

IN VITRO CORRELATE FOR A CLONAL DELETION*
MECHANISM OF IMMUNE RESPONSE GENE-CONTROLLED
NONRESPONSIVENESS‡

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Recent analyses of immune response (*Ir*)¹ gene-controlled responses have led to the conclusion that nonresponsiveness, in the majority, if not all, of the cases, is caused by the absence of functional T cell clones rather than a failure of antigen presentation (1-4). Theoretically, nonresponsiveness at the T cell level can arise in at least two ways, both associated with the individualization of the T cell repertoire. First, some T cell clones are physically or functionally eliminated because they react with self-major histocompatibility complex (MHC) antigens, or with other self antigens in association with self MHC molecules (5). Second, the T cell repertoire is positively selected to recognize self MHC molecules or other self-antigens in the context of self-MHC molecules, and therefore the clones recognizing foreign antigens that are not identical with or closely related to self antigens are not included in the repertoire (6).

To test whether a case can be made for the involvement of one of these two mechanisms in nonresponsiveness, we have used as a model system the secondary in vitro response of T cells depleted of alloreactive cells to antigens presented by allogeneic antigen-presenting cells (APC) (1). In previous experiments testing the responses to three different antigens [poly(glu⁴⁰ala⁶⁰), poly(glu⁵¹lys³⁴tyr¹⁵) (GLT), and lactate dehydrogenase B] in a large number of allogeneic T cell-APC combinations, we have not found a single case of nonresponsiveness (1, 2, 4). Furthermore, we have demonstrated that these responses are restricted by both A(A_αA_β) and E(E_αE_β) molecules, when the APC express both molecules, and only by the A molecule when the APC do not express cell-surface E molecules (7). The only exception in this pattern was the response of allogeneic (*H-2^d*) T cells to GLT presented by APC expressing both A^k and E^k molecules, which was channelled selectively through the A^k molecules (7). Thus, allogeneic T cells did not appear to recognize GLT in the context of E^k molecules. Using *H-2*-recombinant strains that differ from each other only in terms of expression vs. nonexpression of cell-surface E molecules, we demon-

* Here we use the term "clonal deletion" in a functional sense; we do not wish to imply actual physical elimination of clones.

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¹ Abbreviations used in this paper: A molecule, molecule composed of the A_α and A_β chains; APC, antigen-presenting cell; BUdR, 5-bromo-2-deoxyuridine; E molecule, molecule composed of the E_α and E_β chains; GLT, poly(glu⁵¹lys³⁴tyr¹⁵); *Ir*, immune response; MHC, major histocompatibility complex; SI, stimulation index.

strate here that the nonresponsiveness to GLT in the context of E^k molecules results from the elimination of E_β^k -specific alloreactive T cells from the responding cell population. The results illustrate that depletion of T cells reacting to a particular MHC molecule can lead to nonresponsiveness to a synthetic antigen.

Materials and Methods

Mice. 10- to 15-wk-old male and female mice were obtained from our colony at the Max Planck Institute for Biology. The strains and their alleles at *H-2* loci are listed in Table I.

Antigen. The random copolymer GLT was a gift from Dr. P. H. Maurer, Department of Biochemistry, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA. The antigen was dissolved in distilled water adjusted to pH 8.1, aliquoted and stored at -70°C . For tissue culture the antigen was diluted in RPMI 1640 (Gibco, BCK Biocult-Chemie, Karlsruhe, Federal Republic of Germany) to a concentration ranging from 0.2 to 0.4 mg/ml and sterilized by γ -irradiation (3,300 rad).

Monoclonal Antibodies. Ascites fluids containing high-titered monoclonal antibodies were produced using the hybridomas 13/4 R5 (anti-Ia.m7), and B22-277 (anti-Ia.m8) (8) obtained from Dr. G. J. Hämmerling, German Cancer Research Center, Heidelberg, Federal Republic of Germany, and 10-2.16 (anti-Ia.m17) (9) obtained from the Salk Institute, San Diego, CA.

