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Autoimmune Thyroiditis (EAT) in Genetically Resistant Mice Mediated by a T Cell Line

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Experimental autoimmune thyroiditis (EAT) can be induced in genetically susceptible strains of mice by immunization to mouse thyroglobulin (Tg). EAT also can be produced by administration of anti-mouse Tg T cell lines and clones. Previously we were able to raise virulent anti-Tg T cell lines from mice genetically susceptible to EAT. These virulent lines, upon attenuation, were able to vaccinate the susceptible mice against EAT. We now report the isolation of a virulent T cell line from C57BL/6 mice genetically resistant to EAT. The T cell line and its clones recognize a Tg epitope cross-reactive between mouse and bovine Tg. Unexpectedly, the virulent anti-Tg line attenuated in various ways failed to vaccinate C57BL/6 mice against EAT mediated by the line itself. These results shed some light on the regulation of autoimmunity.

Introduction

Mice of the C57BL/6 (B6) strain are low responders to mouse thyroglobulin (Tg) and consequently are resistant to experimental autoimmune thyroiditis (EAT) inducible in other strains by active immunization to mouse-Tg [1]. The H-2K class I major histocompatibility complex (MHC) gene was found to be important to the EAT phenotype; the B6.H-2^{bm1} strain, essentially B6 mice with an H-2K mutation, were susceptible to EAT [2]. The mutant H-2^{bm1} MHC class I gene product was subsequently found to function in presenting mouse-Tg at the level of the target organ [3]. The native H-2^{b} gene products of B6 apparently do not support strong T cell responses to Tg epitopes specific for mouse-Tg.

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Despite their low response to mouse-Tg, B6 mice do respond well to bovine-Tg. However, bovine-Tg is a poor inducer of EAT [4], apparently because the bovine-specific epitopes dominate the T cell response; upon immunization to bovine-Tg there is only a negligible response to mouse-Tg.

This paper reports our success in isolating from B6 mice a line of T cells responsive to an epitope shared by mouse and bovine-Tg. This line was highly virulent in B6 mice. However, unlike virulent lines of high responder T cells reactive to epitopes specific to mouse-Tg, the B6 line could not be used to induce resistance to itself by standard procedures of T cell vaccination.

Materials and methods

Mice

Inbred strains of mice C57BL/6 (B6), B6.H-2^{bm1} (bm1), C3H/eB (C3H) and (C3H × B6)F_1 were supplied by Jackson Laboratories (Bar Harbor, ME, USA). Female mice were used at the age of 2 months. Each experimental group contained five to six mice.

Thyroglobulin

Bovine-Tg was purchased from Sigma Chemicals (St Louis, MO, USA) and mouse-Tg was prepared from thyroid glands of C3H/eB mice as described [4] without the step of column chromatography.

Immunization to Tg and EAT

EAT was induced as described [3] by two weekly subcutaneous inoculations of 300 μg of mouse-Tg or of 50 μg of bovine-Tg, each emulsified in 0.1 ml of incomplete Freund's adjuvant (Difco, Detroit, MI, USA) to which had been added 7 mg/ml killed *Mycobacterium tuberculosis*, H37Ra (Difco). Thyroid glands were removed 4 to 5 weeks later to assay thyroiditis histologically after fixation and staining with hematoxylin and eosin and with cresyl green. Thyroiditis was graded by an observer ignorant of the identity of the experimental group. A Pathology Index (PI) was assigned by the average area of thyroid section infiltrated: 1 = 25% of the gland, 2 = 50%, 3 = 75% and 4 = 100%.

To raise lines of T cells, the draining popliteal lymph nodes were removed 9 days after the second of two weekly injections of mouse-Tg (150 μg) into each hind foot-pad or of bovine-Tg (50 μg) in 0.1 ml of incomplete Freund's adjuvant containing 1 mg of *M. tuberculosis* H37Ra, as described [5].

