INHIBITION OF ANTIGEN-SPECIFIC T LYMPHOCYTE ACTIVATION BY STRUCTURALLY RELATED *Ir* GENE-CONTROLLED POLYMERS

II. Competitive Inhibition of I-E-Restricted, Antigen-specific T Cell Responses

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T inducer cells corecognize foreign antigens in association with class II (Ia) major histocompatibility complex (MHC)[1] molecules on the surface of antigen-presenting cells (APC) (1, 2). These allelic MHC gene products (*Ir* genes) both restrict and control responsiveness to foreign epitopes (3, 4). The mechanisms that account for these phenomena are incompletely understood. It is clear that Ia molecules may affect the specificity of antigen responses, which has been defined as determinant selection (5, 6). This observation, along with the current concepts of the T cell receptor, argues for a close association of antigen with Ia molecules (7). These findings do not address the fundamental nature of this association.

It has been possible to detect competition between related antigens for accessory cell antigen presentation, both in the guinea pig and the mouse (8–10). In the latter system, we have demonstrated (9) that this competition is strongly influenced by the APC class II MHC gene products (9). Further, we have detected (11) in parallel, a modification of an Ia determinant upon antigen association with the APC. These findings are consistent with a specific antigen–Ia molecule association, at least in one case. The present studies were initiated to investigate whether antigen competition at the accessory cell level was generalized, or restricted to a single antigen pair and/or Ia allele.

Materials and Methods

Mice. B10.D2 mice, age 5–7 wk, were purchased from The Jackson Laboratory, Bar Harbor, ME. BALB/c AnN (B/c) mice, age 5–8 wk, were purchased from Charles River Breeding Laboratories, Kingston, NY. B10.GD mice were bred in our animal colony. Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institutes of Laboratory

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[1] *Abbreviations used in this paper:* APC, antigen-presenting cell; MHC, major histocompatibility complex; GL, L-glutamic acid^{60}-L-lysine^{40}; GLA, L-glutamic acid^{55}-L-lysine^{35}-L-alanine^{10}; GLleu, L-glutamic acid^{55}-L-lysine^{35}-L-leucine^{10}; GLϕ, L-glutamic acid^{56}-L-lysine^{35}-L-phenylalanine^{9}; GLT, L-glutamic acid^{55}-L-lysine^{35}-L-tyrosine^{10}; Gϕ, L-glutamic acid^{62}-L-phenylalanine^{38}; GT, L-glutamic acid^{50}-L-tyrosine^{50}; IL-2, interleukin 2; mAb, monoclonal antibody.

Animal Resources, National Research Council (DHEW publication No. (NIH) 78-23, revised 1978).

Antigens. L-glutamic acid^{56}-L-lysine^{35}-L-phenylalanine^{9} (GLϕ), L-glutamic acid^{55}-L-lysine^{35}-L-leucine^{10} (GLleu), L-glutamic acid^{55}-L-lysine^{35}-L-tyrosine^{10} (GLT), L-glutamic acid^{55}-L-lysine^{35}-L-alanine^{10} (GLA), L-glutamic acid^{60}-L-lysine^{40} (GL), L-glutamic acid^{62}-L-phenylalanine^{38} (Gϕ), and L-glutamic acid^{50}-L-tyrosine^{50} (GT) were purchased from Miles Laboratories Inc., Elkhart, IN. L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT) was purchased from Vega Biochemicals, Tucson, AZ. Antigens were prepared as previously described (9).

T Cell Hybridomas. T Cell hybridomas reactive to GLϕ were obtained from the fusion of GLϕ-immune, in vitro restimulated proliferating T cell blasts to BW5147 as previously described (10). Specific hybridomas were identified by their ability to produce the lymphokine interleukin 2 (IL-2) upon stimulation with antigen plus syngeneic accessory cells. The GAT + I-A^{d}–specific T cell hybrids RF7.24 (12) and RF9.140 (9), as well as the I-E^{d}-reactive clone RF26.12 (11), have been previously characterized. All hybrids were passaged in vitro in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) with 4.5 g/l glucose, 10% heat-inactivated fetal calf serum, and antibiotics.

