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HAPTEN-SPECIFIC T CELL RESPONSE TO AZOBENZENEARSONATE-*N*-ACETYL-L-TYROSINE IN THE LEWIS RAT

I. Induction and Suppression of Delayed-Type Hypersensitivity and *in Vitro* Proliferative Responses[1]

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Pretreatment of Lewis rats with a single i.p. injection of ABA-*N*-acetyl-tyrosine in incomplete Freund's adjuvant induced an unresponsiveness for delayed-type hypersensitivity to subsequent immunization with the same antigen in complete Freund's adjuvant. Complete suppression of *in vitro* antigen-induced proliferative responses required repeated pretreatment.

Passive transfer of lymphoid cells from spleen and lymph nodes but not sera from suppressed rats induced unresponsiveness of hapten-specific T cell functions. Nylon wool-nonadherent cells and cells panned on F(ab')$_2$ of rabbit anti-Lewis rat Ig plates suppressed the induction of DTH and *in vitro* antigen-stimulated proliferation.

Adult thymectomy increased DTH and failed to abolish the induction of suppression.

The intensity and duration of an immune response is governed not only by the nature, route, and the dose of antigen but also is regulated by a series of cell interactions that are genetically determined. Because of the complexity of the regulatory network, studies on the genetic, cellular, and molecular aspects of immunoregulation are required in order to understand immunologic circuits.

Suppressor T cells (Ts)[2] exerting demonstrable negative regulation of humoral immune responses have been well defined *in vivo* and *in vitro* (1–5). Recently, several systems of unresponsiveness involving cell-mediated immunity (CMI) including delayed-type hypersensitivity (DTH) (6–10), cytotoxicity (11), mixed lymphocyte reaction (12), and transplantation immunity (13) have all been shown to be influenced by Ts. Genetically programmed Ts subsets in turn are surprisingly heterogeneous with regard to phenotypic markers (14, 15), density, and sensitivity to cyclophosphamide (16). Recently, studies on the receptor of Ts-mediating idiotypic suppression suggested 2 complementary subsets that might be mutually stimulatory (17–19). Moreover, work performed by Miller *et al.* (20, 21) on DNP-modified allogeneic *vs* syngeneic cell-induced unresponsiveness of contact sensitivity confirmed the existence of 2 types of Ts producing afferent or efferent regulation. Whether Ts subsets actually represent distinct subpopulations or reflect different pathways and maturation stages by which the suppressive signal is mediated is still an open question.

To approach the question of whether there is one Ts subset for every T cell function, we have conducted a series of experiments in the Lewis rat using azobenzenearsonate-*N*-acetyl-tyrosine (ABA-tyr), a simple antigen of limited heterogeneity. The capability of inducing DTH, *in vitro* T cell proliferation, T helper cell (Th), and T suppressor cell response all specific for the same simple determinant would provide a potential model for studies on the cellular interaction and molecular requirements of T cell activities within functional subsets in which phenotypic markers remain largely undefined.

We now report an investigation in Lewis rats on the susceptibility of several ABA-specific T cell functions to induced unresponsiveness and the possible role of Ts in these phenomena. Our results indicate similar kinetics for DTH response and *in vitro* antigen-induced proliferation after immunization with ABA-tyr in complete Freund's adjuvant (CFA). ABA-specific unresponsiveness in DTH could be induced by pretreatment by ABA-tyr in incomplete Freund's adjuvant (IFA). However, complete suppression of *in vitro* proliferation required repeated pretreatment. Passive transfer of nylon wool-nonadherent cells and cells panned on F(ab')$_2$ of rabbit anti-Lewis rat Ig plates suppressed the induction of DTH and *in vitro* antigen-stimulated proliferation, strongly suggesting the T-cell nature of this suppression.

MATERIALS AND METHODS

Animals. Female 6- to 8-wk-old Lewis rats (Microbiological Associates, Walkerville, MD) were used.

Antigens and mitogens. The conjugation and purification of ABA conjugates were carried out as described previously (22). Antigens were emulsified in a 1:1 ratio with CFA containing 5 mg killed *Mycobacterium tuberculosis* per milliliter.

Old tuberculin (O.T.) was a gift from Massachusetts State Toxin Lab, Jamaica Plain, MA. Purified protein derivatives

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[1] This work was supported by Grant AI010895 from the National Institutes of Health.

[2] Abbreviations used in this paper: ABA, azobenzenearsonate; Ts, suppressor T cells; DTH, delayed-type hypersensitivity; ABA-tyr, ABA-*N*-acetyl-L-tyrosine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; P.E., peritoneal exudate; O.T., old tuberculin; sIg, surface immunoglobulin; [^{3}H]TdR, tritiated thymidine; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2-(5-phenyloxyzolyl)-benzene.

(PPD) were purchased from Parke Davis Co., Detroit, MI. Concanavalin A (Con A) was purchased from Calbiochem, San Diego, CA, and used at 1 μg per microtiter well (50 μg/ml).

Antisera. No. 4818 is a mouse anti-Ia^k (A·TH anti-A·TL ascites) reagent prepared in Dr. David Sachs' laboratory. The specificity of this antiserum had been checked by cytotoxicity and absorption studies using cells from B10 and A congenic strains of mice. It reacts only with the gene products of I-A and E/C subregions (personal communication). No. 4814 is normal A·TH ascites fluid. Both were kindly provided by Dr. D. Sachs from the Immunology Branch, N.I.H. Fluorescein-conjugated IgG fraction of rabbit anti-rat IgG (H and L chain) was purchased from Cappel Laboratories (Cochranville, PA). Using a 1:2 dilution, 23 to 26% of spleen cells stained positive for surface Ig (sIg). $F(ab')_2$ of rabbit anti-Lewis rat Ig was prepared according to the method of Goding (23).

