl (TNP)-primed B cells in the presence of TNP-KLH to generate an anti-TNP response. These experiments suggest that non-MHC determinants, such as autologous Mls gene products, may play a role in genetically restricted antigen recognition by T lymphocytes.

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