Cell Culture. Cell culture media was RPMI 1640 (M. A. Bioproducts, Walkersville, MD) supplemented as previously described (9). T cell hybrids were stimulated at 5×10^{4} per culture with or without a source of accessory cells in the presence or absence of antigen or combinations of antigen, in 200-μl flat-bottomed microtiter plates. The precise amount of each constituent is detailed in the respective experimental protocols. Either 1,660 rad gamma-irradiated spleen or the cloned, Ia^{d}-bearing, in vitro passaged B lymphoblastoid cell line, A20 (13), (made available by Dr. R. Asofsky), were used as sources of accessory cells. In some experiments, A20 APC or the T cell hybrids were antigen pulsed by incubating 5×10^{4} cells/ml with antigen for 18 h at 37°C, followed by extensive washing. In some cultures, α-Ia monoclonal antibodies (mAb) were added at a final concentration of 1:8 in culture supernatant. These supernatants were prepared from the hybridomas, MKD6 (14) (α-I-A^{d}), kindly provided by Drs. J. Kappler and P. Marrack) or 14.4.4.S (15) (α-I-E) (made available by Dr. D. Sachs), as previously described (16). T cell hybridoma cultures were incubated at 37°C for 18–24 h at which time 100 μl of supernatant was removed, exposed to 8,000 rad gamma irradiation and assayed for IL-2 content.

IL-2 Assay. The T cell lymphokine IL-2 was assayed as previously described (9, 14). Briefly, 5×10^{3} HT-2 cells (an IL-2-addicted T cell line derived by Dr. J. Watson and obtained from Drs. J. Kappler and P. Marrack) were added to supernatants for assay in flat-bottom microtiter plates and incubated for 24 h at 37°C. Cultures were pulsed with 1 μCi of [^{3}H]thymidine over the last 5 h of incubation, after which time they were harvested with the aid of a semiautomated harvester (Cambridge Technology, Inc., Cambridge, MA) and the incorporation of label into DNA measured by liquid scintillation counting. Data is expressed as the arithmetic mean counts per minute (cpm) of duplicate or triplicate cultures.

Results

Specificity of GLϕ-specific T Cell Hybridomas. Two GLϕ-reactive T cell hybridomas were derived from the fusion of antigen-stimulated, proliferating T cells to the T cell lymphoma, BW5147. As shown in Table I, these hybrid clones were stimulated to produce the lymphokine IL-2 upon coculture with the terpolymer GLϕ and syngeneic APC. Incubation of the hybrids with antigen or accessory cells alone did not stimulate their activation (Table I and data not shown). As further illustrated, the T cell–APC interaction is MHC restricted. Using accessory cells from recombinant inbred mice, this restriction maps to the Eα Eβ genetic subregions of the H-2^{d} haplotype (I-E^{d}). Thus, both of the GLϕ hybrids cooperate with B10.D2 but not B10.GD accessory cells. As expected, the latter

TABLE I

MHC Specificity of Copolymer-specific T Cell Hybridomas

Hybrid	Origin	Accessory cell					Antigen	mAb	cpm ± SEM
		Origin	K	A	E	D			
RF21.21	B/c α-GLϕ	B10.D2	d	d	d	d	—	—	333 ± 22
							GLϕ	—	27,227 ± 2,116
		B10.GD	d	d	b	b	—	—	782 ± 117
							GLϕ	—	763 ± 309
RF21.8	B/c α-GLϕ	B10.D2	d	d	d	d	—	—	378 ± 38
							GLϕ	—	11,785 ± 537
		B10.GD	d	d	b	b	—	—	429 ± 48
							GLϕ	—	742 ± 248
RF7.24	B/c α-GAT	B10.D2	d	d	d	d	—	—	373 ± 14
							GAT	—	29,721 ± 362
		B10.GD	d	d	b	b	—	—	931 ± 121
							GAT	—	28,933 ± 489
RF21.21	B/c α-GLϕ	A20	d	d	d	d	—	—	897*
							GLϕ	—	32,119*
							GLϕ	α-I-E^d	3,276*
							GLϕ	α-I-A^d	28,591*
RF21.8	B/c α-GLϕ	A20	d	d	d	d	—	—	1,281 ± 576
							GLϕ	—	38,080 ± 269
							GLϕ	α-I-E^d	958 ± 250
							GLϕ	α-I-A^d	30,674 ± 1,850
RF9.140	B/c α-GAT	A20	d	d	d	d	—	—	491 ± 28
							GAT	—	33,347 ± 2,884
							GAT	α-I-E^d	27,551 ± 1,002
							GAT	α-I-A^d	1,901 ± 486

Microcultures were prepared with 5×10^4 T cell hybridomas and either 10^6 1,660 rad x-irradiated spleen cells or 5×10^4 A20 B lymphoblastoid cells as a source of APC with or without 200 μg/ml (or 33 μg/ml RF21.21 + A20) of the indicated antigen. Some groups contained a 1:8 dilution of mAb containing culture supernatant (α-I-E^d, 14.4.4.S; α-I-A^d, MKD6). 200-μl cultures were incubated for 18 h at 37°C, after which 100 μl supernatant was removed, x-irradiated, and assayed for IL-2 content. Data are expressed as the arithmetic mean cpm obtained from an IL-2 assay on three culture wells, plus or minus SEM.

