CONTACT SENSITIVITY TO AZOBENZENEARSONATE AND ITS INHIBITION AFTER INTERACTION OF SENSITIZED CELLS WITH ANTIGEN-CONJUGATED CELLS*

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When A/J mice are immunized to *p*-azobenzenearsonate (ABA)[1] coupled to a protein, such as keyhole limpet hemocyanin (KLH), 20–70% of the anti-ABA antibodies produced bear a common idiotype, known as the cross-reactive idiotype (CRI; 1, 2). CRI is absent in the serum of A/J mice immune to antigens other than ABA. In many other mouse strains, immunization with ABA-KLH results in high levels of anti-ABA antibodies but these lack the CRI, the expression of which is linked to the C_H gene cluster (3, 4). The CRI is thus a genetic marker for the V regions of immunoglobulin (Ig) of the A/J strain.

The presence of CRI has also been demonstrated on T cells, such as suppressor T cells induced by the injection of ABA-coupled syngeneic spleen cells (5) and on suppressor factors derived from these (6). Mice from strains that lacked the gene coding for the CRI also produced factors specific for ABA but devoid of CRI. The involvement of the specific V_H gene for the CRI in coding not only for antibodies with ABA specificity but also for ABA-specific suppressor T cell factors was thus inferred.

To study the existence of CRI on immune T cells, we produced contact sensitivity to ABA and examined the interaction of sensitized cells with ABA-conjugated spleen cells in vitro. Sensitization produced at least two separate T cell activities restricted by different regions of the major histocompatibility complex (MHC). One of these was H-2I restricted and responsible for the successful transfer of sensitivity to naive recipients. The other was revealed after in vitro interaction of sensitized cells with antigen-conjugated cells. It was H-2K restricted and inhibitable by soluble antigen or by anti-CRI antibody. Sensitization with ABA thus induced CRI^+ T cells able to participate in idiotype-anti-idiotype-driven regulatory mechanisms, regardless of whether T cells mediating contact sensitivity were themselves CRI^+.

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[1] *Abbreviations used in this paper:* ABA, *p*-azobenzenearsonate; ABA diazonium, diazonium salt of *p*-arsanilic acid; ABA-HGG, ABA conjugated to human gammaglobulin; ABA-KLH, ABA conjugated to keyhole limpet hemocyanin; C_H, constant portion of the immunoglobulin heavy chain; CRI, cross-reactive idiotype; DMSO, dimethylsulfoxide; DNFB, 2,4-dinitrofluorobenzene; DTH, delayed-type hypersensitivity; EBSS, Eisen's balanced salt solution; GAT, L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}; HGG, human gammaglobulin; IBC, idiotype-binding capacity; KLH, keyhole limpet hemocyanin; MHC, major histocompatibility complex; NP, 4-hydroxy-3-nitrophenyl acetyl; PBS, phosphate-buffered saline; TNP, 2,4,6-trinitrophenyl; V_H, variable portion of the immunoglobulin heavy chain.

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Materials and Methods

Mice. The inbred strains used in this study were obtained from the specific pathogen-free colony of The Walter and Eliza Hall Institute, Victoria, Australia, where strict sibling mating is maintained.

Sensitization. Mice were injected intraperitoneally with 200 mg/kg cyclophosphamide (Endoxan, Asta; Bristol Laboratories, Crows Nest, Australia) and 2 d later were painted on the clipped thorax, abdomen, and forepaws with either diazotized *p*-arsanilic acid or 4-ethoxymethylene-2-phenyl oxazolone (BDH Chemicals Ltd., Poole, England). After 7 d, they were either challenged on the ears to measure sensitivity or used as donors of sensitized cells. The ABA diazonium was prepared as described below and 0.1 ml of a 100-mM solution in dimethylsulfoxide (DMSO; Ajax, Sydney, Australia) was routinely applied. Oxazolone was dissolved to 30 mg/ml in ethanol just before use and 0.1 ml was applied.

ABA Diazonium. 1.085 g *p*-arsanilic acid (Sigma Chemical Co., St. Louis, Mo.) was mixed with 1.25 ml water and 1.25 ml concentrated hydrochloric acid (BDH Chemicals Ltd.). The mixture was warmed to dissolve the arsanilic acid and then cooled on ice. 0.38 g sodium nitrite was dissolved in 2.5 ml water and added over 10 min to the cooled arsanilic acid. For sensitization, this was diluted to 1:10 to make a 100-mM solution.

ABA-conjugated proteins. Conjugation of ABA to KLH (A-grade; Calbiochem-Behring Corp., American Hoescht Corp., San Diego, Calif.) or to human gammaglobulin (HGG; Commonwealth Serum Laboratories, Melbourne, Australia) was performed as described elsewhere (7). 1 mmol of neutralized diazonium salt was added per 1 g of protein at 10 mg/ml. Unreacted arsanilic acid and derivatives were removed by dialysis.

ABA-coupled Cells. Spleen cells were prepared by pressing cells from the spleen through a stainless steel mesh and washing them in Eisen's balanced salt solution (EBSS). To remove erythrocytes, cells were suspended to 5×10^7 leukocytes/ml in EBSS and an equal volume of water was added with stirring. Osmolarity was immediately restored by adding 3 vol of 3.5% saline. The cells were washed and suspended at 10^8/ml in EBSS at 37°C and incubated with 0.01 mg/ml DNase (beef pancreatic type I; Sigma Chemical Co.). ABA diazonium prepared as above was neutralized with 4 M sodium hydroxide (BDH Chemicals, Ltd.) and made to 500 mM with EBSS; 200 μl was added to each 10-ml cell suspension and the mixture was shaken for 3 min. The suspension was then diluted in 10-fold excess of cold EBSS and the cells were washed three times. This procedure uses a lower pH than generally reported (5, 6). The efficacy of the antigen-presenting function of the cells was, however, demonstrated by their ability to induce sensitivity and stimulate in vitro proliferation of ABA-sensitized cells.