Cell Cultures. The culture medium was RPMI 1640 supplemented with 5% heat-inactivated horse serum (Gibco), L-glutamine, antibiotics, and 5×10^{-5} M 2-mercaptoethanol. Removal of alloreactive cells, priming of T cells in vitro, and secondary culture of T cells were performed as described previously (1). Briefly, splenic T cells from unprimed mice were cultured with allogeneic, glass-adherent peritoneal cells for 3 d, and alloreactive T cells were removed by treatment with 5-bromo-2-deoxyuridine (BUdR) and light (10). The surviving T cells were primed in bulk cultures with GLT (40 $\mu\text{g}/\text{ml}$) in the presence of fresh allogeneic, peritoneal adherent cells (as a source of APC) for 3 d, followed by a "resting" period of 4 d without antigen. The T cells were then distributed in flat-bottomed microculture wells ($1 \times 10^5/\text{well}$) together with 1×10^5 fresh APC, with or without antigen. Monoclonal antibiotics, when present, were included in the same culture volume (0.2 ml) at the initiation of secondary cultures. Proliferation was measured after 3 d by [^3H]thymidine incorporation. All determinations were done in triplicate, and the standard deviation was always $\pm 20\%$ of the mean. The results are expressed as Δcpm (cpm in cultures with APC and antigen minus cpm in cultures with APC, without antigen), and stimulation index (SI: the ratio of cpm in cultures with antigen plus APC, and cpm in cultures with APC, without antigen).

Results

Responsiveness of E-disparate T Cell-APC Combinations to GLT. As has been shown by Jones and her colleagues (11, 12), there are several inbred mouse strains that do not express cell surface E molecules because of a mutation in the E_α locus. Furthermore, there exist pairs of recombinant mouse strains that differ from each other only in terms of expression vs. nonexpression of E molecules (see Table I). We have used two of these strain pairs, 4R-2R and 7R-9R, as T cell-APC combinations to test their responsiveness to GLT, an antigen the response to which is E-restricted in most syngeneic combinations (13, 14). As expected, no response occurred in combinations 2R T cells-4R APC, and 9R T cells-7R APC, where the APC were derived from E-nonexpressor strains (Table II). In the reciprocal combinations, that is, 4R T cells-2R APC and 7R T cells-9R APC, response could only be demonstrated in the latter combination (Table II). Thus, GLT was recognized by 7R T cells in the context of $E_\beta^s E_\alpha^k$ molecules, but not by 4R T cells in the context of $E_\beta^k E_\alpha^k$ molecules. These results fit in with previous typing data that have demonstrated responsiveness to GLT in 9R but not in the other three strains after in vivo immunization (13; Z. A. Nagy, unpublished results).

TABLE I
Mouse Strains Used in this Study and Their Responsiveness to GLT

Strain	Abbreviation	<i>H</i> -2 haplo-type	Alleles at <i>H</i> -2 loci						E molecule expressed on cell surface	Response to GLT*
			<i>K</i>	<i>A</i> _α	<i>A</i> _β	<i>E</i> _β	<i>E</i> _α	<i>D</i>		
B10.A(2R)	2R	<i>h2</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>b</i>	E _β ^k E _α ^k	NR
B10.A(4R)	4R	<i>h4</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>O</i>	<i>b</i>	—	NR
B10.A(5R)	5R	<i>i5</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>k</i>	<i>d</i>	E _β ^b E _α ^k	R
B10.S(7R)	7R	<i>t2</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>O</i>	<i>d</i>	—	NR
B10.S(9R)	9R	<i>t4</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>k</i>	<i>d</i>	E _β ^s E _α ^k	R

* See reference 13; NR, nonresponder; R, responder.

TABLE II
Secondary In Vitro Proliferative Response of E-disparate T Cell-APC Combinations Using GLT as Antigen

T cells	APC	Incompatibility*	Proliferation to APC + GLT: Δcpm (SI)	
			Experiment 1	Experiment 2
7R	9R	E _β ^s E _α ^k	22,329 (13.5)	3,348 (2.7)
9R	7R	—	1,929 (2.0)	ND‡
4R	2R	E _β ^k E _α ^k	1,379 (2.2)	170 (1.2)
2R	4R	—	1,512 (1.9)	405 (1.2)

* Preculture of T cells with APC followed by BUdR and light treatment was performed in all combinations, irrespective of the presence or absence of incompatibility.

‡ ND, not done.

TABLE III
Elimination of Alloreactivity against the E^k Molecule Renders 4R T Cells Nonresponsive to GLT Presented by 5R or 9R Cells

T cells	Alloreactivity removed against	APC in 1° and 2° cultures (+ GLT)	Proliferation to APC + GLT: Δcpm (SI)	
			Exp. 1	Exp. 2
4R	5R	5R	23,871 (15.3)	3,831 (6.6)
4R	5R + 2R	5R	1,425 (2.2)	55 (1.0)
4R	9R	9R	4,881 (3.3)	3,928 (3.4)
4R	9R + 2R	9R	-98 (0.8)	174 (1.2)
4R	5R + 9R	5R	6,999 (8.7)	ND*
4R	5R + 9R	9R	15,273 (10.0)	ND

* ND, not done.

