Brief Definitive Report

IDENTIFICATION OF AN I-J$^+$ ANTIGEN-PRESENTING CELL REQUIRED FOR THIRD ORDER SUPPRESSOR CELL ACTIVATION*

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The ability of class II antigens to serve as restriction elements in immune cell interactions has been demonstrated in a variety of systems (1, 2). Among these molecules, the I-A and I-E subregion-encoded determinants are the best studied (3); a substantial body of experimental evidence suggests that T cell associative recognition of antigenic determinants and I-A (2) or I-E (1) -encoded molecules on antigen-presenting cells (APC) is a source of the genetic restrictions found in the generation of immunity.

Genetic restrictions in antigen-specific immune suppression have also been described (4). In T suppressor (Ts) cell-cell interactions, both Igh (5) and I-J (6) -encoded determinants have been shown to serve as restriction elements. The mechanism responsible for Igh restriction appears to involve V_H-anti V_H interactions between different T cell subsets (5). Although I-J has been detected on Ts cells in many systems (4, 6) and I-J restriction has been demonstrated in many of these systems (6), the cellular mechanism(s) responsible for this I-J restriction is unclear.

Recently, we defined an I-J-restricted event in the activation of Ts effector cells (Ts_3) in the azobenzenearsonate (ABA) system (7). Here, we report that an I-J$^+$, I-A$^-$ APC may be responsible for this restriction. Furthermore, the fact that mice pretreated with low doses of cyclophosphamide are deficient in these cells may help explain how this drug acts to inhibit Ts function in vivo.

Materials and Methods

Mice. A/J, BALB/c, and (BALB/c × A/J)F_1 (CAF_1) mice were purchased from The Jackson Laboratory, Bar Harbor, ME.

Antibody and Complement (C′) Treatment. Monoclonal anti-I-J^k antibody (WF C.12.8) from ascites fluid was the generous gift of Dr. Carl Waltenbaugh, Northwestern University. A hybridoma line producing anti-I-A^k monoclonal antibody (10.2.16) was obtained from the Salk Institute, San Diego, CA. Antibodies were purified on protein A columns and diluted appropriately before use. Erythrocyte-free spleen cells were suspended at a concentration of 10^7 cells/ml of diluted antibody. After 15 min on ice, cells were incubated for 15 min at 37°C in 10% CO_2. After washing, cells were suspended at a concentration of 10^7 cells/ml of 1:15 diluted rabbit C′ (Pel-Freez Biologicals, Rogers, AR) and incubated for 45 min at 37°C in 10% CO_2. The cells were subsequently washed extensively, coupled with the activated diazonium salt of

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TABLE I

Antigen Presentation by I-J$^+$ APC and TsF_2 are Required to Activate Ts_3

Treatment of APC*	TsF_2‡	Percent specific release					
		ABA(CA)F_1§			Uncoupled (CA)F_1		
		100:1	33:1	11:1	100:1	33:1	11:1
C′	−	60	46	40	0	6	5
C′	+	15	9	6	0	5	5
Anti I-J^k + C′	−	51	43	41	3	5	2
Anti I-J^k + C′	+	63	53	45	2	2	4
Negative control	−	5	5	4	7	6	4

* 3×10^7 APC from the spleen of (CA)F_1 mice were treated with rabbit C′ alone or monoclonal anti I-J^k (a gift of C. Waltenbaugh) plus C′. These cells were used to prime (CA)F_1 recipients.

‡ TsF_2 was administered on days 4 and 5 after priming in a total dose of 6×10^7 cell equivalents.

§ ABA-specific cytotoxicity was assayed on Con A (CA)F_1 blast cells that were coupled with ABA or left uncoupled in a standard 4-h ^{51}Cr release assay after restimulation of the primed T cells with ABA-coupled (CA)F_1 cells. Targets were Con A-induced (CA)F_1 blast cells that were coupled with ABA or left uncoupled.

arsanilic acid, and injected as described previously (8).

Immunization of Animals. Mice were immunized subcutaneously with $1–3 \times 10^7$ ABA-coupled spleen cells. This procedure has been described elsewhere (8).

Cytotoxic T Lymphocyte (CTL) Assay and Delayed-type Hypersensitivity (DTH). CTL and DTH assays have been described elsewhere (8, 9). Briefly, mice were challenged in the footpad with 30 μl 10 mM-active ABA solution 5 d after immunization. 24 h later, DTH was measured by an investigator unfamiliar with the experimental groups. 0–1 d later, 7×10^6 responders were cultured with 6×10^6 ABA-coupled x-irradiated (1,500 rad) spleen cells in 2 ml for 5 d. CTL responses were then measured by ^{51}Cr-release assay against ABA-coupled concanavalin A (Con A) blast cells in standard 4-h assay.