Oxazolone-coupled Cells. 10 ml EBSS was stirred at room temperature while 0.25 ml of 10 mg/ml oxazolone in ethanol was added to it through a 27-gauge needle. 10 ml of spleen leukocytes prepared as above, at a concentration of 5×10^7/ml in EBSS was then added and the mixture was incubated for 20 min at room temperature before washing in EBSS.

Trinitrophenyl (TNP)-coupled Cells. Spleen leukocytes, prepared as above, were suspended at room temperature to 5×10^7 cells/ml EBSS, with 3 mg/ml neutralized trinitrobenzenesulfonic acid (BDH Chemicals Ltd.). The mixture was left for 10 min before washing 3 times in EBSS.

Measurement of Delayed-Type Hypersensitivity (DTH). Mice were challenged by injecting 10 μg of 1 mg/ml ABA-KLH intradermally into the left ear or by painting the ear with either 10 μl of 100 mM ABA diazonium in DMSO or 5 mg/ml oxazolone in a 50:50 vol/vol acetone/di-n-butylphthalate mixture (BDH Chemicals Ltd.). After 24 h, the thickness of the left and right ears was measured with an engineer's micrometer and the difference was taken as the amount of swelling caused by challenge.

Anti-Thy-1.2 Treatment. Cells were suspended at 5×10^7/ml in a 1:10 dilution of AKR anti-C3H (anti-Thy-1.2) serum in EBSS on ice for 30 min, then washed in EBSS and resuspended in warm guinea pig complement in EBSS at 5×10^7 cells/ml. After 30 min at 37°C, the cells were washed.

CBA Anti-C57BL Serum. The serum was a generous gift from Ms. J. Gamble, The Walter and Eliza Hall Institute. Cells were treated with this serum in the same way as with anti-Thy-1.2 serum.

Nylon Wool Filtration. Cells were passed through nylon wool columns according to the method of Cantor and Simpson (8). Control preparations were incubated and centrifuged at

the same time. Cell samples recovered after passage were checked for sensitivity to anti-Thy-1.2 and complement.

Preparation and Transfer of Sensitized Cells. Mice were sensitized as above to ABA or oxazolone. After 7 d, the axillary, subcapsular, and inguinal lymph nodes were pooled with the spleen and cell suspensions prepared by passage through a stainless steel sieve in EBSS. Clumps were allowed to settle by incubating on ice for 2 min and the cells were washed twice in EBSS. For transfer they were injected intravenously into naive mice and the mice were challenged on the ear with antigen within 30 min.

Incubation of Sensitized Cells on Monolayers of Antigen-coupled Cells. The method of Stulting and Burke (9) was used to prepare monolayers of cells on dishes. 60-mm tissue culture grade polystyrene petri dishes (Kayline, Thebarton, South Australia) were washed with phosphate-buffered saline (PBS) and then incubated for 1 h with 2 ml of 50 μg/ml poly-L-lysine in PBS (type 1-B, 400,000 mol wt; Sigma Chemical Co.). The dishes were then washed by adding PBS. Antigen-coupled spleen cells, washed twice in PBS, were added so that each dish received 10^7 cells in 2 ml PBS. After incubating for 30 min at room temperature, the dishes were washed gently with PBS to remove nonadherent cells. Sensitized cells, depleted of erythrocytes as described above, were suspended at 5×10^6 cells/ml in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum. 2 ml of cell suspension was added to each dish and incubated at 37°C in 5% CO_2 for 3 h. Nonattached cells were recovered by gentle swirling and, when required, attached cells were recovered by vigorous pipetting. The cells were washed twice in EBSS.

Preparation and Characterization of Anti-CRI Serum. Anti-CRI serum was prepared and characterized as described by Walker and Morahan.[2] Briefly, affinity-purified anti-ABA antibodies from hyperimmune A/J mice were injected into rabbits and the resulting rabbit antiserum was exhaustively absorbed with preimmune A/J immunoglobulin Sepharose to render it CRI specific. This reagent is referred to simply as anti-CRI serum in the text. The specificity of the antiserum was tested in a liquid-phase competitive radioimmunoassay using a subsaturating concentration of anti-CRI serum to precipitate ^{125}I-anti-ABA immunoglobulin. Addition of preimmune A/J serum to the system resulted in only minimal competition (<10%) even at the highest concentration tested (1:10), whereas anti-ABA antiserum competed at least 1,000-fold more effectively. The anti-CRI reagent reacted with anti-ABA antisera from A/J mice (107% inhibition with a dilution of 1:200) but not with anti-ABA antisera from B10.A (5R) or C3H.He strains (9% and 15% inhibition, respectively, at a dilution of 1:20). These data are highly compatible with those of Nisonoff's group and confirm the restricted strain distribution of this particular idiotype (4).