Proliferative responses

T cell responses from the draining popliteal lymph nodes were measured in triplicate wells as described [5] and recorded as the Δ cpm (mean cpm of experimental group − mean cpm of control group) and as the stimulation index (S.I. = mean cpm of

experimental group ÷ mean cpm of control group). Standard deviations were always less than 10% of the means.

T cell lines

The isolation of T cell lines was done essentially as previously described [5] with some modifications. The lymph node cells, washed in culture medium without added serum, were suspended (5×10^6/ml) in 25 ml tissue culture flasks (Nunc Products, Denmark) and incubated with mouse or bovine-Tg (100 μg/ml). After incubation for 72 h in air + 7.5% CO_2 at 37°C, the cells were collected, washed in medium and resuspended (5 to 10×10^6/ml) in 25 ml flasks. Culture medium was replaced every 3 to 4 days and the cultures were restimulated with Tg every 8 to 14 days. T cell lines, 2×10^5/ml, were activated by incubation with Tg (50 μg/ml) and irradiated (1,500R) syngeneic spleen cells (5×10^6/ml) as a source of antigen presenting cells (APC) in 25 ml flasks. After 72 h, the cells were used to produce thyroiditis or to vaccinate against thyroiditis. The virulent T cell line TF1 was raised from (C3H × B6) F1 mice as described [5].

Thyroiditis produced by T cells

Activated line cells, 2 to 3×10^6 per mouse, were intraperitoneally inoculated (ip) into syngeneic mice and the mice were examined 1 week later for the development of thyroiditis as described [5].

T cell vaccination

Mice were inoculated ip with 3×10^6 activated line cells that had been attenuated by gamma irradiation (1,500R) [5], by hydrostatic pressure, or by the chemical cross-linker glutardialdehyde as described [6]. One month later, the mice were challenged to induce EAT either adoptively, by injection of intact activated line cells, or by active immunization to Tg [5].

Results

Isolation of a virulent T cell line from B6 mice

Our strategy was to immunize B6 mice with bovine-Tg and then repeatedly to stimulate *in vitro* the T cells from the draining popliteal lymph nodes using either bovine or mouse-Tg. Table 1 shows the proliferative responses to bovine or to mouse-Tg and the EAT produced by the primed lymph node cell population and by the line cells obtained after 3, 4 or 7 stimulations with either bovine or mouse-Tg.

The primed lymph node cells showed a strong response to bovine-Tg and very little response to mouse-Tg. Transfer of these cells to recipient B6 mice caused no EAT. The virulent control T cell line, TF1 [5], showed a strong response to mouse-Tg and no response to bovine-Tg. The TF1 line was strongly pathogenic in (C3H × B6)F_1 mice *in vivo* causing severe EAT (Table 1).

From the B6 primed lymph node cells two lines were developed. The B6-B line was selected using bovine-Tg. It showed a very strong proliferative response to bovine-Tg and no response to mouse-Tg. This line was avirulent. The virulent B6-M line was obtained by priming B6 mice with bovine-Tg and selecting the T cells *in vitro* with mouse-Tg. A negligible response to mouse-Tg was seen after three

Table 1. *Isolation of thyroiditogenic T cell line from B6 low responder mice*

Responding cells	Proliferative response to Tg $\Delta cpm \times 10^{-3}$ (SI)		
	Bovine	Mouse	EAT (P1)
Bovine-Tg primed lymph node	79 (13)	3 (1.5)	0
Line B6-B: Bovine-Tg Stimulations			
3	43 (4)	0 (1)	0
4	134 (34)	0 (1)	0
7	201 (82)	2 (1.1)	0
Line B6-M: Mouse-Tg Stimulations			
3	N.D.	3 (1.6)	0
4	66 (17)	26 (7.2)	1
7	71 (29)	81 (33)	4
Line TF1	0 (1)	210 (21)	4

B6 mice were immunized to bovine-Tg and two T cell lines were developed using repeated stimulations with bovine-Tg or with mouse-Tg: B6-B (bovine-Tg) and B6-M (mouse-Tg). After 3,4 and 7 stimulations, the proliferative responses to bovine-Tg and to mouse-Tg were measured and the capacity of the cells to cause EAT was assayed in syngeneic B6 mice. Line TF1 had been raised previously from $(C3H \times B6)F_1$ mice (5) and was tested in such mice.