* Duplicate cultures.

APC are active with a GAT-specific, I-A^d-restricted T cell hybrid. These MHC restriction specificities were confirmed using α-Ia mAb. The activation of the GLϕ-specific hybridomas was blocked by α-I-E^d but not α-I-A^d, while the reciprocal pattern of inhibition was observed for a GAT-specific hybrid. This effect is highly specific since both I-A and I-E gene products are coexpressed on the APC. It should be noted that all of these hybrids cooperate with the same cloned Ia^d-positive tumor cell line. These results indicate that both of the T cell hybridomas, RF21.21 and RF21.8, are specific for GLϕ in association with the I-E^d molecule on accessory cells.

The antigen specificity of the T cell hybrids was analyzed using a panel of

structurally related copolymers. As shown in Table II, the fine specificity of the two GLϕ-reactive hybrids was different. RF21.8 recognized the closely related copolymers GLϕ, GLleu, and GLT. It was nevertheless highly specific for these terpolymers, since GL, Gϕ, GT, and GLA failed to activate this clone. RF21.21 was absolutely specific for GLϕ and was not stimulated by any of the related copolymers.

Inhibition of Hybrid Activation by a Structurally Related Copolymer. The results presented above demonstrate that several of the copolymers, e.g., GLϕ and GLleu, can be presented interchangeably to the RF21.8 hybrid. Therefore, for effective antigen presentation, these two antigens may associate at a similar site on an Ia-bearing accessory cell. The distinct antigenic fine specificity of the RF21.21 hybrid allowed us to examine the effect of the nonactivating copolymer, GLleu, on the GLϕ response of this non–cross-reactive hybrid. As shown in Table III, GLleu is capable of inhibiting the accessory cell–dependent, GLϕ-specific activation of this hybrid. The level of inhibition observed is generally less complete than that observed in the previously reported GT-GAT response (9), but it is nevertheless quite clear.

We further tested the specificity of the GLleu inhibitory effect. As shown in Table IV, GLleu does not inhibit the response of RF7.24 to GAT + I-A^d. Aside from their specificity, RF21.21 and RF7.24 were identically constructed. As is further illustrated (Table IV), the RF7.24 hybrid is inhibited by GT, as we have previously described (9). It is therefore not simply an uninhibitable response. Similarly, we have reported (11) that GLleu does not block the activation of several I-A^d-specific alloreactive hybrids, including those that are specific for the putative GAT/GT Ia association site and which are antigen inhibitable. Further-

TABLE II

Fine Specificity of GLϕ-specific, I-E^d-restricted T Cell Hybrids

Hybrid	A20 H-2^d APC	100 μg/ml antigen	cpm
RF21.21	+	—	474
	+	GLϕ	12,197
	+	GLleu	582
	+	GLT	433
	+	GL	559
	+	Gϕ	1,121
	+	GLA	454
	+	GT	1,154
RF21.8	+	—	432
	+	GLϕ	13,111
	+	GLleu	14,072
	+	GLT	11,782
	+	GL	504
	+	Gϕ	1,015
	+	GLA	536
	+	GT	983

Microcultures were prepared in duplicate as described in Table I, except for the indicated additions of antigen.

TABLE III
Inhibition of a GLϕ T Cell Hybrid's Activation by GLleu

Hybrid	A20 APC	GLϕ	GLLeu	cpm ± SEM
RF21.21	+	−	−	296 ± 37
	+	+	−	44,907 ± 1,523
	+	+	+	15,080 ± 971
	+	−	+	232 ± 6
RF21.8	+	−	−	437 ± 54
	+	+	−	25,078 ± 3,875
	+	+	+	31,247 ± 1,182
	+	−	+	34,113 ± 1,272

Microcultures were prepared as described in Table I, except that GLϕ was added at 33 μg/ml in the presence or absence of 200 μg/ml of GLleu.