The idiotype-binding capacity (IBC) of staphylococcus A protein-purified anti-CRI was determined by liquid-phase radioimmunoassay using a monoclonal anti-ABA Ig (7.1.3) as a radioactive tracer. This antibody was shown to express the entire CRI.[2] In brief, the exact specific activity of ^{125}I-7.1.3 Ig was determined as 3.7×10^3 cpm/ng using a competitive radioimmunoassay with ^{125}I-7.1.3 Ig in excess over anti-CRI and unlabelled 7.1.3 Ig as inhibitor. Increasing amounts of ^{125}I-7.1.3 Ig were then incubated with a fixed amount of either anti-CRI IgG or an equal amount of nonimmune rabbit IgG (to control for nonspecific binding). With saturating levels of ^{125}I-7.1.3 Ig, 500 ng of anti-CRI IgG was found to bind 6.5×10^3 cpm (or 1.8 ng) or 7.1.3 Ig. The IBC was therefore 1 μg of 7.1.3/280 μg of anti-CRI.

Results

Hapten-specific Contact Sensitivity to ABA Diazonium. Sensitization of mice to ABA-tyrosine or ABA-protein, using protocols similar to those used in guinea pigs, has proved difficult (10). ABA-DTH has, however, been readily induced by ABA-conjugated spleen cells given subcutaneously (11). Although reliable, this method requires that ABA-coupled spleen cells from at least one mouse are needed to sensitize a single

[2] Walker, I. D., and G. Morahan. Monoclonal anti-azobenzenearsonate antibodies expressing the cross-reactive idiotype: immunochemical studies show that all idiotypic determinants reside on a single molecule. *Scand. J. Immunol.* In press.

recipient. We therefore attempted to produce contact sensitivity to ABA by skin painting with ABA in DMSO. Initial experiments were performed in CBA mice. They were injected with cyclophosphamide and skin painted with ABA diazonium dissolved in DMSO. Doses used ranged from 1 to 20 μM. After 7 d, the ears were challenged by painting with ABA diazonium in DMSO or by injecting ABA-KLH intradermally. Mice sensitized with doses of 2.5 μM or more ABA responded to both the sensitizer and to ABA on the foreign carrier, KLH (Table I). Sensitivity was thus hapten specific.

The sensitivity could be transferred to naive mice by injecting a mixture of spleen and peripheral lymph node cells taken from donors 7 d after sensitization. The transfer could be abrogated by treating the cells with anti-Thy-1.2 serum and complement, and it could be mediated by cells passed through nylon wool, 94% of which could be killed by anti-Thy-1.2 serum and complement (Table II). The ABA diazonium must thus have activated a population of T cells that mediated contact sensitivity to the ABA determinant.

Transfer of ABA Contact Sensitivity Is Restricted by H-2I. Transfer of DTH to protein antigens was shown to be restricted by the I-A region of the MHC, whereas transfer of contact sensitivity to DNFB was restricted by H-2K, H-2D, or H-2I (12). To

TABLE I

Hapten-specific Contact Sensitivity Induced by ABA Diazonium

ABA diazonium painted on each CBA mouse	Ear increment (10^{-2} mm) 6 d after challenge with	
	1 μM ABA diazonium	10 μg ABA-KLH
μM		
20.0	20.2 (6.2)	15.3 (3.5)
10.0	27.6 (4.3)	15.5 (1.8)
5.0	15.1 (5.7)	13.9 (1.2)
2.5	13.1 (6.5)	9.7 (2.2)
1.0	3.5 (2.5)	5.7 (1.2)
None	6.0 (1.7)	6.2 (2.1)

Five mice/group; arithmetic mean (SD).

TABLE II

T Cell Dependence of Transfer of Sensitivity to ABA

Number of cells transferred* ($\times 10^{-6}$)	Ear increment (10^{-2} mm) 24 h after transfer of cells pretreated as follows‡			
	Complement only	Anti-Thy-1.2 serum and complement§	Incubation control for nylon filtration	Nylon wool filtration‖
90	9.6 (1.8)	3.7 (1.7)	14.6 (2.6)	—
50	8.4 (0.8)	2.8 (1.5)	9.7 (4.0)	12.2 (2.0)
25	8.8 (1.0)	3.4 (1.2)	—	12.5 (0.8)

* Spleen and lymph node cells from mice sensitized to ABA in DMSO 6 d earlier.

‡ Five mice/group; arithmetic means (SD). Value in naive mice not given cells was 4.4 (2.0).

§ All these values are significantly different from those in recipients of cells treated with complement only ($P < 0.001$).

‖ 94% of cells filtered were killed by anti-Thy-1.2 and complement.

determine which region of the MHC restricts transfer of sensitivity to ABA, mice were immunized as above (see Tables I and II) and cells were transferred to appropriate recipients, which were then challenged with ABA diazonium. Transfer was accomplished only when donors and recipients shared H-2I, not when they shared the H-2K or H-2D regions alone (Table III). This result is therefore unlike that previously obtained with contact sensitivity to DNFB. A re-examination of the MHC regions restricting transfer of sensitivity to DNFB did, however, confirm the original results (data not shown).