Immunization. Fifty to 100 μg ABA-tyr emulsified in a total volume of 0.1 ml adjuvant was distributed among the 4 footpads.

Assay of DTH. Intradermal skin tests were performed on the shaved flank with the appropriate antigen (usually 15 μg ABA-bovine-insulin and a 1:60 dilution of O.T.) in 0.1 ml saline. The skin site was observed at 4 and 24 hr. Induration and erythema were measured in millimeters by vernier calipers (No. 12, Glogau Co., Chicago, IL). A negative DTH response was considered to be <2.5 mm induration and <4 mm erythema, which was the average cutaneous reaction of normal rats to test antigens. For comparisons, an index of DTH was obtained by multiplying the diameter of erythema in millimeters and the degree of induration (+ = 3.1 to 3.5 mm, ++ = 3.6 to 4 mm, +++ = 4.1 to 4.5 mm, etc). Histologic examination of cutaneous reaction utilized hematoxylin-eosin-stained paraffin section of formalin-fixed material.

Measurement of in vitro proliferative response. Rats were injected i.p. with 10 ml peptone broth (Bactopeptone, Difco, Detroit, MI) 3 days before sacrifice. Peritoneal exudate (P.E.) cells were obtained by lavage with 50 ml cold RPMI 1640 supplemented with 1% fetal calf sera (FCS; Flow Laboratories, Rockville, MD) and 10 U sodium heparin/ml (GIBCO Diagnostics, Madison, WI). After washing, cells from each rat were suspended in 3 ml warm RPMI 1640 supplemented with 5% FCS and 0.02 M HEPES[2] buffer (Microbiological Associates) followed by incubation for 1 hr at 37°C, 5% CO_2 on medium prewashed glass wool column (Fischer Scientific Co., Pittsburgh, PA), 0.6 μg per column. Nonadherent cells were eluted with 50 ml warm RPMI 1640 supplemented with 10% FCS. The glass wool-nonadherent P.E. cells were 99% viable and contained an average of less than 2% sIg^+ cells and 3% neutral red positive cells. Culture conditions were modified according to Soulillou *et al.* (24). Briefly, 10^5 P.E. cells (5×10^5 cells/ml) in RPMI 1640 supplemented with 5% FCS, 0.02 M HEPES buffer, and 5×10^{-5} M 2-mercaptoethanol were cultured for 4 days in flat-bottomed Falcon Micro Test II tissue culture plates with appropriate antigen concentration. On the 3rd day of culture, cells were pulsed with 1 μCi [^{3}H]TdR[2] (Schwartz/Mann, Orangeburg, NY) per well. Cells were harvested on a glass filter using an M12V Cell Harvester (Brandel Co., Rockville, MD). The glass filter pads were dried and placed in plastic vials containing 4 ml of scintillation fluid (Fischer Scientific Co., 1 gallon toluene with 16 μg PPO[2] and 1.6 μg POPOP[2]) and counted in a Beckman liquid scintillation counter. Cultures were usually run in triplicate, and the results are expressed as mean cpm.

Enrichment of T cells. Separation of nylon wool-nonadherent and -adherent cells was done according to Julius *et al.* (25). Briefly, spleen cells were treated with 0.155 M Tris-ammonium chloride buffer to lyse red blood cells (RBC). RBC-free spleen and lymph node cells (10^8) were loaded on the nylon wool column. After incubation for 1 hr, the nonadherent cells were eluted with warm medium, and yields averaged 30 to 35% of total cells loaded. Approximately 4 to 7% were sIg^+ cells. Negative selection of T cells was done on plastic Petri dishes coated with $F(ab')_2$ of rabbit anti-rat Ig according to Wysocki and Sato (26). Briefly, 100 μg of antibody in 10 ml phosphate-buffered saline (PBS) was applied on dishes overnight at 4°C. Unbound antibody was removed, and dishes were washed with PBS. All the dishes were saturated with 10% FCS for 1 hr at 4°C before used. Spleen and lymph node cells pretreated with low ionic strength filtration (5% glucose, 2% Hanks'-HEPES, and 0.001% DNase) to remove dead cells were panned on antibody-coated plates (3×10^7 cells per plate) for 1 hr at 4°C. The nonadherent spleen cells usually contained less than 2% sIg^+ cells with about a 36% cell recovery on anti-rat Ig plates, and 9% sIg^+ with a 60% cell recovery on $F(ab')_2$ of anti-rat Ig plates.

RESULTS

Induction of DTH. Since skin reaction varied mainly in intensity of induration rather than extent of erythema, an index of reaction that is the product of erythema (in millimeters) and induration (1+, 2+, etc.) was used. The time course of DTH responses in Lewis rats is shown in Figure 1. Rats injected with 900 μg ABA-tyr in CFA expressed a maximum ABA-specific DTH response at 2 to 3 wk, which decreased markedly by 5 wk. Reaction to O.T. peaked later at about 3 to 4 wk and remained at maximum levels longer. Dose-response studies (Fig. 2) showed that 90 μg ABA-tyr per rat produced maximal DTH reactions. From the gross appearance and histologic analysis, it was noticed that the induration seen in DTH of rats is more significant then erythema. That is, low DTH responses sometimes had the same diameter erythema, yet the intensity of induration was substantially less than that of a high response. Palpable, deep indurated lesions were generally maximal at 24 hr. Both diffuse and focal aggregates of mononuclear cells accompanied by few polynuclear leukocytes massively infiltrated in the deep dermis, subcutaneous fat, and muscle were seen.