Removal of T Cells Alloreactive against 2R Renders 4R T Cells Unresponsiveness to GLT Presented by Allogeneic APC. The nonresponsiveness observed in the 4R T cell-2R APC combination can be explained in three different ways: first, the T cell repertoire of 4R lacks clones recognizing GLT + E_β^kE_α^k; second, 2R APC cannot present GLT in an immunogenic form to 4R T cells; and third, the E_β^kE_α^k-specific alloreactive cells removed from the 4R cell population included also the GLT-specific clones. Our experimental system allowed us to test the last of these three possibilities. The results in Table III demonstrate that 4R T cells, after removal of cells alloreactive against

5R or 9R, can respond to GLT presented by 5R or 9R APC. In contrast, removal of cells alloreactive against 5R + 2R abolishes the response of 4R T cells against GLT presented by 5R cells and, similarly, removal of T cells reacting to 9R + 2R eliminates the response of 4R cells against GLT presented by 9R cells. In control experiments, removal of alloreactivity against 5R + 9R did not abolish the response of 4R T cells to GLT presented by either 5R or 9R APC. Thus, the removal of cells alloreactive against molecules controlled by $H-2^{i5}$ ($K^bA_\beta^bA_\beta^bE_\beta^bE_\alpha^kD^d$), $H-2^{i4}$ ($K^sA_\alpha^sA_\beta^sE_\beta^sE_\alpha^kD^d$) or both these haplotypes has no effect on the response of 4R T cells to GLT, whereas the removal of $E_\beta^kE_\alpha^k$ -specific T cells abolishes the anti-GLT response. An alternative explanation of the data would be that the encounter of 4R T cells with $E_\beta^kE_\alpha^k$ molecules leads to the generation of suppression, which prevents the subsequent response of these cells to GLT. The data in Table IV demonstrate, however, that the response of 4R T cells to GLT + 9R APC is not inhibited by mixing them with 4R T cells that previously encountered 2R cells, indicating that the observed nonresponsiveness is apparently not due to suppression. Taken together, the results demonstrate that among the 4R T cells alloreactive against the E_β^k chain are also cells recognizing GLT presented by allogeneic APC.

4R T Cells Involved in the Response to GLT Presented by 5R and 9R APC. We have recently shown that the response of T cells to an antigen presented by allogeneic APC is restricted by both A and E molecules; in contrast, in syngeneic T cell-APC combinations, *Ir* gene-controlled responses are selectively channelled through either A or E molecules (7). The results in Table V demonstrate that the responses of 4R T cells to GLT + 5R and GLT + 9R APC follow the same rule: they are both A and E restricted, as shown by the inhibition of T cell proliferation by both A- and E-specific monoclonal antibodies. We must, therefore, assume that the removal of E_β^k

TABLE IV
Unresponsiveness of 4R T Cells to GLT Presented by 9R Cells after Depletion of Alloreactivity to 2R + 9R Cells Is Not Due to Suppression

Group	T cells		APC in 1° and 2° cultures (+ GLT)	Secondary proliferation	
	Strain	Alloreactivity removed against		Δ cpm	SI
I*	4R	9R	9R	4,427	4.0
II*	4R	9R + 2R	9R	-70	0.8
III‡	1:1 mixture of T cells from I and II		9R	6,040	4.5

* 1×10^5 T cells in secondary cultures.

‡ 2×10^5 T cells in secondary cultures.

TABLE V
Restriction Molecules Involved in the Response of 4R T Cells to 5R + GLT and 9R + GLT

T cell	APC (+ GLT)	Response		Percent inhibition of response by*	
		Δ cpm	SI	Anti-A	Anti-E
4R	5R	6,442	4.6	80 (Ia.m8)‡	34 (Ia.m7)
4R	9R	4,928	3.4	61 (Ia.m17)	51 (Ia.m7)

* Percent reduction of cpm in the presence of antibodies (final dilution 1:600).

‡ The specificity recognized by each antibody is given in parentheses.

alloreactive cells abolishes anti-GLT responses restricted by four molecules, $A_\alpha^b A_\beta^b$, $E_\beta^b E_\alpha^k$, $A_\alpha^s A_\beta^s$, and $E_\beta^s E_\alpha^k$ (Table III). One can explain this finding by assuming that the four GLT-specific cell populations are cross-reactive. However, the data in Table VI demonstrate that this is not the case: 4R T cells primed to GLT + 9R APC exhibit no cross-reactivity with GLT + 5R APC. Similar results were obtained in the reciprocal combination (i.e., 4R anti-GLT + 5R did not cross-react with GLT + 9R; data not shown).