Preincubation of ABA-sensitized Cells with ABA-conjugated Spleen Cells Inhibits Transfer of Sensitivity. Previous work in this laboratory (13) has shown that antigen-specific suppressor T cells could be enriched considerably by incubation on antigen-coated dishes. In view of the MHC restricitions imposed on DTH T cells, it is possible that enrichment could be achieved by incubation, not on antigen-coated dishes, but on antigen-conjugated cells of appropriate genotypes. To investigate this, ABA-sensitized cells were obtained from mice as in the experiments on transfer of sensitivity. Before transfer, however, the cells were incubated at 37°C on dishes with a monolayer of ABA-conjugated spleen cells, using poly-L-lysine as an adhesive. Cells in the supernatant fluid (nonattached cells) were removed after 3 h by gentle swirling and cells from the bottom (attached cells) were removed by vigorous washing. Approximately equal numbers of nonattached cells from the supernate and attached cells from the bottom were recovered, and 10^7 cells from each population were injected intravenously into each mouse. It should be noted, however, that the attached population consisted of antigen-coupled cells as well as some sensitized cells adhering to the monolayer. For comparison, ABA-sensitive cells were incubated on dishes coated with oxazolone-coupled spleen cells. The ABA-sensitive cells recovered from either the supernate or from the bottom of dishes coated with ABA-spleen cells had a much reduced ability to transfer ABA-sensitivity as compared with nonattached cells obtained from dishes coated with oxazolone-coupled spleen cells (Table IV). Because the cells obtained from the bottom of the dishes contained both antigen-coupled cells and some cells from the sensitized mice, it was necessary to eliminate the antigen-coupled cells before

TABLE III

Transfer of Contact Sensitivity to ABA Is H-2I Restricted

Donor strain*	Recipient strain‡	Ear increment (10^{-2} mm)§
A.SW (ssssssss)	A.TL (*s*kkkkkkd)	4.4 (2.8)‖
A.SW	A.TH (*sssssss*d)	13.2 (1.0)
B10.A(3R) (bbbbbddd)	B10 (*bbbbb*bbb)	20.0 (3.6)
B10.A(3R)	B10.D2 (ddddd*ddd*)	8.2 (6.2)‖
B10.A(3R)	B10.BR (kkkkkkkk)	2.7 (0.6)‖
CBA (kkkkkkkk)	A.TL (*s*kkkkkkd)	19.0 (1.8)
CBA	C3H.SW (bbbbbbbb)	3.9 (2.0)‖
CBA	CBA (*kkkkkkkk*)	17.3 (3.6)

* 5×10^7 cells were transferred. MHC regions indicated are: K, I-A, I-B, I-J, I-E, I-C, S, and D.

‡ Letters in italics indicate regions of compatibility between donor and recipient mice.

§ Arithmetic mean (SD). Mean value in nonsensitized mice not receiving cells ranged from 2.2 (1.4) to 4.8 (0.8).

‖ These values are not significantly different from those in corresponding nonsensitized mice ($P > 0.05$).

TABLE IV

Inhibition of Sensitivity Transfer after 3 h Incubation of ABA-sensitized CBA Cells on Plates Coated with Antigen-coupled Spleen Cells

Group	Antigen-coupled cells		Cells transferred*	Ear increment (10^{-2} mm) after ABA challenge‡
	Strain	Antigen		
a	CBA	Oxazolone	Attached	3.7 (0.7)
b	CBA	Oxazolone	Nonattached	12.3 (0.7)
c	CBA	ABA	Attached	4.7 (1.4)
d	CBA	ABA	Nonattached	3.6 (1.4)
e	None	None	None	3.8 (1.2)
f	(CBA × C57BL)F_1	Oxazolone	Attached	3.3 (1.3)
g	(CBA × C57BL)F_1	Oxazolone	Nonattached	12.1 (3.7)
h	(CBA × C57BL)F_1	ABA	Attached	1.6 (1.2)
i	(CBA × C57BL)F_1	ABA	Nonattached	5.9 (1.4)
j	(CBA × C57BL)F_1	ABA	Attached; treated with CBA anti-C57BL serum and complement	2.8 (0.9)
k	None	None	None	2.0 (1.0)

* Attached cells include antigen-coupled cells and some adhering sensitized cells. 10^7 cells were transferred intravenously except in (j), where 2×10^6 cells were injected because the antigen-coupled cells were killed.

‡ Five mice/group; arithmetic mean (SD). *P* values: (b) (d), <0.001; (g) (h), <0.001; (g) (i), <0.01; (g) (j), <0.001.

transfer, as these might in some way have interfered with the expression of the sensitivity after transfer. To do this, (CBA × C57BL)F_1 mice provided cells for antigen coupling and CBA mice provided the ABA-sensitized cells. Before transfer, the attached cells, washed off the bottom of plates, were treated with CBA anti-C57BL antiserum and complement to lyse the ABA-conjugated cells. After this treatment, however, the attached cells still did not transfer sensitivity (Table IV). Several possible reasons can be offered to explain the inability of sensitized cells in this system to transfer sensitivity. For instance, they could have become attached to the antigen-coupled cells, but their function could not be demonstrated because of some inhibition associated with their interaction with the coupled cells, an inhibition initiated either in vitro or in vivo. The experiments with parental sensitized cells and F_1 monolayers provided a means of removing the antigen-coupled cells before injection but introduced the additional complication of a possible allogeneic effect. Another possibility is the activation of some suppressor cells present in the sensitized cell population that are able to inhibit the transfer of DTH. The following experiments provide some support for such a suppressor effect.