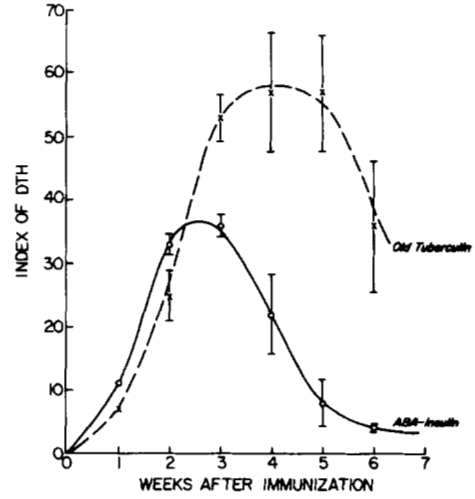

Figure 1. Time course of DTH to ABA-*N*-acetyl-tyrosine (ABA-tyr). Rats were immunized with 900 μg ABA-tyr. Skin tests were performed with 50 μg lightly conjugated ABA-insulin (○——○), and a 1:20 dilution of O.T. (×-----×). Normal rats have no cutaneous response to both antigens. Vertical bars represent SEM. n = 3.

Induction of in vitro proliferation. Spleen, lymph node, and glass wool-purified P.E. cells of immune rats were studied for their ability to give [^{3}H]Tdr incorporation in response to ABA-tyr. The highest stimulation capacity was shown by P.E. cells. Lymph node cells showed a low but significant response, whereas spleen cells were generally nonstimulatable or even suppressive (data not shown).

Table I gives a typical example of stimulation by ABA-tyr. P.E. cells injected with 90 μg ABA-tyr in CFA showed a high extent of *in vitro* [^{3}H]Tdr uptake when incubated with ABA-tyr, whereas cells from rats injected with saline in CFA showed no stimulation. The dose-response curve seen in Figure 3 shows the optimal concentration of ABA-tyr for the strongest stimulation in culture to be 50 μg per 10^5 cells (2.5 mg/ml). Table II shows the fine specificity of the ABA-tyr-primed cells and demonstrates that stimulation *in vitro* is in part hapten specific. A definite cross-reactivity between primed cells and heterologous conjugate is seen, but the differences in stimulation with homologous or heterologous conjugate show the partial carrier specificity for tyrosine or histidine seen by others (27). It is of interest, too, that phosphanylazo-tyrosine, a structurally related hapten, did not cross-react.

Inhibition of in vitro proliferation by a cross-reactive mouse anti-Ia^k. Many lines of evidence had confirmed that Ia-positive accessory cells play an obligatory role with regard to antigen processing and presentation in immune phenomena, including the induction of T cell proliferation. To ascertain the requirement for macrophage-like cells in the proliferative response of rat P.E. cells, attempts to deplete accessory cells were made using nylon wool or Sephadex G-10 columns. However, repeated passage over nylon wool columns gave only a 43% decrease in proliferative response with no significant decrease in neutral red positive cells. Complete loss of proliferative response by passage over G-10 columns was only occasionally seen, even though in most experiments the neutral red positive cells decreased to less than 0.2%. Another approach in defining the participation of accessory cells was to use anti-Ia sera to block the induction of T cell proliferation. Numerous studies on immune responses under Ir gene control had demonstrated that antisera to Ia specificity inhibit recognition of macrophage-associated antigen by T lymphocytes (28). Since Sachs *et al.* (29) were able to detect Ia cross-specificities shared by rats and mice with serologic and biochemical methods, we used mouse anti-Ia^k in our studies. Table III demonstrates the ability of mouse anti-Ia^k to block ABA-tyr-induced proliferation. Although the addition of normal mouse ascites fluid to cultures produced some nonspecific stimulation, anti-Ia^k serum produced 61% inhibition in a typical experiment. Although antigen-induced stimulation could be suppressed, Con A stimulation was less affected at the 1:92 dilution used.

Suppression of ABA-specific T cell responses by administration of immunogenic doses of ABA-tyr in IFA. To establish a tolerance model that might be mediated by suppressor T cells,

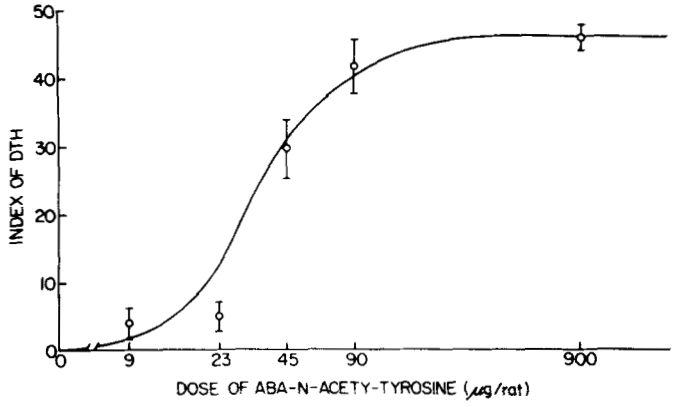

Figure 2. Dose response of DTH to ABA-tyr. Rats were skin-tested with 15 μg ABA-bovine-insulin 2 wk after immunization. n = 3.