cycles of stimulation. However, after four stimulations a response to mouse-Tg was detectable. This response increased until after seven cycles of selection it equalled the response to bovine-Tg. The equal response of the B6-M line to both bovine and mouse-Tg has remained stable and four clones isolated from the B6-M line by limiting dilution also responded to both bovine and to mouse-Tg (Table 2). Line B6-M, unlike line B6-B, was found to be markedly virulent (Table 1). Three of the four B6-M clones were also virulent (Table 2). Thus, we isolated three lines of anti-Tg T cells, each recognizing different Tg epitopes: line B6-M recognizes a bovine-Tg specific epitope(s) and is not virulent; virulent line TF1 recognizes a mouse-Tg specific epitope(s); and line B6-M recognizes a bovine-mouse cross-reactive epitope(s) and is virulent.

Genetic restrictions in Tg presentation

Lines B6-B and B6-M were found to have a $CD4^+$ and $CD8^-$ phenotype similar to line TF1, measured as described (data not shown) [5]. To find out whether there were genetic restrictions on the interaction with antigen presenting cells (APC), proliferation of the lines to their specific Tg antigen was measured using APC from various sources. The results are shown in Table 3. It can be seen that line TF1 responded to

Table 2. *Clones of Line B6-M*

Clone	Proliferative response Δcpm × 10^{-3} (SI) Bovine-Tg	Mouse-Tg	EAT (PI)
a1	9.6 (9)	5.6 (6)	0
b2	5.0 (6)	8.3 (8)	3
e1	4.2 (4)	2.7 (3)	4
f1	2.4 (3)	4.2 (5)	4

Line B6-M was cloned by limiting dilution; 0.3 cells/well. The resulting clones were expanded and tested for proliferation and for their ability to cause EAT (5).

Table 3. *Genetic restrictions of APC on proliferation of anti-Tg lines*

Line	Proliferative responses to Tg Δcpm × 10^{-3}(SI) APC B6	bm1	$(C^3H \times B6)F_1$	C3H
B6-B	245 (12)	337 (8)	268 (10)	0 (1)
B6-M	262 (18)	204 (11)	141 (9)	0 (1)
TF1	0 (1)	0 (1)	208 (9)	282 (12)

The responses of lines to bovine (B6-B) or to mouse-Tg (B6-M, TF1) were measured using splenic APC derived from the indicated mouse strains.

mouse-Tg in the presence of syngeneic $(C3H \times B6)F_1$ or semi-syngeneic C3H spleen cells as a source of APC. There was no response when spleen APC originated from B6 or $B6^{bm1}$ strains. Thus line TF1 appeared to be restricted by the $H\text{-}2^k$ high responder parental alleles, but not by the low responder parental $H\text{-}2^b$ alleles of its F_1 MHC genotype. This finding is compatible with the notion that MHC molecules of $H\text{-}2^k$, but not of $H\text{-}2^b$, are efficient presenters of mouse-Tg epitopes.

Lines B6-B and B6-M, in contrast, appeared to be restricted to $H\text{-}2^b$ gene products; they responded to Tg in the presence of APC of B6, bm1 or (C3H × B6)F1 spleen cells, but not to APC of C3H origin.

Genetic restrictions in mediation of EAT

Table 4 shows the ability of the lines to mediate EAT in various strains of recipient mice. Line TF1 showed essentially the same restrictions in producing disease as it did in its *in vitro* response to mouse-Tg: it caused EAT in $(C3H \times B6)F_1$ mice and in C3H mice, but not in B6 or bm1 mice. As expected, the avirulent line B6-B caused no disease in any of the mice.