Interaction between ABA-sensitized Cells and ABA-conjugated Spleen Cells In Vitro Induces a Nonspecific Suppressive Effect. To test for a suppressive effect, experiments were performed in which oxazolone-sensitized cells were mixed with ABA-sensitized cells and incubated on a monolayer of ABA-coupled cells. After 3 h, the nonattached cells in the supernatant fluids were tested for their ability to transfer sensitivity to either ABA or oxazolone. The results in Table V indicate that an antigen-specific interaction is required to inactivate the sensitive cells, but once initiated, a nonspecific inhibition occurs. Thus, for example, ABA-sensitive cells were inactivated after incubation with

TABLE V

*Specificity of Inhibition of Sensitivity Produced after Incubation of ABA-sensitized Cells on ABA-conjugated Cells**

Group	Antigen to which CBA cells were sensitized	Antigen-coupled cells	Antigen challenge	Ear increment (10^{-2} mm)‡
a	None	None	ABA	4.9 (1.1)
b	ABA	Oxazolone	ABA	14.2 (3.0)
c	ABA	ABA	ABA	8.6 (1.8)
d	ABA + oxazolone§	ABA	ABA	5.5 (2.9)
e	None	None	Oxazolone	3.3 (1.8)
f	Oxazolone	Oxazolone	Oxazolone	1.7 (1.5)
g	Oxazolone	ABA	Oxazolone	6.4 (1.4)
h	Oxazolone + ABA§	ABA	Oxazolone	3.4 (0.9)

* 10^7 cells were transferred after in vitro incubation at 37°C for 3 h, except in (d) and (h), where 2×10^7 cells were transferred.

‡ Five mice/group: arithmetic mean (SD). *P* values: (c) (b), <0.01; (d) (b), <0.01; (f) (g), <0.01; (g) (h), <0.01.

§ A mixture of 10^7 cells from ABA-sensitized mice and 10^7 cells from oxazolone-sensitized mice was incubated and transferred.

ABA-coupled cells, not with oxazolone-coupled cells. Furthermore, when both ABA-sensitive and oxazolone-sensitive cells were incubated with ABA-coupled spleen cells, sensitivity to both ABA and oxazolone was inhibited. After an antigen-specific interaction, therefore, a nonspecific suppressive effect is observed.

H-2K Restricts the Induction of Suppressive Activity That Follows the Interaction of ABA-sensitive Cells and ABA-coupled Cells. Because the transfer of sensitivity to ABA is H-2I restricted, it was of interest to determine whether an MHC region might be involved in governing the suppressive effect obtained after the interaction of ABA-sensitized cells and ABA-coupled cells. ABA-sensitive cells were thus incubated in dishes coated with ABA-conjugated spleen cells of various genotypes. After 3 h, the nonattached cells in the supernatant fluids were transferred to recipients syngeneic to the sensitized cell donors and ABA sensitivity was tested. The inhibitory phenomenon was observed only when the ABA-sensitive cells and the ABA-coupled spleen cells shared H-2K; the sharing of H-2I alone, or of H-2D alone, did not provoke inhibition (Table VI). These results suggest that the cells responsible for the inhibition are H-2K restricted and are thus likely to be different from the T cells responsible for the transfer of ABA-sensitivity, as these are H-2I restricted.

T Cells Mediate the Nonspecific Suppression Induced by the Interaction of ABA-sensitive Cells and ABA-coupled Spleen Cells. The restriction imposed by the MHC on the cells mediating the suppression of the sensitivity indicates that T cells may be responsible. Two types of experiments did show involvement of T cells. First, ABA-sensitive cells were passed through nylon wool and >90% of a sample of the cells obtained was sensitive to anti-Thy-1.2 serum and complement. This enriched T cell population could transfer sensitivity to ABA but this was severely diminished after incubation with ABA-coupled spleen cells (Table VII). Second, ABA-sensitive cells were mixed with oxazolone-sensitive cells, incubated on plates coated with ABA-coupled cells, and tested for their ability to transfer sensitivity. The ABA-sensitive cells prevented the ability of the oxazolone-sensitive cells to transfer sensitivity to oxazolone, but this

TABLE VI

MHC Restriction of Suppression of Sensitivity Resulting from Interaction of Sensitized Cells and Antigen-coupled Cells

Group	Strain providing ABA-sensitized cells	Strain providing antigen-coupled cells	Ear increment (10^{-2} mm)*
a	None	None	4.3 (1.0)
b	A/J (kkkkkddd)‡	Uncoupled A/J	10.1 (0.1)
c	A/J	ABA-coupled A/J	4.8 (0.9)
d	A/J	ABA-coupled BALB/c (ddddd*ddd*)§	13.0 (1.8)
e	A/J	ABA-coupled A.TL (s*kkkkkkd*)	10.5 (2.8)
f	A.TL (skkkkkkd)	Uncoupled A.TL	15.4 (1.8)
g	A.TL	ABA-coupled A.TL	8.1 (2.0)
h	A.TL	ABA-coupled CBA (*kkkkkkk*k)	15.3 (3.2)
i	A.TL	ABA-coupled A.SW (*s*sssssss)	5.8 (2.1)
j	None	None	6.6 (1.0)

* Five mice/group syngeneic with donors of sensitized cells; arithmetic mean (SD). *P* values: (c) (b), <0.001; (g) (f), <0.001; (i) (f), <0.001.

‡ MHC regions indicated are K, I-A, I-B, I-J, I-E, I-C, S, and D.

§ Letters in italics indicate regions of compatibility between donors of sensitized cells and donors of antigen-coupled cells.