TABLE I

In vitro [^{3}H]TdR incorporation in antigen-primed P.E. cells

Immunization	Background	[^{3}H]TdR Incorporation[a] (Δ cpm)		
		Con A[b] (1 μg)	PPD (2 μg)	ABA-tyr (50 μg)
Saline/CFA	3,769 ± 223	45,160 ± 5,789	24,750 ± 1,314	−2,701 ± 355
90 μg ABA-tyr/CFA	3,071 ± 390	30,363 ± 5,171	17,645 ± 569	65,980 ± 2,137

[a] 10^5 pooled, glass wool-nonadherent P.E. cells were cultured 3 wk after immunization. n = 2.

[b] Con A response is lower than usual in this experiment.

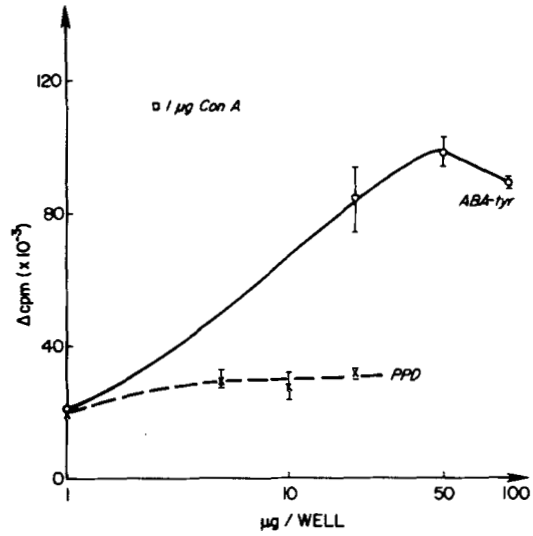

Figure 3. Dose responses of *in vitro* proliferation to ABA-tyr 2 × 10^5 pooled glass wool nonadherent P.E. cells were cultured 3 wk after immunization with 90 μg ABA-tyr.

TABLE II

Hapten specificity and cross-reactivity of ABA-conjugates in in vitro proliferation

Immunization	Background	[^{3}H]TdR Incorporation[a] (Δ cpm)				
		Con A (1 μg)	PPD (2 μg)	ABA-tyr (50 μg)	ABA-his (50 μg)	Phosphanilic-tyr (50 μg)
45 μg ABA-tyr/CFA	1,161 ± 47	111,605 ± 3,726	56,616 ± 887	98,542 ± 3,957	19,930 ± 967	−128 ± 17
45 μg ABA-his/CFA	3,841 ± 307	89,425 ± 5,136	31,547 ± 1,911	17,113 ± 1,416	112,219 ± 6,225	N.T.[b]

[a] 2 × 10^5 pooled, glass wool-nonadherent P.E. cells were cultured 3 wk after immunization. n = 3.

[b] N.T., not tested.

we chose ABA-tyr pretreatment in IFA as a suppressive regimen, since previous studies showed that when given in saline, very large repeated doses were required for unresponsiveness in guinea pigs (30). Table IV shows that pretreatment with as little as 9 μg ABA-tyr begins to suppress, and 90 to 900 μg produced an almost complete suppression of DTH. It is of interest that animals suppressed for ABA-specific DTH response had increased O.T. reactivity compared with immune controls, suggesting some form of antigenic competition.

TABLE III

Inhibition of in vitro proliferation by mouse anti-Ia^k ascites[a]

Culture Treatment	[^{3}H]TdR Incorporation (Δ cpm)			
	Background	Con A (1 μg)	ABA-tyr (50 μg)	% Suppression of ABA-tyr
Anti-Ia^k	784 ± 200	45,920 ± 7,143	11,502 ± 830	61 ± 3 ($p < 0.001$)
NMA	2,722 ± 722	49,323 ± 3,328	33,757 ± 1,124	
—	804 ± 134	45,742 ± 5,293	29,622 ± 788	

[a] 10^5 pooled, glass wool-nonadherent P.E. cells from rats immunized with 45 μg ABA-tyr/CFA were cultured with 10 μl anti-Ia^k (A·TH anti-A·TL) ascites or normal A·TH ascites at 1:4 dilution (end dilution 1:92). n = 3.

Although a single injection of ABA-tyr in IFA was sufficient to induce a complete unresponsiveness with respect to DTH skin reactivity, the results in Figure 4 demonstrate that a similar regimen induced only a 26% decrease in *in vitro* proliferation. In order to achieve total suppression of *in vitro* responsiveness, it was found necessary to give 2 injections of ABA-tyr in IFA 1 wk apart and to use a challenge dose of 45 μg ABA-tyr in CFA. Again, as in skin reactivity, ABA-suppressed rat P.E. cells showed somewhat higher reactivity to PPD in some cases.

In an effort to determine the kinetics of unresponsiveness induction, groups of rats were treated with ABA-tyr in IFA at various intervals before or after immunization. As shown in Table V, suppression occurred if treatment was given as much as 12 wk before immunization. Simultaneous injections (0 day) produced 45% suppression. However, 1 day after immunization was already too late to achieve suppression. Therefore, the suppressive effect appeared to be working on the induction phase of DTH.

