

## Control of Experimental Autoimmune Encephalomyelitis by T Cells Responding to Activated T Cells

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T cell vaccination against experimental autoimmune disease is herein shown to be mediated in part by anti-ergotypic T cells, T cells that recognize and respond to the state of activation of other T cells. The anti-ergotypic response thus combines with the previously shown anti-idiotypic T cell response to regulate autoimmunity.

**A**UTOIMMUNE DISEASES SUCH AS experimental autoimmune encephalomyelitis (EAE) are caused by T cells expressing two attributes: receptors for the specific self antigen, which identify the target tissue, and a state of functional activation, which is a prerequisite for attack. The self-antigen in EAE is the basic protein (BP) of central nervous system myelin (1). EAE can be suppressed by anti-idiotypic T cells that recognize the specific anti-BP receptors (the idiotypes) of the autoimmune T cells (2, 3). We now report the control of EAE by T cells that recognize not the idiomorph of the autoimmune T cells, but a marker of their state of activation. These T cells, which we term anti-ergotypic T cells [ $\epsilon\rho\gamma\omega$  (ergon) = work, action] were induced by vaccinating Lewis rats with activated cells of syngeneic T cell clones lacking receptors for BP. In contrast to anti-idiotypic T cells, defined by a response to the specific idiomorph borne by the anti-BP T cells, the anti-ergotypic T cells responded to activated T cells in general, without regard for their idiomorph specificities. Anti-ergotypic T cells could also be detected after immunization with antigen *in vivo*, suggesting that they may function physiologically. Administration of anti-ergotypic T cells to syngeneic rats protected the rats against EAE induced either by adoptive transfer of activated anti-BP clones or by active immunization to BP.

We undertook this investigation as a result of our study of the use of T cell vaccination to induce resistance to EAE and other

experimental autoimmune diseases in rats and mice (4). We found that autoimmune diseases such as EAE (5), thyroiditis (6), or adjuvant arthritis (7) could be prevented or treated by administering virulent autoimmune T cells specific for the target antigens under circumstances in which the T cells were rendered avirulent. T cell vaccination was also found to be effective against collagen II arthritis (8) and against experimental autoimmune neuritis (9). Anti-idiotypic immunity to the autoimmune T cell receptor was associated with the resistance induced by T cell vaccination (2). Indeed, a clone of anti-clonotypic T cells was shown to mediate resistance to EAE (3).

However, several observations suggested that immunity to T cell receptors might not be the only protective element induced by T cell vaccination. First, T cells were efficient vaccines only after they had been activated by incubation with specific antigen or a T cell mitogen before injection (4, 10). For example, as few as  $10^4$  activated anti-BP T cells (2) were more effective than  $5 \times 10^7$  idiomorph-positive anti-BP T cells that had not been activated. Therefore, some change in T cells associated with activation is important in the introduction of T cell vaccination; the presence of the idiomorph alone is insufficient.

Second, in addition to the high degree of disease-specific protection mediated by T cell vaccination, we observed a mild degree of nonspecific protection. For example, Lewis rats are most effectively vaccinated against EAE by use of activated Z1a T cell clones. Z1a recognizes the immunologically dominant epitope present in the 68- to 88-amino acid sequence of BP (11). This clone can either produce EAE or vaccinate rats against EAE, depending on treatment of the cells and the number of cells administered (10, 12). In contrast to Z1a, clone A2b (13) recognizes a nine-amino acid sequence in

the 65-kD heat shock protein of *Mycobacterium tuberculosis* (14). A2b causes arthritis in irradiated Lewis rats or, when suitably treated, induces resistance to adjuvant arthritis (15). Thus, each clone recognizes a different epitope and is associated with a different autoimmune disease. Nevertheless, when groups of five rats each were or were not vaccinated with  $2 \times 10^7$  glutaraldehyde-treated (15) Z1a or A2b clones, the A2b-treated rats clearly showed some resistance to EAE produced by  $2 \times 10^6$  activated Z1a cells. All five of the unvaccinated control rats died of EAE, whereas the rats vaccinated with the specific anti-BP Z1a clone were markedly resistant and developed barely detectable clinical disease. Vaccination with clone A2b did not prevent severe EAE, but it did prevent lethal EAE; none of the A2b-treated rats died. Thus, nonspecific vaccination with activated A2b induced significant resistance, albeit a resistance that was not as effective as that obtained by vaccination with clone Z1a.