TABLE VII

T Cells Are Involved in the Suppression Following Interaction of Sensitized Cells and Antigen-coupled Cells

Group	Sensitized cells*	Antigen-coupled cells	Antigen challenge	Ear increment (10^{-2} mm)‡
a	None	None	ABA	6.3 (2.0)
b	ABA, nylon filtered	Oxazolone	ABA	22.4 (2.9)
c	ABA, nylon filtered	ABA	ABA	13.2 (3.1)
d	None	None	Oxazolone	4.8 (2.3)
e	Oxazolone + unsensitized§	ABA	Oxazolone	9.1 (0.6)
f	Oxazolone + ABA treated with complement§	ABA	Oxazolone	5.4 (0.9)
g	Oxazolone + ABA treated with anti-Thy-1.2 and complement§	ABA	Oxazolone	7.8 (0.6)

* 10^7 cells were transferred after in vitro incubation as shown.

‡ Five mice/group; arithmetic mean (SD). *P* values: (b) (c), <0.01; (e) (f), <0.001; (f) (g), <0.01.

§ 10^7 cells from each cell population were added to dishes.

inhibition was abolished when the ABA-sensitive cells were pretreated with anti-Thy-1.2 and complement (Table VII).

Soluble Antigen Abolishes the Suppression That Follows Interaction of ABA-sensitive Cells and ABA-coupled Cells. Because suppressor T cells can be enriched by incubation on dishes coated with antigen alone (13), it was of interest to determine the effect of soluble antigen in the present system. ABA-sensitive cells were incubated for 30 min at 37°C with 1 or 10 μg/ml ABA-HGG and subsequently incubated in dishes coated with ABA-coupled spleen cells for 3 h. The ABA-HGG was present throughout the entire in vitro incubation. The ability of the nonattached cells in the supernate to transfer sensitivity after incubation was tested. As before, incubation with ABA-coupled spleen cells decreased transfer but this was abrogated by the presence of 1 μg/ml ABA-HGG. The soluble antigen did not exert a nonspecific effect because it did not interfere with the interaction of oxazolone-sensitive cells and oxazolone-

coupled spleen cells. Furthermore, soluble antigen alone did not activate the suppressive effect, because ABA-sensitive cells incubated with soluble antigen and oxazolone-coupled spleen cells could transfer ABA sensitivity (Table VIII).

Anti-ABA CRI$^+$ Antibody Inhibits the Suppression Produced by the Interaction of ABA-coupled Spleen Cells and ABA-sensitized Cells from A/J Mice. Because some T cells from A/J mice bear the CRI found on A/J anti-ABA antibodies (5, 6), the effects of anti-CRI sera on the interaction of ABA-sensitized A/J cells and ABA-coupled spleen cells were studied. ABA-sensitive A/J cells were incubated with 1 μg/ml IBC rabbit anti-CRI for 30 min at 37°C and were then incubated on dishes coated with ABA-coupled spleen cells. The anti-CRI reagent was present throughout the in vitro incubation period. After 3 h, the nonattached cells in the supernate were tested for transfer of ABA sensitivity. The suppresive effect induced by the interaction of ABA-sensitive A/J cells with ABA-coupled spleen cells did not occur in the presence of anti-CRI. By contrast, anti-CRI did not affect the suppression induced by the interaction of ABA-sensitive CBA cells and ABA-coupled cells. In addition, anti-CRI also abrogated the suppression following interaction of A/J cells enriched for T cells by nylon wool passage (Table IX).

To exclude the possibility that anti-CRI serum might be acting through non-CRI determinants or via CRI$^+$ antibody passively adsorbed on T cells, A/J mice were simultaneously painted with ABA and oxazolone and the interaction of their sensitive cells with spleen cells conjugated with ABA, oxazolone, and TNP was examined. When the cells were incubated on plates coated with spleen cells conjugated with ABA or oxazolone, their ability to transfer sensitivity to both contact sensitizers was inhibited. No such inhibition occurred when incubation was performed on plates coated with cells conjugated with TNP. This shows a nonspecific suppressive effect produced by an antigen-specific interaction similar to that in the mixing experiments described in Table V. Anti-CRI prevented the inhibition for both agents, but only when the suppression was induced by incubation on ABA-conjugated spleen cells, not

TABLE VIII

Effect of Soluble Antigen on the In Vitro Interaction of Sensitized Cells and Antigen-coupled Cells.

Group	Antigen to which CBA cells were sensitized*	ABA-HGG added in vitro	Antigen-coupled cells	Antigen challenge	Ear increment (10^{-2} mm)‡
		μg/ml			
a	None	0	None	ABA	3.3 (2.5)
b	ABA	0	Oxazolone	ABA	14.4 (3.3)
c	ABA	1	Oxazolone	ABA	16.2 (2.7)
d	ABA	10	Oxazolone	ABA	12.4 (2.3)
e	ABA	0	ABA	ABA	6.4 (1.2)
f	ABA	1	ABA	ABA	14.5 (2.3)
g	ABA	10	ABA	ABA	10.8 (1.7)
h	None	0	None	Oxazolone	0.9 (0.6)
i	Oxazolone	0	ABA	Oxazolone	8.9 (1.8)
j	Oxazolone	1	ABA	Oxazolone	8.2 (3.3)
k	Oxazolone	0	Oxazolone	Oxazolone	5.4 (0.9)
l	Oxazolone	1	Oxazolone	Oxazolone	3.7 (1.2)

* 10^7 cells were transferred after incubation in vitro as shown.

‡ Five mice/group; arithmetic mean (SD). *P* values: (e) (f), <0.001; (k) (l), NS.