Passive transfer of suppression by cells. As observed previously in guinea pigs (31), ABA-tyr in CFA or IFA produced no significant antibody response in rats measurable by passive hemagglutination (data not shown). Nevertheless, attempts

TABLE IV

Dose-dependent induction of ABA-specific suppression on DTH

Pretreatment ABA-tyr/IFA[a] − 7 day	Immunization ABA-tyr/CFA 0 day	No. Animals	Skin Test (14 Day)		% of ABA-Specific Suppression
			ABA-insulin 20 μg	O.T. 1:40	
μg	μg				
900	90	3	7[b] ± [c] (3 ± 1)[d]	13 +++ (37 ± 7)	93 ± 2 ($p < 0.01$)
90	90	3	11 ± (7 ± 2)	12 ++ (26 ± 7)	83 ± 5 ($p < 0.02$)
9	90	3	13 ++ (22 ± 10)	7 ± (3)	48 ± 24 ($p > 0.1$)
0.9	90	2	14 ++++ (49 ± 7)	12 + (8 ± 5)	0
—[e]	90	5	12 ++++ (42 ± 7)	11 + (9 ± 1)	

[a] Rats injected with ABA-tyr/IFA alone showed no DTH response.
[b] Erythema (mm).
[c] Induration.
[d] Index of DTH.
[e] Rats pretreated with saline/IFA had no effect on DTH.

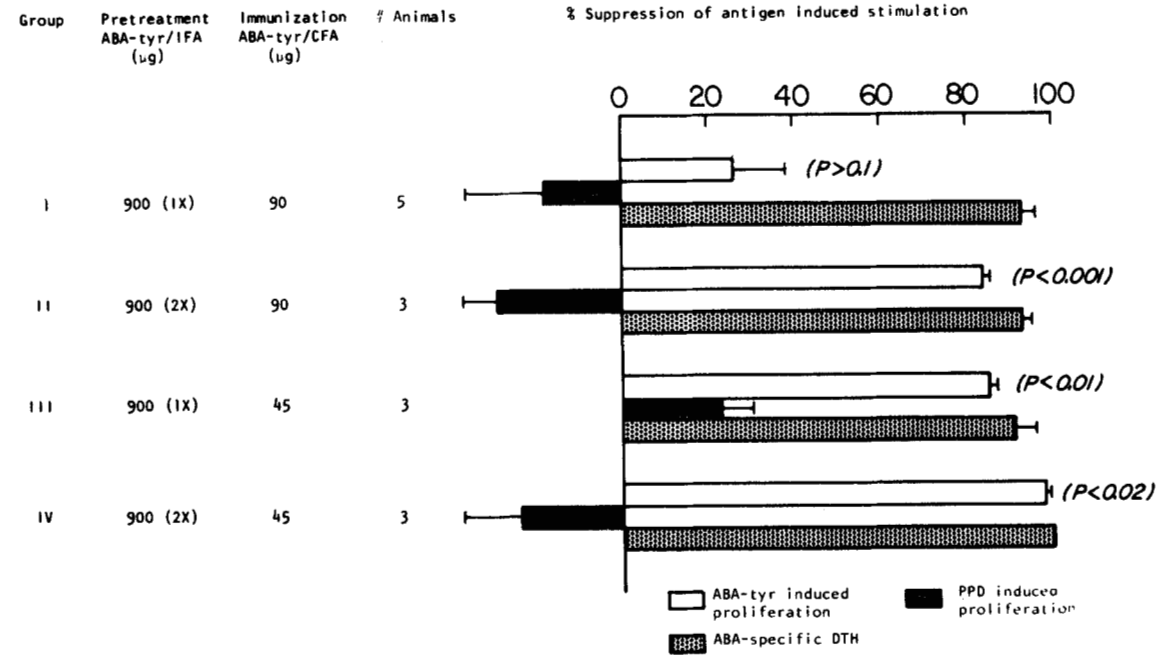

Figure 4. Induction of ABA-specific suppression on antigen-induced *in vitro* proliferation. Rats were injected with ABA-tyr/IFA, 1× at 7 days or 2× at 7 and 14 days before immunization. Glass wool nonadherent P.E. cells were assayed for proliferation 3 wk after immunization. Pooled P.E. cells were used in Groups II and III.

were made to see whether suppression was transferable by serum. The results (see Tables VI, VIII, and IX) demonstrate the failure of such attempts. On the contrary, the results in Table VI demonstrate that suppression was transferable by pooled spleen and lymph node cells taken from suppressed donors 2 wk after the last suppressive injection. Suppressor cells could suppress the induction of ABA-specific DTH by 88% and *in vitro* proliferative response to ABA-tyr by 82%.

To analyze the cell type involved in this active suppression in the absence of a specific T cell reagent for rat cells, cells negatively selected by passage over nylon wool columns or by panning on Petri dishes coated with $F(ab')_2$ of rabbit anti-rat Ig were transferred. As seen in Table VII, nylon wool-nonadherent cells, which represent about 30% of the unfractionated population, are seen to contain the major suppressive activity (76% *vs* 91% suppression). Whether the difference in degree of suppression seen is due to a nylon wool-adherent population (such as macrophages) involved as a minor suppressive component or is simply an experimental artifact of cell loss due to incomplete eluting is unknown. In hopes that the clone of Ts might expand after immunization after a suppressive regimen, as previously reported (19), we altered our experimental protocol for passive transfer by adding an additional immunization to the donor rats. In Table VIII, it is evident that the 40% sIg^- cells after panning on anti-rat IgG plates produced a 98% decrease in ABA-specific DTH. Control cells from saline-injected animals had no effect. Again, Table IX shows a typical experiment on *in vitro* proliferation, in which 97% suppression was observed after transfer of sIg^- cells.