The results, therefore, can be interpreted in two ways. First, if the T cell receptors recognize a single combinational determinant formed by GLT and the restricting class II molecule, one must assume that the determinants formed by the GLT + A_α^b , GLT + E_β^b , GLT + A_α^s , and GLT + E_β^s combinations are carried by the E_β^k chain. Second, if the T cell receptors recognize GLT and the restricting class II epitope as separate entities, then either the four class II molecules share an epitope or, more likely, one GLT epitope is recognized in four different MHC contexts and this epitope resembles a determinant on the E_β^k chain.

7R T Cells Recognizing GLT Presented by 9R Cells Do Not Cross-React with the E_β^k Chain. The results so far have demonstrated that 4R T cells specific for GLT + 5R or GLT + 9R APC cross-react with alloantigenic determinants on the E_β^k chain. It remained to be determined whether T cells with the same specificity can also be generated in response to GLT in other *H-2* disparate strains or alternatively, whether the presence of GLT-specific T cells that cross-react with the E_β^k chain is a feature of the T cell repertoire in the 4R strain. We tested, therefore, whether the removal of alloreactivity against 2R cells would influence the response of 7R T cells to GLT presented by 9R APC. The experiments in Table VII demonstrate that the elimination of alloreactive cells specific for 2R alloantigens ($K^k A_\alpha^k A_\beta^k E_\beta^k D_\alpha^k D^b$) does not affect the response of 7R T cells to GLT presented by 9R APC. We conclude, therefore, that two mouse strains of different *H-2* haplotypes generate T cells with different specificity in response to the same antigen, even when the antigen is a synthetic polypeptide that, presumably, possesses a small number of epitopes. These results provide evidence for *H-2*-dependent individualization of the T cell repertoire.

TABLE VI

4R T Cells Specific for GLT Presented by 9R APC Do Not Cross-react with GLT Presented by 5R Cells

T cells	Alloreactivity removed against	APC in 1° culture (+ GLT)	APC in 2° culture (+ GLT)	Proliferation to 2° APC + GLT	
				Δ cpm	SI
4R	5R + 9R	9R	9R	8,213	7.3
4R	5R + 9R	9R	5R	586	1.6

TABLE VII

Elimination of Alloreactivity against 2R Does Not Affect the Response of 7R T Cells to GLT Presented by 9R Cells

T cells	Alloreactivity removed against	APC in 1° and 2° cultures (+ GLT)	Proliferation to APC + GLT: Δ cpm (SI)	
			Exp. 1	Exp. 2
7R	9R	9R	16,951 (8.1)	2,918 (4.9)
7R	9R + 2R	9R	16,923 (6.6)	3,584 (2.9)

Discussion

The results reported in this communication demonstrate that 4R T cells, which are nonresponders to GLT presented by either syngeneic or 2R APC, can mount a proliferative response to this antigen presented by either 5R or 9R cells. However, elimination of cells reactive against 2R alloantigens ($E_{\beta}^k E_{\alpha}^k$) renders 4R T cells nonresponders to GLT presented by either 5R or 9R cells, demonstrating that these T cells cross-react with the E_{β}^k chain.