TABLE IX

Effect of Anti-CRI on Suppression after Interaction of ABA-sensitized Cells and Antigen-coupled Cells

Group	ABA-sensitized cells*	Anti-CRI in vitro‡	Antigen-coupled cells	Ear increment (10^{-2} mm) after ABA challenge§
a	None	None	None	2.7 (1.0)
b	A/J spleen and lymph nodes	−	Oxazolone	9.1 (1.1)
c	A/J spleen and lymph nodes	+	Oxazolone	9.5 (3.1)
d	A/J spleen and lymph nodes	−	ABA	2.7 (2.2)
e	A/J spleen and lymph nodes	+	ABA	10.5 (2.0)
f	None	None	None	4.6 (0.8)
g	A/J nylon wool filtered cells‖	−	Oxazolone	13.8 (2.2)
h	A/J nylon wool filtered cells‖	−	ABA	8.6 (2.4)
i	A/J nylon wool filtered cells‖	+	ABA	16.8 (3.6)
j	None	None	None	3.5 (2.5)
k	CBA spleen and lymph nodes	−	Oxazolone	12.6 (1.3)
l	CBA spleen and lymph nodes	−	ABA	8.0 (2.8)
m	CBA spleen and lymph nodes	+	ABA	8.4 (1.6)

* 10^7 cells were transferred after incubation as shown.
‡ Plus sign: 1 μg/ml IBC anti-CRI, minus sign: normal rabbit serum.
§ Five mice/group; arithmetic mean (SD). *P* values: (d) (e), <0.001; (h) (i), <0.01; (k) (l), <0.01; (k) (m), <0.01.
‖ Nylon-filtered cells were 97% sensitive to anti-Thy-1.2 + complement.

TABLE X

*Effect of Anti-CRI on Suppression after Interaction of Cells from A/J Mice Simultaneously Sensitized to Both ABA and Oxazolone and Antigen-coupled Cells**

Antigen-coupled cells	Anti-CRI in vitro‡	Ear increment (10^{-2} mm) after challenge with§	
		Oxazolone	ABA
TNP	+	13.9 (3.8)	10.0 (1.8)
TNP	−	11.0 (2.4)	8.1 (2.0)
ABA	+	10.6 (3.3)‖	9.5 (2.6)¶
ABA	−	6.8 (0.6)‖	5.2 (1.3)¶
Oxazolone	+	5.5 (1.5)	2.7 (0.7)
Oxazolone	−	5.3 (1.0)	4.5 (1.7)

* 10^7 cells were transferred after incubation as shown.
‡ Plus sign: 1 μg/ml IBC anti-CRI; minus sign: normal rabbit serum.
§ Five mice/group; each mouse was challenged on separate ears; arithmetic mean (SD). Values in nonsensitized mice not given cells were 2.7 (0.9) for oxazolone and 3.2 (1.4) for ABA.
‖ $P < 0.05$.
¶ $P < 0.02$.

on oxazolone-coupled cells (Table X). This experiment reveals the target of anti-CRI action: (*a*) the anti-CRI acts on the cell, which interacts with ABA-coupled spleen cells to initiate the suppressive effect; (*b*) it does not act on the A/J oxazolone-sensitive cells; and (*c*) it does not act via passively adsorbed CRI^+ antibody because after simultaneous painting with ABA and oxazolone, the oxazolone interactions were unaffected by anti-CRI.

It seems from the results of Table IX that most of the suppressive activity that

follows the interaction of ABA-sensitive A/J T cells and ABA-coupled spleen cells is produced by CRI^+ T cells, because it is so easily abolished by anti-CRI. To confirm this, cell titration experiments were performed. 10^7 cells from A/J mice sensitized to oxazolone were mixed with varying numbers of ABA-sensitive A/J cells. The total cell number in each mixture was normalized by adding normal spleen cells. The ability of the cells to transfer sensitivity to oxazolone was then measured and compared to the effect obtained when anti-CRI had been added during the incubation. The results are shown in Fig. 1. Significant inhibition of transfer of oxazolone sensitivity could be achieved with 6×10^6 but not 2×10^6 ABA-sensitive cells. Inhibition was not observed when ABA-sensitive cells were present with anti-CRI in the incubation. This suggests that at least 40% of the measurable suppressive activity resulted from CRI^+ T cells.

Discussion

The major finding described in this paper is that painting the skin of mice with ABA diazonium induced two types of T cells: one transferred contact hypersensitivity to ABA and was H-2I restricted; the other produced a nonspecific suppressor effect after activation in vitro with ABA-coupled cells. The interaction of the suppressor T cell with the ABA-conjugated cells was antigen specific, H-2K restricted, and abrogated by soluble antigen or anti-idiotype.

Transfer of contact hypersensitivity to ABA was restricted by the I region of the MHC. This is in contrast to DNFB contact sensitivity, which could be transferred when donors and recipients shared either the H-2K, H-2I, or H-2D regions (12). One possibility for the difference is suggested by experiments that show that contact sensitizers with different physicochemical properties form immunogenic complexes with different cell populations and therefore may involve presentation in association with different H-2 gene products (14). It should, however, be noted that sensitization with ABA diazonium did induce cells that were restricted by H-2K but did not mediate contact hypersensitivity.