It had been reported in many systems (32) that induction of active suppression could be abolished by adult thymectomy, indicating that precursors of suppressor T cell have a relatively short life span. However, as seen in Table X, thymectomy up to 9 wk before suppression had no effect on the induction of suppression. However, it is of interest that thymectomy actually enhanced DTH response and that animals thymectomized as little as 1 wk before immunization had markedly elevated DTH reactions.

DISCUSSION

In murine studies, much information concerning regulatory T cells and soluble products involved in antibody response suggests the existence of a complex network of self-governing circuits integrated by virtue of shared components. As proposed by Herzenberg *et al.* (33), a core regulatory circuit (CRC)

TABLE V

ABA-specific suppression of the inductive phase of DTH[a]

Pretreatment (IFA)	Time of Pretreatment	Immunization (CFA) 0 Day	No. Animals	Skin Test (2 wk)		% of ABA-Specific Suppression
				ABA-insulin 15 μg	O.T. 1:80	
	+1 day		3	12 +++ (36 ± 2)	13 +++ (36 ± 6)	10 ± 5
900 μg	0 day	45 μg	3	11 ++ (22 ± 11)	N.T.	45 ± 28 ($p > 0.1$)
	−1 day		3	9 ++ (17 ± 8)	N.T.	57 ± 20 ($p < 0.1$)
	−1 week		4	5 − (3 ± 1)	16 +++++ (82 ± 26)	92 ± 3 ($p < 0.01$)
	−2 week		2	3 − (2 ± 2)	14 ++++ (49 ± 7)	95 ± 5 ($p < 0.02$)
	−3 week		3	0 − (0)	15 +++ (37 ± 8)	100 ($p < 0.01$)
	−6 week		2	5 − (3 ± 3)	13 ++ (26 ± 14)	92 ± 8 ($p < 0.05$)
	−12 week		1	4 − (1)	13 ++ (26)	97
—	—	45 μg	10	12 +++ (40 ± 6)	11 + (15 ± 9)	

[a] Rats were pretreated with 900 μg ABA-tyr in IFA at various times, then immunized with 45 μg ABA-tyr in CFA.

TABLE VI

Suppressor cell-mediated ABA-specific suppression of DTH

Donor Treatment −21 and −14 Day	Material[a] Transferred 0 Day	No. Recipients[b]	Skin Test (14 Day)		% of ABA-Specific Suppression	% Suppression of ABA-tyr Induced Proliferation
			ABA-insulin 15 μg	O.T. 1:60		
900 μg ABA-tyr/IFA (2X)	10^8 cell	7	6 ± (5 ± 2)	11 ± (17 + 5)	88 ± 5 ($p < 0.01$)	82 ± ($p < 0.01$)
	4 ml sera	5	13 +++ (43 ± 11)	11 ± (8 + 2)	14 ± 22 ($p > 0.1$)	
Saline/IFA (2X)	10^8 cell	7	13 +++ (41 ± 7)	11 ± (20 + 6)		
	4 ml sera	4	14 +++ (50 ± 18)	11 + (8 ± 3)		

[a] Pooled cells from spleen and lymph nodes of donors were transferred i.v. into 300 R-irradiated recipients. 300 R-irradiation had no effect on DTH. Pooled sera from donors were injected i.v. and i.p. into normal recipients.

[b] Recipients were immunized with 45 μg ABA-tyr/CFA in the footpads 2 days after the transfer.

TABLE VII

Suppressor activity of nylon-wool nonadherent cells

Donor Treatment −21 and −14 Day	Cells[a] Transferred 0 Day	Skin Test[b] (14 Day)		% of ABA-Specific Suppression
		ABA-Insulin (15 μg)	O.T. (1:60)	
900 μg ABA-tyr/IFA (2X)	3×10^7 Nylon-wool[c] nonadherent fraction	8 + (7 ± 2)	12 +++ (35 ± 7)	76 ± 6 ($p < 0.05$)
900 μg ABA-tyr/IFA (2X)	10^8 Unfractioned	5 ± (3 ± 2)	12 +++ (31 ± 6)	91 ± 6 ($p < 0.02$)
Saline/IFA (2X)	10^8	12 +++ (34 ± 9)	12 +++ (32 ± 8)	

[a] Pooled cells from spleen and lymph nodes of donors were transferred i.v. into 300 R-irradiated recipients.

[b] Recipients were immunized with 45 μg ABA-tyr/CFA in the footpads 2 days after the transfer. Skin tests were performed 2 wk after immunization. n = 5.

[c] Thirty to 35% recovery from a nylon wool column loaded with 10^8 cells.