These results illustrate that a "blind spot" in the T cell repertoire can be generated artificially by removing cells alloreactive against a single chain of a class II MHC molecule, and that this "blind spot" mimicks the effects of *Ir* gene-controlled nonresponsiveness. There is some uncertainty as to whether the T cell clones removed are in fact E_{β}^k -specific, or whether they recognize a non-H-2 self-antigen in the context of E_{β}^k . This issue is difficult to clarify, first, because the *H-2* haplotype of 4R (*H-2^{h4}*) is not available on a background other than C57BL/10, and second, because the hypothetical non-H-2 antigen may be monomorphic. However, since these clones proliferate vigorously within 3 d of encounter with $E_{\beta}^k E_{\alpha}^k$ -incompatible cells, it is very likely that they are truly alloreactive, that is, directed against the incompatible MHC molecules of the stimulator cells. The results, therefore, suggest that strains expressing the $E_{\beta}^k E_{\alpha}^k$ molecule are nonresponders to GLT because of tolerance of this molecule. In fact, many such strains (*H-2* haplotypes *a*, *a1*, *aq1*, *h*, *h1*, *h2*, *k*, *m*, *t1*, *w8*, and *w26*) have been tested, and all were found to be nonresponders to GLT (15–17; N. Ishii and Z. A. Nagy, unpublished results). It should be pointed out that the *H-2* haplotypes listed above differ from one another at the *K* and/or *D* loci, but they all share, in addition to E_{β}^k and E_{α}^k , the A_{α}^k and A_{β}^k alleles (only one recombinant has been found thus far that separates the *A* and *E* gene duplexes but it does not carry the *k* alleles; see reference 18). One can conclude, therefore, that the class I loci are irrelevant for the nonresponsiveness to GLT, but a possible role of the A^k alleles cannot be excluded. It is known that in syngeneic combinations all *A* alleles, except A^q and A^{w17} , are nonresponders to GLT; that is, the response in all responder haplotypes, except *H-2^q* and *H-2^{w17}*, is channelled through the *E* molecules (11, 12). The reason for this selective restriction remains one of the unanswered intriguing questions of the *Ir*-gene studies. However, the data presented here (Table VII) indicate that the *A* allele carried by a given strain does influence the clonal composition of the response although it itself cannot confer responsiveness to the strain. Thus, strain 7R that carries the A^s allele generates different T cells in response to GLT than does the 4R strain carrying the A^k allele (both strains are *E*-nonexpressors). We predict, therefore, that a hypothetical recombinant strain carrying the A^s and $E_{\beta}^k E_{\alpha}^k$ alleles would respond to GLT presented on 9R APC, and that the response would not exhibit cross-reactivity with E_{β}^k . A similar prediction is that (2R × 9R)_{F1} T cells would respond to GLT + 9R but would lack the clones cross-reacting with E_{β}^k . Whether the influence of *A* alleles is exerted through tolerance or positive selection is not known. Nevertheless, the results of this study suggest that tolerance of self MHC antigens is one of the mechanisms that can cause *Ir* gene-controlled nonresponsiveness. A similar conclusion concerning the mechanism of nonresponsiveness to the H-Y antigen has recently been reached by Müllbacher (19).

Our results also point out potential complications one can encounter when studying the effect of MHC molecules on the responsiveness to antigens. It is clear from the

data, first, that both class II molecules (A and E) influence the composition of the responding clones even when only one of them serves as a restricting molecule and, second, that tolerance of certain MHC molecules may not totally abolish an antigen-specific response but may lead to the elimination of certain clones without changing the overall responder phenotype of the strain. Therefore, findings similar to the one described here might be exceptional, occurring only when alloreactive cells specific for a certain MHC molecule include the complete clonal spectrum of a response to antigen. This situation may arise when one selects responses involving narrow clonal spectra, or when one studies the reactivity of individual antigen-specific T cell clones with defined allogeneic MHC epitopes.

Summary

We used T cell-antigen-presenting cell (APC) combinations from two pairs of recombinant mouse strains, B10.A(4R)-B10.A(2R) and B10.S(7R)-B10.S(9R) (abbreviated 4R, 2R, 7R, 9R, respectively), which differ from each other only in the nonexpression vs. expression of cell-surface E molecules, to study the mechanism of the *Ir* gene-controlled (E-restricted) response to the terpolymer poly(glu⁵¹lys³⁴tyr¹⁵) (GLT). No response to GLT occurred when the APC were from E-nonexpressor strains 4R and 7R. When APC from E-expressor strains were used and alloreactivity against the incompatible E molecules was removed by BUdR + light treatment, 7R T cells responded to GLT presented by 9R APC, but 4R T cells failed to respond to GLT presented by 2R APC. However, 4R T cells mounted a proliferative response to GLT presented by fully allogeneic 5R or 9R APC. The latter response was completely abolished by the depletion of cells alloreactive against 2R and 5R or 2R and 9R. Since removal of alloreactivity against 5R plus 9R did not affect the response of 4R T cells to GLT presented by either 5R or 9R cells, we conclude that the 4R T cells generated in response to GLT cross-react with the additional incompatibility presented by 2R cells, that is, the E_β^k chain. In contrast, 7R T cells recognizing GLT presented by 9R APC do not cross-react with E_β^k. These results demonstrate that "blind spots" in the T cell repertoire produced by depletion of cells alloreactive against a single chain of a class II MHC molecule can render a strain nonresponsive to a synthetic polypeptide antigen, and that this nonresponsiveness corresponds to that attributed to the MHC-linked *Ir* genes.

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