TABLE IV
Specificity of GLleu Inhibition of Hybridoma Activation

Exp.	Hybrid	Specificity	B/c APC	Primary antigen	Inhibiting antigen	cpm ± SEM
1	RF21.21	GLϕ + I-E^d	+	—	—	522 ± 27
			+	GLϕ	—	6,341 ± 142
			+	GLϕ	GLLeu	1,437 ± 129
			+	GLϕ	GT	16,115 ± 514
			+	—	GLleu	441 ± 35
			+	—	GT	1,385 ± 59
1	RF7.24	GAT + I-A^d	+	—	—	578 ± 20
			+	GAT	—	17,743 ± 1,003
			+	GAT	GLleu	13,528 ± 1,773
			+	GAT	GT	1,970 ± 298
			+	—	GLleu	509 ± 4
			+	—	GT	1,323 ± 58
1	RF21.8	GAT + I-A^d	+	—	—	716 ± 40
			+	GLϕ	—	12,243 ± 1,888
			+	GLϕ	GLleu	17,735 ± 968
			+	—	GLleu	18,011 ± 583
2	RF26.12	I-E^d	−	−	−	1,551 ± 66
			10×10^5	−	−	23,197 ± 652
			2.5×10^5	−	−	15,013 ± 631
			10×10^5	−	GLleu	22,997 ± 696
			2.5×10^5	−	GLleu	12,365 ± 1,077

Microcultures were prepared as described in Table I, except that in experiment 1, hybrids were stimulated with 100 μg/ml of GLϕ or GAT in the presence or absence of either 200 μg/ml of GLleu or GT. In experiment 2, the number of BALB/c (B/c) APC were titrated in the presence or absence of 500 μg/ml GLleu. RF26.12 is a B10 α-BALB/c alloreactive hybrid.

more, the RF21.21 is not simply more susceptible to inhibition with any inhibiting antigen. As can be seen (Table IV), the GLϕ response of RF21.21 is not inhibited by GT, which is a highly potent inhibitor of the T hybrid response to GAT. The results of these reciprocal inhibitions demonstrate a high degree of specificity to

this phenomenon. The GAT-specific and alloreactive control hybrids are restricted by the I-A^d molecule, while the GLϕ hybrids corecognize I-E^d. To test whether GLleu might be nonspecifically interfering with I-E molecule function, we assessed the effect of this antigen on the activation of a hybrid with specificity for I-E^d. As shown in Table IV, the activation of the I-E^d-specific alloreactive hybrid, RF26.12, was not affected by GLleu even under limiting stimulation and higher concentration of this antigen. Moreover, RF21.8 which corecognizes I-E^d, is activated by GLleu with or without GLϕ. Clearly, I-E molecule function is not nonspecifically affected. Taken together, these results demonstrate that GLleu is not simply toxic in culture and does not generally interfere with APC activity. Further, I-E molecule function on the accessory cells is not nonspecifically inhibited. Moreover, highly selective and reciprocal inhibition of antigen-specific activation occurs between pairs of structurally related antigens even with the same clonal APC.

Fine Specificity of Antigenic Inhibition. The fine specificity of the antigen inhibition was next investigated. As noted above, several of the GL-related copolymers (GLϕ, GLleu, and GLT) will activate RF21.8. Having observed an inhibitory affect of GLleu on the activation of RF21.21, we therefore tested both GLT and GL for their affect on this hybrid's response. As shown in Table V, both of these copolymers inhibited the GLϕ-specific activation of RF21.21. It therefore appears that an amino acid sequence involving L-glutamic acid and L-lysine is critical to this inhibitory effect. There is, however, some difference between the various GL copolymers, with GLleu being the strongest and most efficient inhibitor (data not shown). The inhibition observed with both GLT and GL was highly specific. Neither of these antigens inhibited the GAT response of RF7.24 (Table V), which was inhibitable with GT. Further, GLT activated RF21.8. Since GL is specifically inhibitory for the GLϕ response of RF21.21 and does not activate the RF21.8 hybrid, we could test its inhibitory potential on this more cross-reactive clone. Surprisingly, GL failed to inhibit the response of RF21.8. This finding raised the possibility that the difference in antigen reactivity of this hybrid clone would require a difference in the nature of the corresponding antigenic inhibitor. We therefore tested an additional structurally related but nonactivating copolymer, Gϕ, for an inhibitory effect. As shown in Table VI, Gϕ inhibited the response of RF21.8 to GLϕ. Again, GL was ineffective in blocking this hybrid's response and GLleu caused activation. Interestingly, the reciprocal pattern was seen with the other GLϕ-reactive hybridoma, RF21.21. In this case, Gϕ failed to inhibit (or activate) while both GL and GLleu blocked the response. Together, the results of this selective and reciprocal inhibition of responses to the same antigen on the same clonal APC again demonstrate a very high degree of specificity of this phenomenon.