TABLE VIII

Suppressor T cell-mediated ABA-specific suppression of DTH

Donor Treatment[a]	Material Transferred[b]	Skin Test[c]		% of ABA-Specific Suppression
		ABA-insulin (15 μg)	O.T. (1:60)	
900 μg ABA-tyr/IFA 2× plus	9×10^7 T cells	− (1 ± 1)	12 ± (6)	98 ± 2 ($p < 0.01$)
45 μg ABA-tyr/CFA	4 ml sera	15 ++++ (62 ± 12)	10 − (3)	0
Saline/IFA 2× plus	8×10^7 T cells	13 +++ (47 ± 6)	11 − (3)	
45 μg ABA-tyr/CFA	4 ml sera	14 ++++ (52 ± 8)	9 − (2)	

[a] Donors were injected with ABA-tyr/IFA 21 and 14 days before immunization of ABA-tyr/CFA, then sacrificed 10 days after immunization.

[b] Pooled cells from spleen and lymph nodes of 3 donors were panned on $F(ab')_2$ of rabbit anti-rat Ig plates 2 times. Transfer of nonadherent T cells was based on 1 donor to 1 300 R-irradiated recipient. Pooled sera from donors were injected i.v. and i.p. into normal recipients.

[c] Recipients were immunized with 45 μg ABA-tyr/CFA in the footpads 2 days after the transfer. Skin tests were performed 2 wk after immunization. n = 3.

TABLE IX

Suppressor T cell-mediated ABA-specific suppression of T cell proliferation

Donor Treatment[a]	Material[b] Transferred	[^{3}H]TdR Incorporation[c] (Δcpm)				% Suppression of ABA-tyr
		Background	Con A (1 μg)	PPD (2 μg)	ABA-tyr	
900 μg ABA-tyr/IFA 2×	9×10^7 T	1,654 ± 75	30,158 ± 1,869	14,590 ± 471	445 ± 456	97 ± 3 ($p < 0.001$)
45 μg ABA-tyr/CFA	4 ml sera	2,631 ± 751	50,908 ± 2,041	9,682 ± 2,022	23,117 ± 4,006	6 ± 17
Saline/IFA 2× plus	8×10^7	737 ± 64	20,890 ± 578	12,039 ± 841	14,269 ± 680	
45 μg ABA-tyr/CFA	4 ml sera	4,463 ± 806	48,897 ± 3,973	13,625 ± 2,875	24,694 ± 9,516	

[a] Donors were injected with ABA-tyr/IFA 21 and 14 days before immunization of ABA-tyr/CFA, then sacrificed 10 days after immunization.

[b] Pooled cells from spleen and lymph nodes of 3 donors were panned on $F(ab')_2$ of rabbit anti-rat Ig plates 2 times. Transfer of nonadherent T cells was based on 1 donor to 1 300 R-irradiated recipient. Pooled sera from donors were injected i.v. and i.p. into normal recipients. n = 3.

[c] Recipient was immunized with 45 μg ABA-tyr/CFA in the footpads 2 days after the transfer. 10^5 P.E. cells were cultured 3 wk after immunization. Recipients receiving cells or sera were assayed at different date.

TABLE X

Thymectomy shows no effect on the induction of suppression on DTH

Thymectomy Week	Injection for Suppression[a] 0 Day	Immunization[b] 7 Day	No. animals	Skin Test (2 wk)		% of ABA-Specific Suppression
				ABA-Insulin (15 μg)	O.T. (1:80)	
−	+	+	6	6 ± (3)	10 + (10)	85
−	−	+	6	12 ++ (20 ± 5)	7 + (8 ±)	
−1	+	+	4	9 + (7 ± 2)	4 + (2)	89 ± 2 ($p < 0.001$)
−1	−	+	4	14 ++++ (62 ± 9)	5 ± (3 ± 2)	
−3	+	+	4	9 + (10 ± 4)	14 +++ (39 ± 4)	79 ± 8 ($p < 0.001$)
−3	−	+	3	12 ++++ (48 ± 2)	14 +++ (42 ± 16)	
−5	+	+	4	12 + (9 ± 2)	12 ++ (21 ± 8)	84 ± 3 ($p < 0.001$)
−5	−	+	4	14 ++++ (58 ± 5)	13 + (10 ± 2)	
−9	+	+	4	12 +[c] (13 ± 6)	14 + (19 ± 5)	87 ± 6 ($p < 0.001$)
−9	−	+	5	18 ++++++[c] (103 ± 9)	12 + (12 ± 6)	

[a] Suppression with 900 μg ABA-tyr in IFA.

[b] Immunization with 90 μg ABA-tyr in CFA.

[c] Skin test with a different batch of 15 μg ABA-insulin.

determines the activation of Th or Ts. Auxillary regulatory circuits link the cooperative events among accessory cells, antibody (idiotype, allotype), and soluble factors to the CRC. Recently, studies on unresponsiveness induced to cell-mediated functions as contact sensitivity (20, 21) and DTH (8, 18) utilized hapten or idiotype-derivatized spleen cells to probe the genetically restricted cell interactions and have shown various Ts involved in antigen- or receptor-driven regulation. In hopes of further clarifying this apparent heterogeneity of Ts subsets participating in the immune response, we have been investigating the cellular elements and interactions of various Ts cell reactivities in the Lewis rat using a well-characterized simple antigen, ABA-tyr, which does not induce any detectable antibody to ABA.