Induction and Administration of Second Order Suppressor Factor (TsF_2). Naïve A/J mice received 1.5×10^8 cell equivalents over 5 d of conventional or monoclonal TsF_1. TsF_2 was prepared from spleen cell freeze-thaw lysates from these mice as described previously (7). Mice receiving TsF_2 were injected with 3×10^7 cell equivalents/day on days 4 and 5.

Cyclophosphamide Treatment. Mice receiving cyclophosphamide (Cytotoxan; Mead Johnson & Co., Evansville, IN) were injected intraperitoneally with 20 mg/kg (as noted in text) 2 d before they were killed. Spleen cells were subsequently ABA coupled and injected subcutaneously into two separate dorsal sites as described previously (8).

Results and Discussion

To generate ABA-specific Ts_3 effector cells, I-J-restricted presentation of hapten is required (7). To characterize the cell (or cells) responsible for this restriction, we treated ABA-coupled APC with monoclonal anti-I-J antibody and C′ before immunization. This treatment removed APC required for Ts3 but not for CTL induction (Table I) or for DTH priming (data not shown). It should be noted that the same monoclonal antibody has been used to lyse an I-J$^+$ APC in another experimental system.[1] Because there is no representation of ABA in the immunized animal (see Table II, ref. 7), we were confident that these immunizing cells were the only source of ABA presentation for Ts3 induction in the recipient mice.

To characterize the expression of I-A encoded determinants on the I-J$^+$ APC, we adopted a slightly different protocol. Immunization with monoclonal anti-I-A^k + C′-treated ABA-coupled spleen cells fails to prime for anti-ABA DTH or CTL responses (Table II and Table III); in the absence of a positive response it is impossible to

[1] T. L. Delovitch et al. Manuscript submitted for publication.

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TABLE II

The APC Required for Activating Ts_3 May Be I-A Negative

Immunization	A/J TsF_2	Percent specific release					
		ABA-$(CA)F_1$ Con A blasts			Uncoupled $(CA)F_1$ Con A blasts		
		100:1	33:1	11:1	100:1	33:1	11:1
ABA/A/J	−	40.6	32.8	21.8	0.9	7.8	2.8
ABA/BALB/c	+	52.8	38.0	24.9	7.6	11.4	3.9
ABA-A/J + ABA/BALB/c	−	52.6	47.8	37.4	12.8	14.0	17.1
ABA/A/J + ABA/BALB/c	+	14.2	9.2	8.7	4.3	2.7	4.5
ABA-A/J (anti-I-A^k + C′) + ABA/BALB/c	+	8.1	6.9	2.4	2.1	0.8	3.3
ABA-A/J (anti-I-A^k + C′) + ABA/BALB/c	−	46.2	31.7	19.4	1.6	3.0	7.2
Negative control	−	4.6	1.1	8.3	0.6	8.6	4.1

$(CA)F_1$ were immunized with either 2×10^7 ABA-A/J subcutaneously 2×10^7 ABA/BALB/c subcutaneously, or both (in separate dorsal sites). In some groups A/J cells were treated with monoclonal anti-I-A^k (10.2.16) and C′ before ABA coupling. A/J TsF_2 was administered on days 4 and 5 after priming in a total dose of 6×10^7 cell equivalents.

TABLE III

I-J^+ APC is I-A^- and x-Ray (1,500 rad) Resistant

Immunization	A/J TsF_2	DTH	*P* values
		10^{-2} *mm*	
A/J-ABA + BALB-ABA	−	29.3 ± 3.0	—
A/J-ABA + BALB-ABA	+	19.0 ± 3.9	<0.02
(Anti-I-A^k + C′)-A/J-ABA + BALB-ABA	−	33.3 ± 1.3	NS*
(Anti-I-A^k + C′)-A/J-ABA + BALB-ABA	+	14.5 ± 3.0	<0.002
(Anti-I-A^k + C′)-A/J ABA	−	11.3 ± 2.1	<0.001

$(CA)F_1$ were immunized with 10^7 ABA/A/J and 10^7 ABA-BALB/c spleen cells that had been subjected to 1,500 rad gamma radiation. In some groups, A/J spleen cells were treated with monoclonal anti-I-A^k (10.2.16) and complement before ABA coupling. Mice were challenged in the footpad with 30 μl 10 mM-activated ABA solution 5 d after priming. DTH was measured 24 h later. *P* values were determined by Student's *t* test.