Table 4. *Genetic restrictions on production of EAT* in vivo

	EAT in recipient mice (PI)			
Lines	B6	$B6^{bm1}$	$(C^{3H} \times B6)F_1$	C3H
B6-B	0	0	0	0
B6-M	4	1.25	0.6	0.4
TF1	0.25	1	4	4

T cell lines were activated by incubation with Tg and then injected into recipient mice of various strains to test their ability to produce EAT.

Virulent B6-M, in contrast to line TF1, showed a pattern of disease restriction that differed from its APC restriction *in vitro*. It caused severe EAT in syngeneic B6 mice, mild EAT in mutant bm1 mice, and no EAT in semi-syngeneic $(C3H \times B6)F_1$ mice or in allogeneic C3H mice. Thus, the ability of a virulent T cell to proliferate in response to mouse-Tg *in vitro* does not necessarily correlate with its ability to mediate disease *in vivo*.

Line B6-M does not vaccinate against EAT

It was previously demonstrated that line TF1 could be used to induce resistance to EAT by vaccinating syngeneic mice with irradiated TF1 line cells [5]. Experiments were performed to find out whether lines B6-B or B6-M could also serve as vaccines. The results are shown in Table 5.

Unvaccinated $(C3H \times B6)F_1$ mice were susceptible to EAT induced by active immunization with mouse-Tg in CFA or produced adoptively by inoculation with activated TF1 T cells. Note that adoptive EAT was much more severe than was actively induced EAT. T cell vaccination with attenuated TF1 line cells completely prevented EAT induced in either way. Vaccination with B6-M T cells did not protect the mice.

In contrast to the $(C3H \times B6)F_1$ mice, unvaccinated B6 mice were susceptible only to EAT mediated by the B6-M line. As expected for low-responder mice, B6 mice did not develop EAT following active immunization with mouse-Tg.

Vaccination with B6-M or B6-B line cells treated by irradiation failed to induce resistance to adoptive EAT caused by virulent B6-M T cells. B6-M T cells attenuated by treatment with hydrostatic pressure or with glutaraldehyde [6] also failed to induce resistance to EAT (not shown). Thus, the low responder B6 mice, unlike the high responder $(C3H \times B6)F_1$ mice, could not be effectively vaccinated against EAT using the T cells capable of inducing the disease.

Discussion

The results presented here support two conclusions: T cells responsive to epitopes common to mouse and bovine-Tg are capable of causing EAT and B6 mice develop

Table 5. *Vaccination against EAT: adoptive or active*

Mouse	T Cell vaccination	EAT challenge	EAT (PI)
$(C3H \times B6)F_1$	None	TF1	3
		Tg/CFA	1.5
	TF1	TF1	0
		Tg/CFA	0
	B6-M	TF1	3
		Tg/CFA	1.5
B6	None	TF1	0
		B6-M	3
		B6-B	0
		Tg/CFA	0
	B6-M	B6-M	3
		B6-B	0
	B6-B	B6-M	4
		B6-B	0

Mice of the (C3H × B6)F1 or B6 strains were challenged to induce EAT by active immunization or by adoptive transfer (2×10^6 T cells) after some had been vaccinated with irradiated line cells (5×10^6 T cells).

such virulent T cells following immunization to bovine-Tg. Two questions follow: why is bovine-Tg not thyroiditogenic in mice, and why do B6 mice not develop EAT following immunization with either mouse or bovine-Tg?

Inspection of Table 1 reveals that bovine-Tg specific epitopes are dominant over epitopes cross-reactive with mouse-Tg: bovine-Tg primed lymph node cells of B6 mice showed a strong response to bovine-Tg and hardly any response to mouse-Tg. We previously demonstrated that EAT high responder strain CBA ($H\text{-}2^k$) mice primed with bovine-Tg also responded more strongly to bovine-Tg than they did to mouse-Tg [5]. CBA mice also failed to develop EAT after such immunization. Thus, the dominance of the specific bovine-Tg epitopes diverts the T cell response to epitopes that are not present on mouse-Tg and consequently EAT does not develop.