Competitive Inhibition of Hybrid Activation. The effect of the inhibiting antigen was tested over a range of GLϕ concentration. As can be seen in Fig. 1, at limiting concentration of GLϕ, the response of RF21.21 was almost completely inhibited by GLleu. However, as the concentration of GLϕ was increased, the inhibition was reversed. Identical concentrations of Gϕ or GT failed to cause inhibition even at limiting concentrations of GLϕ. A similar analysis of the effect of Gϕ on the response of RF21.8 is illustrated in Fig. 2. The Gϕ inhibitory effect

TABLE V

Fine Specificity of Antigen Inhibition of Hybrid Activation

Hybrid	B/c APC	Primary antigen	Inhibiting antigen	cpm ± SEM
RF21.21	+	—	—	512 ± 35
	+	GLϕ	—	15,821 ± 332
	+	GLϕ	GLleu	2,039 ± 187
	+	GLϕ	GLT	1,293 ± 109
	+	GLϕ	GL	3,471 ± 604
	+	GLϕ	GT	24,088 ± 449
	+	—	GT	826 ± 62
RF21.8	+	—	—	419 ± 60
	+	GLϕ	—	10,308 ± 967
	+	GLϕ	GLleu	30,514 ± 2,614*
	+	GLϕ	GLT	39,278 ± 1,637*
	+	GLϕ	GL	14,247 ± 2,515*
	+	—	GLleu	25,662 ± 194
	+	—	GLT	51,956 ± 3,207
	+	—	GL	694 ± 49
RF7.24	+	—	—	480 ± 29
	+	GAT	—	38,912 ± 1,110
	+	GAT	GLleu	31,192 ± 1,250
	+	GAT	GLT	37,075 ± 2,596
	+	GAT	GL	39,199 ± 2,131
	+	GAT	GT	1,602 ± 150
	+	—	GLleu	589 ± 63
	+	—	GLT	412 ± 10
	+	—	GL	838 ± 167

Microcultures were prepared as described in Table IV, experiment 1, except that GAT was used at 50 μg/ml. All inhibiting antigens were added at 200 μg/ml.

* The increased responses of RF21.8 observed when GLleu or GLT were added with GLϕ are due to the ability of this hybrid to cross-react with the two former compounds (note responses to GLleu or GLT alone). The response of RF21.8 to GLϕ in the presence of GL was not inhibited (with GL, 10,308; without GL, 14,247).

could also be reversed by increasing the concentration of GLϕ in culture, and as shown, was specific (Figs. 1 and 2).

Identification of the Cellular Site of Antigen Inhibition. To more precisely localize the cellular site of this competitive antigenic inhibition, we performed antigen pulsing experiments. As shown in Tables VII and VIII, the preincubation of APC with GLϕ resulted, as expected, in the immunogenic association of this antigen as assessed in subsequent culture with the GLϕ-specific hybrids. If, however, the competing antigen was present during this initial incubation, successful antigen pulsing was blocked. Several specificity controls are illustrated. As in the preceeding experiments, this effect was both antigen-specific (compare the effects of GLleu vs. GT and of Gϕ) and specific for the appropriate hybridoma (compare RF21.21, RF21.8, and RF7.24). When GLϕ and GLleu, or Gϕ, are

TABLE VI

Reciprocal Inhibition of GLϕ Hybrid Activation by Structurally Related Copolymers

Hybrid	A20 APC	GLϕ	Inhibitor	cpm
RF21.21	+	—	—	1,422
	+	+	—	20,957
	+	+	GLleu	5,558
	+	+	GL	12,110
	+	+	Gϕ	24,855
	+	−	GLleu	1,319
	+	−	GL	1,198
	+	−	Gϕ	2,976
RF21.8	+	−	—	1,362
	+	+	—	10,640
	+	+	GL	14,877
	+	+	Gϕ	2,310
	+	−	GLleu	23,245
	+	−	GL	1,862
	+	−	Gϕ	2,126

Microcultures were prepared in duplicate as described in Table III, except GLϕ was added at 50 μg/ml in the presence or absence of 400 μg/ml of GLleu or GL or 200 μg/ml of Gϕ.