Our experiments on DTH induction have produced results different from those of Bullock (34). In the present case, no detectable DTH response was seen when Lewis rats were injected with antigen in IFA; on the contrary, such treatment produced suppression. There are 2 possible explanations. One is that using ABA-bovine-insulin as the skin test antigen, induration is the major DTH indicator in rats, not erythema and induration both, as seen in guinea pigs. Thus, precise measurement of induration is required to express the actual DTH response and might have been overlooked previously. Another possibility is that the lower dose as well as the type of carrier used by us was not sufficient to induce significant DTH reactions with erythema and induration. Nevertheless, in our hands, ABA-tyr in IFA always induced suppression and never immunity. Additionally, using an assay for *in vitro* T cell proliferation, very low or no stimulation was ever seen in rats injected with antigen in IFA, whereas a specific high stimulation was observed when rats immunized with antigen CFA were used. This hapten-specific proliferative response provides a sensitive assessment of T cell activity to ABA-tyr.

As shown in many systems of immune response, accessory cells play a central role in antigen processing and presentation. However, it has been suggested that the generation of plaque-forming cells and cytolytic T lymphocytes are relatively macrophage independent in the rat (35). Efforts to remove adherent accessory cells by nylon wool filtration, glass adherence, and G-10 column were relatively unsuccessful in reducing stimulability when P.E. cells were used. The successful blocking of stimulation with anti-Ia, however, suggests the possible involvement of an antigen-presenting Ia-positive macrophage-like cell, as in the mouse (36). In fact, the low numbers of macrophages present in glass wool-passed reactive P.E. cells indicates that, if present, this must be a highly efficient antigen-presenting cell. However, since a subpopulation of nylon wool-nonadherent T cells reacted with mouse anti-Ia^k serum (37), blocking response by anti-Ia at the level of T cells is also a possibility. Further studies to resolve this are in progress.

Administration of antigen in IFA to induce and activate Ts effects on DTH was first described by Bullock *et al.* (38) in a guinea pig system. Utilizing the same technique in rats, Welch *et al.* (39) showed suppressor macrophages and Ts could protect rats from experimental allergic encephalomyelitis, a T cell-mediated autoimmune disease. Indeed, in our case, we had observed that suppression could be easily induced *in vivo* with ABA-tyr in IFA on DTH. Yet the complete suppression of DTH was not accompanied by a detectable ability to passively transfer suppression to naive recipients (data not shown). It seemed that the number of suppressor cells transferred in a total dose of 10^8 cells in the former situation was probably insufficient to affect sensitization with ABA-tyr in CFA. However, results with passive transfer of suppressor cells obtained after repeated injection of antigen in IFA and boosting with antigen in CFA showed that the suppressive activity was now more evident, indicating that clonal expansion was possible.

With respect to the cell type involved in the active suppression, it appeared evident that the nonadherent population of nylon wool-passed or rabbit anti-rat Ig-panned cells possessed the suppressive activity, suggesting the suppressor cells were of T cell origin. Unfortunately, our anti-rat thymocyte sera were not clean enough to be used as specific T cell reagents. Further characterization of Ts regarding their ability to bind antigen is currently underway.

A comparison of the kinetics of induction and susceptibility to suppression among ABA-specific T cell functions, such as proliferation, DTH, and cooperation with B cells, was undertaken to ascertain the possible relation of one to the other. In general, they had similar kinetics (manuscript in preparation), yet a direct correlation between degrees of DTH, T cell proliferation, and T helper effects was not always evident. With regard to suppression, DTH was more susceptible to suppression than T cell proliferation. Suppression of ability to cooperate with B cells was also more difficult to achieve. From a functional standpoint, it is still uncertain whether distinct subsets of suppressor cells are involved or an overlapping population requiring different numbers to suppress different T cell functions.

As mentioned previously, Ts differing in their H-2 restriction for generation and expression have been reported to act on the inductive or effector phage in DNP-induced suppression. Other studies on picryl sulfonic acid induced-Ts by Thomas *et al.* (40) also showed that precursors of Ts for each phase differed in their susceptibility to thymectomy and cyclophosphamide treatment. In addition, they found that unfractionated Ts could only inhibit the passive transfer of DTH with sensitized cells, whereas enriched Ts were necessary for the inhibition of DNA synthesis by lymph node cells after sensitization. Our results (Table V) indicated that Ts work only on the afferent limb of sensitization in DTH. In direct mixing experiments using enriched Ts and T effector cells for *in vitro* proliferation, we failed to observe suppressive effects even with 2:1 mixtures. The failure of enriched Ts to suppress the antigen-induced proliferative response suggested the difference found by others in the effect of Ts on efferent or afferent limbs apparently was a matter of quality rather than quantity.

It is worth pointing out that adult thymectomy, which produced a dramatic increase in DTH after immunization, was without effect on subsequent ability to induce suppression. Thus, it would seem that adult thymectomy did not lead to loss of sufficient precursors of Ts (at least after 9 wk) to lead to failure in suppression. Alternatively, the unresponsiveness seen might be mediated by other mechanisms, such as clonal abortion, shown for contact sensitivity (41). Experiments designed to confirm whether there is still detectable Ts activity after thymectomy will resolve the above argument.

In summary, pretreatment of Lewis rats with ABA-tyr in IFA induced an unresponsiveness for DTH and *in vitro* proliferation to subsequent immunization with the same antigen in CFA. DTH was more susceptible to the induction of unresponsiveness. Passive transfer of nylon wool-nonadherent spleen and lymph node cells and cells panned on Petri dishes coated with $F(ab')_2$ of rabbit anti-rat Ig suppressed the induction of both T cell reactivities. This suppressive activity could be observed only on the afferent limb of sensitization, and adult thymectomy, which increased DTH, failed to abolish the induction of suppression.

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