Nevertheless, the quantitatively weak response to the bovine-mouse-Tg cross-reactive epitopes could be amplified artificially by repeated stimulation of the T cells with mouse-Tg *in vitro*. Apparently, the initial few T cells recognizing the mouse bovine cross-reactive epitope responded, proliferated and, eventually, predominated over the bovine-Tg specific T cells which were deprived of stimulation by bovine-Tg epitopes. In this way repeated stimulation with mouse-Tg can provide an advantage to the minority population of T cells reactive to the cross-reactive Tg epitope. B6 T cells reactive to mouse-Tg specific epitopes did not emerge, probably because such cells did not exist in the initial population of bovine-Tg primed lymph node cells. Indeed, cloning of line B6-M produced only T cells that responded to both bovine and mouse-Tg. This finding argues that the response of line B6-M to both types of Tg reflected the presence of T cells with receptors for a common Tg determinant and was not due to a mixture of clones of which some were specific for only one or the

other of the Tg species. Epitopes shared between mouse and heterologous Tg have been shown to be involved in suppression of the T cell response to Tg [7].

It is reasonable to suppose that the bovine-mouse-Tg cross-reactive clones in the B6-M line were virulent in B6 mice because they could recognize epitopes present on endogenous Tg. Apparently, the number of such cells developing *in vivo* in the wake of active immunization to Tg is too small to cause disease. Indeed we have demonstrated that clones of autoimmune effector T cells will not produce disease unless a sufficient number are administered [8,9]. Thus, the expression of an autoimmune disease depends on the numbers of autoimmune T cells as well as on their specificities.

Why are mouse-Tg specific epitopes or bovine-mouse-Tg cross-reactive epitopes so weak in B6 mice compared with other strains such as $H\text{-}2^k$ mice? The observation that bm1 mice but not B6 mice are susceptible to induction of EAT suggests that the MHC class I H-2K molecule is involved in the presentation of mouse-TG [2]. The bm1 effect could be assigned to the level of the target thyroid gland: $(B6 \times bm1)F_1$ mice were susceptible to induction of EAT, but parental strain B6 thyroid glands implanted under the kidney capsule were not damaged, while parental strain bm1 thyroid implants were damaged [3]. Thus, the target for EAT effector cells was composed of Tg together with a suitable H-2K class I molecule.

Mapping of a critical H-2 gene effect to the H-2K locus suggests that recognition of endogenous Tg is class I restricted, and hence Tg is likely to be recognized by $CD8^+$ T cells [10]. Paradoxically, however, our Tg-specific virulent lines showed the $CD4^+$ $CD8^-$ phenotype of helper T cells and therefore should be restricted by class II MHC molecules [10]. It may be that the $CD4^+$ anti-Tg T cells are themselves class I restricted, or they may recruit or activate endogenous class I restricted $CD8^+$ anti-Tg T cells [11]. However, line TF1 was able to cause EAT in T cell deprived nude mice [5], suggesting that $CD4^+$ T cells can see Tg and damage the thyroid without the assistance of class I restricted, endogenous $CD8^+$ T cells. It is conceivable that EAT developing after active immunization is mediated primarily by class I restricted T cells of the $CD8^+$ phenotype. Nevertheless a sufficient number of $CD4^+$, class II restricted T cell lines may also produce EAT. In other words, active EAT and EAT mediated adoptively by T cell lines might have different effector T cells.

Another paradoxical observation was the divergence between the genetic restrictions of the APC required to induce proliferation of the T cells *in vitro* and the genetic restrictions for mediation of EAT *in vivo*. Line TF1 produced EAT in semisyngeneic C3H mice as well as in syngeneic $(C3H \times B6)F_1$ mice (Tables 3 and 4). Line B6-M, in contrast, responded *in vitro* to mouse-Tg presented by APC from semi-syngeneic $(C3H \times B6)\ F_1$ or by class I mutant bm1 mice; as expected, line B6-M responded to mouse-Tg presented by APC from syngeneic B6 mice. However, line B6-M produced strong EAT only in the syngeneic B6 recipient mice. There was much weaker disease noted in bm1 mice and hardly any in $(C3H \times B6)F_1$ mice. The T cell proliferation results indicated that the B6-M line cells should have been able to recognize mouse-Tg in the bm1 and F_1 recipients; why then were the cells not able to produce EAT in the bm1 or F_1 mice to the same extent as they did in B6 mice? Mice of the bm1 and $(C3H \times B6)F_1$ strains are susceptible to actively induced EAT, so their thyroids must be able to present mouse-Tg. It is conceivable that some form of