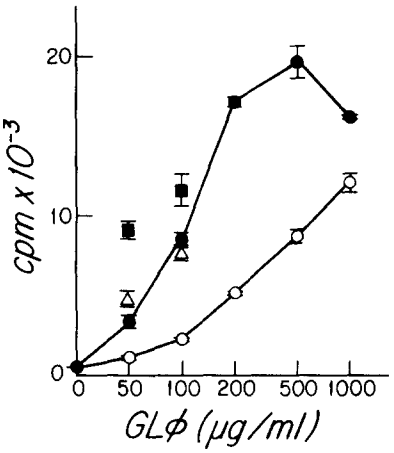

FIGURE 1. GLleu inhibition of the GLϕ response of RF21.21 is reversible. Microcultures were prepared as described in Table I with 5×10^4 RF21.21 with 10^6 irradiated BALB/c splenocytes with or without the indicated amount of GLϕ alone (●) or in the presence of 100 μg/ml of either GLleu (○), Gϕ (△), or GT (■).

added to culture on separately pulsed APC, there was no inhibition of responses. This demonstrates that the inhibitions observed above were not due to the simple carryover of inhibitor into the hybrid cultures, which then might act at some other site, e.g., the T cell hybridoma. Moreover, these results suggest that the blocking effect is not due to the independent association of the nonactivating antigens with the APC, resulting in impotent or tolerizing determinants.

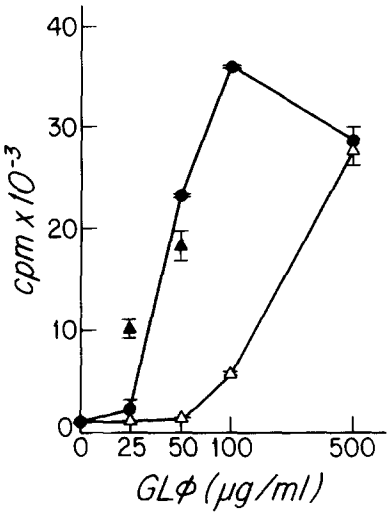

FIGURE 2. Gϕ inhibition of the GLϕ response of RF21.8 is reversible. Microcultures were prepared as described in Table I with 5×10^4 A20 cells with or without the indicated amount of GLϕ alone (●) or in the presence of 200 μg/ml of Gϕ (△) or 400 μg/ml of GL (▲).

The effect of prepulsing the T cell hybrids with the inhibiting antigens previous to stimulating with GLϕ + APC was also tested. Exposing the hybrids to high concentrations of the appropriate inhibitor did not affect their subsequent responses (Table IX). Taken together, these results demonstrate that exposure of the APC to the inhibitors during antigen pulsing is both necessary and sufficient for the inhibitory effect.

The observation that Gϕ and the GL-related copolymers are ineffective at inhibiting GLϕ presentation to RF21.21 and RF21.8, respectively, strongly argues that these inhibitors are not competing at a nonspecific uptake or antigen-processing step. If they were, GLϕ in the presence of either inhibiting copolymer should fail to be presented to both GLϕ-reactive hybrids, which is not observed. This is also suggested by the finding that the competing copolymers can specifically block responses to 18-h GLϕ-prepulsed APC (Table X). This preincubation with GLϕ is sufficient for uptake and processing of this antigen (data not shown). Together, these results suggest that the locus of inhibition in the APC may be distal to the events of antigen uptake and processing.

Discussion

These studies were undertaken to further analyze the effect of structurally related antigens on T cell responses. The major findings of this report are as follows: (*a*) Several structural analogues of GLϕ inhibit the T cell response to this copolymer. (*b*) The inhibition is competitive. (*c*) These effects are highly antigen specific, both in terms of the effective inhibitors and the response that they inhibit. Specific reciprocal inhibitions were demonstrated. (*d*) The antigenic competition occurs at the APC. (*e*) The competition does not appear to be for a nonspecific APC antigen uptake and/or processing step. (*f*) The presentation of

TABLE VII
Inhibition of Antigen Pulsing of Accessory Cells by Competing Copolymers

Exp.	Hybrid	18-h APC pulse		cpm + SEM
		Primary antigen	Inhibiting antigen	
1	RF21.21	—	—	369 ± 24
		GLϕ	—	33,536 ± 1,402
		GLϕ	GLleu	7,195 ± 724
		GLϕ	GT	52,165*
1	RF7.24	—	—	710 ± 117
		GAT	—	44,400 ± 740
		GAT	GLleu	37,810 ± 2,022
		GAT	GT	4,018*
2	RF21.8	—	—	358 ± 42
		GLϕ	—	20,923 ± 1,536
		GLϕ	Gϕ	5,063 ± 336
		—	Gϕ	311 ± 55
2	RF21.21	—	—	345 ± 110
		GLϕ	—	53,462 ± 1,634
		GLϕ	Gϕ	48,469 ± 2,022
		—	Gϕ	471*

Microcultures were prepared as described in Table I, except that the APC was prepulsed with antigen for 18 h at 37°C and washed before hybrid culture to which they were added at 10^5/well. In experiment 1, A20 was pulsed at 5×10^4/ml with or without either 50 μg/ml GLϕ or 20 μg/ml GAT in the presence or absence of 100 μg/ml GLleu or Gϕ. In experiment 2, A20 was pulsed with 50 μg/ml (RF21.8) or 35 μg/ml (RF21.21) of GLϕ in the presence or absence of 300 μg/ml of Gϕ. Multiple independent experiments show that A20 pulsed with the inhibiting antigens alone is not stimulatory for these hybrids (data not shown).
* Duplicate culture.

distinct GLϕ determinants requires antigenically distinct competing antigens for inhibition.