Our next experiments were undertaken to identify the mediators of resistance to EAE induced by vaccination with T cells that do not recognize the BP antigen. We assayed the delayed type hypersensitivity (DTH) reaction of rats to T cell clones after vaccination (Table 1). DTH reactions are a convenient *in vivo* measure of T cell reactivity to antigens or idiotypes (15). Lewis rats were vaccinated with clone D9, a subclone of line Z1a, to induce optimal resistance to EAE, and the responses of the rats were tested by eliciting DTH reactions to either nonacti-

**Table 1.** DTH reaction of D9-vaccinated rats to activated and nonactivated T cell clones D9 and A2b. Groups of five Lewis rats each were vaccinated with three weekly injections of  $2 \times 10^7$  antigen-activated, glutaraldehyde-treated D9 cells (a subclone of Z1a) as described (15). DTH was elicited by injecting  $3 \times 10^5$  irradiated (2500 R) cells in 50  $\mu$ l of phosphate-buffered saline into the pinna. Activated cells were taken after 72 hours of antigen stimulation. Activation was assessed microscopically and confirmed by [ $^3$ H]thymidine incorporation. Nonactivated cells had been kept in IL-2-containing medium without antigen for at least 5 days. Ear swelling was determined after 48 hours by use of an isotonic caliper. Values are means  $\pm$  SE.

State	Clone	DTH as measured by change in ear size ( $10^{-2}$ mm)	
		Naïve rats	D9-vaccinated rats
Nonactivated	D9	5.2 $\pm$ 2.1	12.3* $\pm$ 2.1
	A2b	5.4 $\pm$ 1.8	5.5 $\pm$ 2.0
Activated	D9	14.1 $\pm$ 3.0	33.1 $\pm$ 2.9
	A2b	14.8 $\pm$ 3.2	26.2 $\pm$ 3.2

\* $P < 0.05$  compared to response to A2b.

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vated or activated cells of clones D9 or A2b. When nonactivated T clones were used to elicit the DTH reaction, the DTH response appeared to be clonotypically specific; the response to resting D9 was significantly greater than the response to resting A2b. In contrast, the response was not clonotypically specific when activated T cell clones were used to elicit DTH; there was no difference between activated D9 and activated A2b.

Naïve rats responded more strongly to the activated clones than they did to the nonactivated clones, and the response to the acti-

vated clones was enhanced after T cell vaccination (Table 1). The strength of this response to activated T cells probably obscured the clone-specific response to D9 detectable with resting D9 cells. These results suggested that rats vaccinated with activated T cell clones may develop responses to activation markers (anti-ergotypic) as well as to clone-specific markers (anti-idiotypic).

We designed experiments to determine what triggers anti-ergotypic reactivity, the identity of the anti-ergotypic cells, and

whether anti-ergotypic cells can regulate an autoimmune disease in vivo. To investigate the induction of anti-ergotypic activity, we injected rats in the hind footpads with activated T cell clones A2b or D9, with mitogen-activated syngeneic spleen cells, or with the immunogen *M. tuberculosis*. Seven days later we detected the development of anti-ergotypic activity by measuring the proliferative responses of the draining lymph node cells in vitro to irradiated stimulator cells composed of activated or nonactivated clones or spleen cells (Table 2). Lymph node cells from naïve rats (group 1) showed a background proliferative response to irradiated syngeneic D9 cells and to spleen cells activated or not. Previous administration to the rats of T clones A2b or D9, concanavalin A (Con A)-activated spleen cells, or *M. tuberculosis* antigen led to an increase in the background proliferation of the popliteal lymph node cells measured in the absence of added stimulator cells. The addition of nonactivated stimulator cells, either D9 or spleen cells, led to only a modest increase in the proliferative response above the spontaneous background. However, the addition of activated stimulator cells triggered a marked response. The rats that had been vaccinated with activated A2b, D9, or spleen cells (groups 2, 3, and 4) showed a four- to eightfold increase in proliferation compared to that induced by the nonactivated stimulator cells. The responses of the lymph node cells from the rats primed with *M. tuberculosis* antigen (group 5) were relatively weaker but were still significantly higher than the background stimulation obtained either without stimulator cells or with nonactivated stimulator cells. Endogenous T cells activated by the *M. tuberculosis* antigen most probably served to induce the anti-ergotypic response.

To test whether the anti-ergotypic cells were stimulated by factors produced by activated T cells or by their structural components, we measured responses to D9 cells disrupted by shock freezing, to culture medium obtained from activated D9 cells, or to culture medium from Con A-activated spleen cells, which contain interleukin-2 (IL-2). Neither the supernatants of irradiated activated D9 (7,943 ± 859 cpm) nor spleen cells (8,843 ± 1,012 cpm) could stimulate anti-ergotypic cells (background 7,819 ± 689 cpm), but the disrupted D9 cells did stimulate these cells (37,212 ± 3,087 cpm). Therefore, it is likely that the anti-ergotypic cells responded to a structural component of activated cells and not to some extracellular lymphokine.