hybrid resistance [12] prevented the B6 T cells from functioning in the (C3H × B6)F_1 mice. It is also possible that the bm1 mice may have rejected the B6 T cells [13]. Whatever the mechanisms turn out to be, the results are yet another example of the differences between the behaviors of T cells *in vitro* and *in vivo*.

A final paradox was the ability of the TF1 line to vaccinate (C3H × B6)F_1 mice against EAT, either active or adoptive, and the inability of the B6-M line to vaccinate B6 mice against itself. One might have expected the EAT-resistant B6 mice to be easier subjects to vaccinate than the EAT-susceptible (C3H × B6)F_1 mice, since T cell vaccination ought to be more efficient in adding induced resistance to genetic resistance than it is in adding induced resistance to genetic susceptibility. Nevertheless, the genetically susceptible strain responded to vaccination and the genetically resistant strain did not.

There are at least two possible explanations for the reversal. An unlikely explanation is that line B6-M, for unknown reasons, is uniquely unsuitable as a vaccine and that other, yet to be isolated, B6 lines will function as vaccines. Thus, it is conceivable that B6-M is the exception to our general experience that T cell lines can be made to function as vaccines, if not after irradiation, then after hydrostatic pressure or chemical cross-linking [6]. Line B6-M failed to vaccinate following all of these manouvers.

A more interesting possibility is that the failure of line B6-M to vaccinate is not a unique property of the B6-M line, but that B6 mice are biologically incapable of being vaccinated against EAT using their own virulent T cells.

It appears that the immunological dominance of the autoantigens in experimental animals may be related to endogenous anti-idiotypic T and B cell networks [14–16]. We have found that rats and mice susceptible to autoimmune responses to insulin, to myelin basic protein and to the 65 kDa heat shock protein have preformed networks of lymphocytes recognizing these antigens, together with anti-idiotypic lymphocytes responsive to the antigen-specific autoimmune lymphocytes. Moreover, the effectiveness of T cell vaccination in these disease models seems to be based on amplification of the natural anti-idiotypic T cells that exist even before we vaccinate the animals with their own autoimmune T cells. In other words, T cell vaccination may work by exploiting natural regulatory networks that center around particular dominant self-antigens [14–18]. Indeed, Charreire and her colleagues have shown that a cytotoxic T cell hybridoma clone specific for mouse-Tg could vaccinate H-2^k mice against EAT [19]. The mechanism of vaccination was shown to involve the induction of anti-Tg antibodies and anti-anti-Tg anti-idiotypic antibodies. Thus the anti-idiotypic network centered around Tg seems to include both T cells and B cells. Flynn and Kong demonstrated that T cell vaccination against EAT probably activates both $CD4^+$ and $CD8^+$ regulatory T cells [20].

It is possible that B6 mice may be refractory to T cell vaccination precisely because they are naturally refractory to EAT. B6 mice lack the MHC molecules required for optimal presentation of mouse-Tg; hence they are low responders. If B6 mice cannot respond easily to mouse-Tg, they may fail to develop the natural anti-idiotypic T cells activated by T cell vaccination. Hence, T cell vaccination may not be able to work in animals that, because they are not natural responders, have not been stimulated to organize natural anti-idiotypic regulatory cells. Exposure to the autoantigen itself can induce resistance to development of autoimmune disease

[21–24]; this acquired resistance presupposes the capacity to recognize and respond in some manner to the self antigen. In other words, benign autoimmunity may be a safeguard against autoimmune disease [15,18]. This hypothesis predicts that virulent T cells isolated from genetic low responders in other disease models will also fail to vaccinate.

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