We have previously (9) described the competitive inhibition of the copolymer GT for the presentation of GAT in association with I-A^d on accessory cells. The current studies were undertaken to determine if these observations could be extended to other antigen responses. We chose to study the terpolymer GLϕ because it allowed the analysis of antigen presentation in association with a genetically distinct Ir gene product (I-E^d) relative to that controlling the GAT response (I-A^d) (17). Further, it is a simple antigen, for which a number of structural analogues exist. Therefore, we derived two T cell hybridomas that produced the lymphokine IL-2 specifically upon challenge with GLϕ + I-E^d on APC. The antigenic fine specificity of the two hybridomas was different. One T cell hybrid (RF21.21) recognized only GLϕ while the second (RF21.8) cross-reacted with several GL-related antigens (GLϕ, GLleu, GLT).

To detect antigen competition by a functional T cell assay, the competing

TABLE VIII

Competing Antigens Present on Separately Pulsed APC Are Not Inhibitory

Hybrid	18-h APC pulse			cpm ± SEM
	Primary antigen	Inhibiting antigen		
RF21.21	—	—		2,058 ± 89
	GLϕ	—		14,954 ± 667
	GLϕ	GLleu		4,697 ± 333
	GLϕ	—		
	—	GLleu	MIX	19,623 ± 839
	GLϕ	—		
	—	—	MIX	18,205 ± 1,152
RF21.8	—	—		1,902 ± 88
	GLϕ	—		24,014 ± 594
	GLϕ	Gϕ		12,588 ± 473
	GLϕ	—		
	—	Gϕ	MIX	25,329 ± 976
	GLϕ	—		
	—	—	MIX	27,363 ± 2,659

Microcultures were prepared as described in Table VII. A20 APC were prepulsed with or without 50 μg/ml of GLϕ in the presence or absence of 100 μg/ml GLleu or 200 μg/ml Gϕ and 5×10^4 A20 were added to hybrid culture. Where indicated, 5×10^4 of separately pulsed A20 were added (total 10^5) to the same well.

antigen cannot, itself, be activating. In our previous studies of the GAT response, T cells were genetically unresponsive to the inhibitor GT (9). Since the cross-reactive GL copolymers could associate with the APC, yet not activate RF21.21, they could be tested for their effects on the GLϕ response of this hybrid. We also tested the inhibitory potential of the nonactivating antigens GL and Gϕ on the responses of both hybrids. When added to culture, these antigens could block the GLϕ response of one or the other T cell hybridomas. The nature of this phenomenon appeared identical to that observed in the GT/GAT system. First, the inhibition caused by a constant amount of inhibitor could be reversed by increasing the concentration of GLϕ. The effects were therefore not toxic and the nature of the interaction appears to be competitive. Second, the effect of the competitive inhibitors is highly specific. They do not block the responses of other I-A- or I-E-restricted, accessory cell–dependent T cell hybridomas. Therefore, neither general accessory cell function nor that of its Ia molecules is depressed. Third, the hybrids that are inhibited are not more generally susceptible to inhibition by any antigen. For example, RF21.21, which is inhibited by GL-related antigens, is not affected by either GT or Gϕ despite these later antigens being excellent inhibitors of RF7.24 and RF21.8, respectively. In fact, reciprocal selective inhibitions could be demonstrated between the various hybrid responses. Fourth, the site of the competitive inhibition was at the APC. Thus, the inhibitors