To identify the anti-ergotypic cells, we isolated by density gradient the blast cells responding to syngeneic activated T cells

**Table 2.** Lymph node cells from rats injected with activated cells or *M. tuberculosis* antigen respond to syngeneic-activated T cells in vitro. Clones A2b and D9 were activated for 72 hours by incubation with their respective antigens, *M. tuberculosis* (MT) and BP (10 µg/ml), in the presence of irradiated (2500 R) autologous thymocytes. Spleen cells were stimulated for 48 hours by Con A (1.25 µg/ml). All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 1% autologous rat serum, 1 mM glutamine,  $5 \times 10^{-5}$  M 2-mercaptoethanol, and antibiotics (22). Activated cells were enriched by Ficoll gradient centrifugation (22). After being washed,  $2.5 \times 10^6$  living activated cells were injected into each hind footpad of groups of five 8- to 12-week-old female Lewis rats, and the popliteal lymph nodes were removed on day 7. The lymph node cells of each group were pooled, suspended in the above medium, and placed in U-shaped 96-well microtiter plates at a concentration of  $2 \times 10^5$  cells per well. Stimulator cells were irradiated with 3000 R and added at  $2 \times 10^4$  cells per well. Nonactivated cells had been kept in antigen-free medium for at least 5 days after antigen stimulation. This medium was supplemented with 10% (v/v) supernatant of Con A-stimulated splenocytes as a source of IL-2 and 10% heat-inactivated horse serum (22). The generation of anti-ergotypic cells after immunization to *M. tuberculosis* was tested by injecting 10-week-old female Lewis rats with 50 µl of complete Freund's adjuvant (Difco) into each hind footpad. Popliteal lymph nodes were removed 9 days later and the cells were cultured quadruplicate in 96-well U-shaped microtiter plates at  $2 \times 10^5$  cells per well as above. Values are counts per minute ( $\times 10^{-3}$ ) ± SD.

Group	Induction in vivo	Proliferative response to stimulator cells				
		Nonactivated			Activated	
		None	D9	Spleen	D9	Spleen
1	None	1.4 ± 0.2	1.5 ± 0.2	1.7 ± 0.1	1.4 ± 0.2	1.4 ± 0.3
2	A2b	7.9 ± 0.9	12.3 ± 2.1	7.3 ± 1.1	47.2 ± 3.4	51.0 ± 4.8
3	D9	9.9 ± 1.2	11.3 ± 1.1	8.4 ± 1.0	65.5 ± 7.0	68.2 ± 10.9
4	Spleen	12.4 ± 1.0	18.4 ± 1.3	10.2 ± 1.4	67.2 ± 6.4	66.7 ± 7.8
5	MT antigen	14.5 ± 1.3	15.1 ± 1.6	10.9 ± 0.6	29.5 ± 2.2	35.2 ± 3.1

**Table 3.** Anti-ergotypic cells protect against EAE. Rats were treated by adoptive transfer of  $5 \times 10^6$  anti-ergotypic or control (A2b) T cells administered intraperitoneally. The rats were challenged on the day of treatment to measure their susceptibility to EAE, either adoptive or active. Adoptively transferred EAE was produced by injecting  $3 \times 10^6$  BP-activated D9 cells intravenously. Active EAE was induced by injecting 25 µg of BP in complete Freund's adjuvant into the hind footpads (5). In active EAE a second injection of  $5 \times 10^6$  anti-ergotypic cells was done 3 days later. The incidence and clinical severity of EAE was measured on a scale of 0 to 4 as described (22). Passively transferred anti-ergotypic cells were generated as follows: naïve rats were injected with  $2.5 \times 10^6$  activated A2b cells into each hind footpad. Popliteal lymph node cells were removed 7 days later and restimulated in vitro ( $5 \times 10^6$  cells per milliliter) with Con A-activated irradiated (3000 R) syngeneic splenocytes ( $5 \times 10^5$  cells per milliliter). After 72 hours the cells were harvested, enriched by Ficoll gradient separation, washed, and injected intraperitoneally ( $5 \times 10^6$  cell per animal). Values are mean ± SE.