The interaction of sensitive cells with antigen in vitro was studied. Incubation with antigen-coupled spleen cells prevented transfer of sensitivity, provided the same antigen was used to sensitize and to couple the cells. Loss of transfer capacity was not caused by adsorption of antigen-sensitive cells on antigen-coated cells. Thus, when

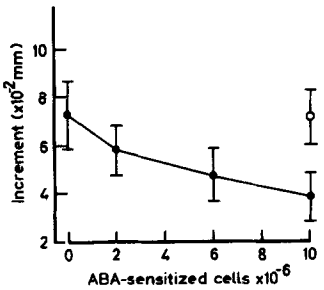

FIG. 1. Effect of anti-CRI on the suppressive activity induced by the interaction of ABA-sensitized cells with ABA-coupled spleen cells. O, + anti-CRI; ●, − anti-CRI. 10^7 oxazolone sensitized A/J cells incubated with 2×10^6–10^7 ABA-sensitized A/J cells on monolayers of ABA-coupled cells. Results shown mean (SD) of groups of five mice; unsensitized mice 4.4 (1.0); inhibition obtained with 10^7 and 6×10^6 ABA cells $P < 0.01$ and 0.02, respectively.

the sensitive cells were recovered and freed from the antigen-coated cells (by using F_1 antigen-coupled cells and an appropriate anti-H-2 serum), transfer was still not observed. Furthermore, the loss of the ability to transfer extended to cells sensitized to other antigens, indicating that although the induction of suppression was antigen-specific, its effect was nonspecific.

The cell responsible for suppression was a T cell as shown by experiments with anti-Thy-1.2 serum and nylon wool-filtered cells. However, suppression could only be produced when the ABA-sensitized T cells and the ABA-conjugated spleen cells had the same H-2K genes. If only H-2I region genes were shared, transfer was not inhibited. Hence the interaction of the H-2I-restricted T cells responsible for transfer of ABA sensitivity with ABA-coupled spleen cells of the same H-2I genotype did not affect the ability to transfer sensitivity. The cells responsible for the suppressor effect in this system resembled the suppressor cells, which are induced after tolerization and inhibit the effector phase of DTH. These may be H-2K, H-2I, or H-2D restricted (15, 16), or cyclophosphamide resistant (17, 18), and they may release nonspecific inhibitors after incubation with antigen in vitro (19). As has been found with other suppressor T cells (13, 20), the cells here reacted with free (non-cell-bound) antigen, but this prevented their activation by ABA presented in association with the H-2K gene product of the antigen-coupled cell.

When A/J mice were used, the suppressive activity was abolished by adding anti-CRI serum in the in vitro incubation. The specificity of the effect of anti-CRI was revealed by its inability to influence CBA cells sensitized to ABA, or A/J cells sensitized to oxazolone. By examining the interaction between ABA-sensitive cells and ABA-coupled cells for its ability to prevent transfer of oxazolone sensitivity by oxazolone sensitive cells, it was shown that anti-CRI affected the cells producing the inhibition, not the target cells of the inhibition. In addition, experiments performed with cells from mice sensitized simultaneously with ABA and oxazolone showed that anti-CRI affected only the interaction of ABA-sensitive cells and ABA-conjugated cells, which indicates that the involvement of passively adsorbed CRI^+ antibody was unlikely. Because T cells enriched by nylon wool filtration were also affected by anti-CRI, a direct interaction between the anti-CRI and the T cell appears plausible.

It is notable that a large proportion of the suppressive activity could be inhibited by anti-CRI. This result is similar to that reported for ABA (6), 4-hydroxy-2-nitrophenyl acetyl (NP) (21), the synthetic polypeptides poly(Tyr,Glu)-poly(D,L,Ala)--poly(Lys) (22), and L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT) (23), and lysozyme (24), where T cell activity has been restricted to a population of cells bearing the major idiotypic determinant. The amplification of T cell function may thus depend to a large extent on idiotype-anti-idiotype interactions that would tend to select for cells bearing the major idiotypes. Experimental evidence in favor of this process has been collected for suppressor T cells active against NP (18), GAT (23), and ABA (5). With ABA, it has been shown that suppressor T cells expressing idiotype can indeed induce other suppressor T cells with anti-idiotype actiivity.

The large contribution by CRI^+ cells in the suppressive effect in the present system could also be accounted for by idiotype-anti-idiotype interactions at the effector phase. Thus, an interaction between a CRI^+ receptor and antigen would initiate a series of reactions between idiotype- and anti-idiotype-specific cells, which would

increase the suppression. Anti-CRI or CRI would then block both interaction with antigen and subsequent idiotype-anti-idiotype reactions.

In summary, it can be said that A/J mice sensitized to ABA possess T cells that express the CRI of A/J antibodies and, after interaction with ABA in association with H-2K gene products of ABA-conjugated cells, can exert a suppressive activity on the effector phase of contact sensitivity. It is not known whether these cells are physiological suppressors, nor is it known yet whether they bear surface markers similar to those on other suppressor T cells. The cells, however, appear to have the ability to participate in idiotype-anti-idiotype interactions irrespective of the idiotypes present on the cells mediating contact hypersensitivity.

Summary

Painting mice on the skin with the diazonium salt of *p*-arsanilic acid elicited two types of T cell activity. One was restricted by the I region of the major histocompatibility complex and was responsible for the transfer of azobenzenearsonate (ABA) sensitivity to naive mice. The other was H-2K restricted and could be demonstrated by its ability to interact specifically with ABA-coupled cells in vitro and to inhibit nonspecifically the transfer of sensitivity by cells sensitized either to ABA or to another antigen. Free antigen, or antibody directed against the cross-reactive idiotype on the anti-ABA antibodies of A/J mice, could inhibit the H-2K-restricted suppressive activity induced in the ABA immune A/J cells.

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