* Not significant.

measure suppression. However, we have recently shown that F_1 mice immunized with P_1 ABA-coupled spleen cells are suppressed by P_1 but not P_2 TsF_2 (7). Exploiting this, we simultaneously immunized CAF_1 mice with both anti-I-A^k + C′-treated A/J ABA-coupled spleen cells and normal BALB/c ABA-coupled spleen cells (Table II). In this way the BALB/c-ABA cells prime for CTL and DTH responses in the CAF_1 mouse, while the A/J-ABA cells prime the pre-Ts3, which can then be fully activated only by A/J TsF_2 (7). As shown in Table II, A/J TsF_2 can suppress CAF_1 mice primed with anti-I-A^k + C′-treated A/J ABA-coupled cells and BALB/c ABA-coupled cells, but not mice primed with BALB/c ABA-coupled cells alone. The effectiveness of the anti-I-A^k + C′ treatment is shown by the inability of treated cells themselves to prime CAF_1 mice (Table III). Thus the critical A/J cell needed to effect suppression in concert with A/J TsF_2 may be I-A^k negative. Although distinct from the I-A^+ APC in its expression of class II antigens, the I-J^+ APC is similar in its resistance to 1500 rad of x radiation (10) (Table III).

Because of the well-established cyclophosphamide sensitivity of the suppressor cell pathway (11), we investigated whether mice pretreated with low doses of cyclophosphamide were deficient in this I-J^+ APC. We found that normal mice immunized with ABA-coupled spleen cells obtained from cyclophosphamide pretreated donors

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TABLE IV

Cyclophosphamide Pretreated Mice are Deficient in Antigen Presentation for Suppression

A. Delayed Type Hypersensitivity

Immunization	TsF_2	Footpad swelling	Percent suppression	P value
		10^{-2} mm		
ABA spleen cells	−	22.0 ± 1.7	−	−
Negative control	−	3.6 + 1.5	100	< 0.001
ABA spleen cells	+	13.1 + 2.2	48	< 0.01
Cy* pretreated ABA spleen cells	−	21.2 ± 3.7	5	NS‡
Cy pretreated ABA spleen cells	+	22.6 ± 3.2	0	NS

B. Cytotoxicity

Immunization	TsF_2	Percent specific release of ABA target		
		100:1	33:1	11:1
ABA spleen cells	−	87.1	61.9	27.1
Negative control	−	15.8	9.7	6.5
ABA spleen cells	+	1.2	4.7	5.7
Cy-pretreated ABA spleen cells	−	97.1	66.0	41.7
Cy-pretreated ABA spleen cells	+	89.3	68.0	46.7

$(CA)F_1$ mice were immunized with either ABA-coupled normal $(CA)F_1$ spleen cells or ABA-coupled spleen cells from $(CA)F_1$ mice pretreated with 20 mg/kg cyclophosphamide 2 d before being killed. The data in Table IVA were normalized and pooled from two independent experiments; each group contained a total of eight animals. Significance was determined using the Student's t test.

* Cyclophosphamide.

‡ Not significant.

(20 mg/kg on day 2) were primed for normal CTL and DTH responses to ABA (Table IV). Thus the cell involved in the presentation of antigen for activation of Ts_3 appears to be sensitive to cyclophosphamide. This deficiency of I-J APC function in cyclophosphamide-pretreated animals might also be explained by a cyclophosphamide-sensitive event required for expression of I-J determinants. Finally, the presentation of antigen in a manner that is resistant to suppression is similar to the phenomenon reported by Britz et al. (12).

The data presented here supports the work of Nakamura et al. (13) who found that an $I\text{-}J^+$ $I\text{-}A^-$ adherent cell was required for in vitro induction of Ts effector cells. There is also evidence that a special set of APC is required to trigger mixed leukocyte response suppressor activity (Susan Rich, personal communication). Further, it appears likely that I-J-restricted events in other Ts pathways (4, 14) may be due to I-J-associated antigen presentation. Elimination of the $I\text{-}J^+$ APC may explain why free antigen, usually not immunogenic when injected intravenously, can immunize mice pretreated with cyclophosphamide (15). Collectively, these experiments indicate that antigen can be processed by two distinct APC for activation of either suppressor or immune cells. Selective manipulation of these APC subsets may permit modulation of immunity in a number of pathological processes (16–18).

Summary

We have found that an $I\text{-}J^+$ $I\text{-}A^-$ antigen-presenting cell (APC) is required for Ts_3 activation in vivo. Together with the I-J restriction previously reported for Ts_3 induction (Takaoki, M., M.-S. Sy, A. Tominaga, A. Lowy, M. Tsurifiji, B. Benacerraf, R. Finberg, and M. I. Greene, 1982, *J. Exp. Med.*, **156:**1325), it appears that this $I\text{-}J^+$ APC is responsible for I-J restriction in the triggering of Ts_3. This restriction may be

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exerted via a pre-Ts_3 associative recognition of antigen and I-J encoded determinants, analogous to the T helper recognition of antigen in the context of I-A and I-E determinants.

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