EAE induction	Group	Cell treatment	EAE	
			Incidence	Maximum clinical score
D9	1	None	10/10	3.1 ± 0.11
	2	Control T cells (A2b)	5/5	3.2 ± 0.2
	3	Anti-ergotypic T cells	0/5	0 ± 0
BP/CFA	4	None	5/5	2.8 ± 0.2
	5	Anti-ergotypic T cells	5/5	1.2* ± 0.49

\*P < 0.05 compared to group 4 (Student's t test).

and assayed them by fluorescence cytometry for the membrane markers of helper (CD4) or suppressor-cytotoxic (CD8) T cells (13). The responding population included T cells positive for CD4 (61%) and T cells positive for CD8 (32%). Although the responding population probably included more than anti-ergotypic T cells, these results suggest that the anti-ergotypic cells may be a mixed population of T cells with both types of markers.

The ability of anti-ergotypic T cells to regulate immune reactions in vivo was tested by measuring their effects on EAE. Anti-ergotypic cell populations were selected in vitro and transferred to recipient rats, which were then challenged to induce EAE, either by active immunization to BP or by adoptive transfer of anti-BP T cells. Rats were inoculated in the hind footpads with A2b cells, and 1 week later the popliteal lymph node cells were removed and cultured in vitro with Con A-activated irradiated spleen cells to stimulate the anti-ergotypic T cells. The activated spleen stimulator cells were lethally irradiated to cause them to die and disintegrate during culture. The responding anti-ergotypic T cells were enriched by separation on a density gradient and then injected intraperitoneally into Lewis rats. As control T cells, we used the A2b clone, which is not anti-ergotypic (does not respond to other activated T cells). The susceptibility of the rats to subsequent induction of EAE, adoptively produced by clone D9 or actively produced by immunization to BP, is shown in Table 3. The Lewis rats that were challenged by D9 T cells without having been treated with anti-ergotypic T cells developed severe EAE (groups 1 and 2). In contrast, the rats that were treated with anti-ergotypic T cells were completely resistant to adoptive EAE (group 3). Thus, intraperitoneal administration of preformed anti-ergotypic T cells suppressed the transfer of EAE by intravenous D9 T cells. The anti-ergotypic cells suppressed equally well the transfer of EAE when mixed directly with the D9 anti-BP cells (16).

Administration of clone A2b at the time of challenge with anti-BP clone D9 did not protect rats against EAE (group 2 in Table

3). However, as illustrated in the experiment described above, repeated administration of clone A2b for several weeks before EAE challenge did induce measurable resistance. Thus activated A2b cells are not directly anti-ergotypic, but like other activated T cells, activated A2b can induce a protective anti-ergotypic response (16).

Intraperitoneal inoculation of anti-ergotypic T cells led to a mild but significant suppression of active EAE (group 5 in Table 3).

The observations that, on the one hand, preformed anti-ergotypic T cells can suppress endogenously generated T effector cells mediating active EAE and that, on the other hand, anti-ergotypic T cells can be generated in the course of immunization in vivo suggest that these cells might fulfill a physiological function in regulating the immune system. Because the activation of a T cell clone, and not only its existence, needs control, the immune system would be expected to contain elements such as anti-ergotypic T cells that can recognize activated T cells. The mechanism by which the anti-ergotypic reaction suppressed the anti-BP effector T cells is not clear; in vitro such cells did not lyse the activated T cells that induced their proliferation (16). It is conceivable that the EAE effector T lymphocytes might be neutralized in vivo by an anti-ergotypic DTH reaction similar to that illustrated in Table 1.

Autologous mixed-lymphocyte reactive (AMLR) T cells may be another example of a class of regulatory T cells responsive to signals other than conventional antigens or idiotypes (17). It has been proposed that AMLR T cells recognize self-class II major histocompatibility complex (MHC) gene products (18). However, the anti-ergotypic response was not MHC-restricted, and preincubation of activated stimulator T cells with either class I or class II monoclonal antibodies did not inhibit the proliferation of the responding anti-ergotypic cells in vitro (16). Similarly, treatment of activated stimulator T cells with antibodies to the IL-2 receptor did not inhibit the proliferation of responding anti-ergotypic T cells. The activation signal (the ergotope) recognized

by anti-ergotypic T cells and how these cells might function as physiological regulators of immunity need further study. Human T cell clones proliferate in vitro in response to autologous activated T cells (19). This proliferative response was inhibited by the presence of antibodies to cell adhesion molecules (20). Investigation of the role of cell adhesion molecules as possible ergotopes regulating EAE awaits the characterization of these markers in the rat. Vaccination with activated T cells can induce resistance to experimental autoimmune disease by a combination of anti-ergotypic and anti-idiotypic mechanisms. The contribution of these control mechanisms to therapeutic human T cell vaccination remains to be seen (21).

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