TABLE IX

Pulsing of T Cell Hybridomas with Competing Copolymers Fails to Cause Inhibition

Exp.	Hybrid	18-h hybrid pulse	A20 APC	GLϕ APC pulse	GLϕ in culture	cpm ± SEM
1	RF21.21	MEDIA	+	−	−	453 ± 4
			+	+	−	32,766 ± 1,158
			+	−	+	31,180 ± 3,662
	RF21.21	GLleu	+	−	−	444 ± 27
			+	+	−	31,714 ± 1,233
			+	−	+	32,977 ± 1,651
2	RF21.8	MEDIA	+	−	−	344 ± 16
			+	+	−	7,070 ± 234
			+	−	+	21,188 ± 644
	RF21.8	Gϕ	+	−	−	388 ± 18
			+	+	−	5,590 ± 165
			+	−	+	18,666 ± 834
	RF21.8	GL	+	−	−	405 ± 72
			+	+	−	5,528 ± 320
			+	−	+	20,724 ± 371

T cell hybrids were incubated at 5×10^4 cells/ml with or without 200 μg/ml GLleu (experiment 1) or 200 μg/ml Gϕ or GL (experiment 2), for 18 h at 37°C. Subsequently, these clones were washed and tested for responsiveness to soluble or APC-pulsed GLϕ as follows. Experiment 1: 10^5 50 μg/ml GLϕ 18-h-pulsed A20 or 5×10^4 unpulsed A20 with or without 25 μg/ml of GLϕ. Experiment 2: 5×10^4 200 μg/ml GLϕ 18-h-pulsed A20 or unpulsed A20 with or without 100 μg/ml GLϕ. Microcultures were otherwise prepared as described in Table I. The data in experiment 1 was obtained from the same cultures as in Table VII.

TABLE X

Specific Inhibition of GLϕ-prepulsed APC by Related Copolymers

Hybrid	A20 APC	18-h GLϕ APC pulse	Antigen in culture	cpm ± SEM
RF21.21	+	−	−	226 ± 34
	+	+	−	14,837 ± 2,269
	+	+	GL	7,245 ± 322
	+	+	Gϕ	16,348 ± 906
RF21.8	+	−	−	294 ± 33
	+	+	−	26,198 ± 2,182
	+	+	GL	20,033 ± 871
	+	+	Gϕ	10,490 ± 897

Microcultures were prepared as described in Table I except 10^5 A20 cells that had been preincubated with 200 μg/ml of GLϕ for 18 h at 37°C were added to cultures in the presence or absence of 400 μg/ml of GL or 200 μg/ml of Gϕ.

were active when added to the antigen pulsing of the APC and this effect was again highly specific. Exposure of the APC was both necessary and sufficient for the blocking effect. On the other hand, preincubation of the hybrid with the inhibiting antigen failed to affect subsequent responses. Fifth, the site of antigen

competition is not at antigen uptake or processing by the accessory cell. In the GLϕ system, this was strongly suggested by the reciprocal inhibitions observed with the GL or Gϕ inhibitors on the two distinct hybridoma responses. Thus, if uptake or a common processing step was blocked, the GLϕ response of both hybrids should have been inhibited, which was clearly not the case. Since GLϕ requires processing for presentation (L. Falo, B. Benacerraf, and K. L. Rock, manuscript in preparation), it is possible that responses to distinct epitopes could require differential processing which then might be independently competed. This is unlikely since GLϕ presentation by prepulsed APC that have already processed antigen is still inhibited by the competing analogues. Further, this can also be observed with metabolically inactivated APC, providing the inhibitor does not require antigen processing (L. Falo, B. Benacerraf, and K. L. Rock, manuscript in preparation). As in the GAT system, this observation argues against a covalent antigen association. Collectively, these observations parallel almost exactly the findings of antigen competition in the GAT response. In this latter system, the site of competition appears to be closely associated or identical with the Ia molecule (11). Our observations in the GLϕ system demonstrate a specific association but do not allow such a precise localization. Considering the similarities of the findings in the two antigenic systems, it would seem reasonable to conclude that the underlying mechanism is identical.

The present findings significantly extend our previous observations. They demonstrate that the phenomenon of antigen competition is not isolated. It has now been identified for one pair of antigens in the guinea pig and two distinct antigens in the mouse (8, 9). Whether it will be demonstrable in all antigen responses, particularly for peptides with nonrepeating sequences, needs to be investigated. The absence of reports of competition with conventional proteins could be related to the requirements of native antigens to be processed for antigen presentation (18). The processing step may limit the concentration of the appropriate antigenic fragment available for competition, which is not the case, where examined, for the competing amino acid copolymers (L. Falo, B. Benacerraf, and K. L. Rock, manuscript in preparation). Another advantage of the copolymer antigens as compared with most proteins may be their multivalency and/or charge.

The present results also demonstrate that competition is not limited to I-A-restricted antigens but can be seen with responses controlled by both class II MHC gene loci. Further, our results show that Ir gene–controlled antigens can function as competitive inhibitors for selected T cells of responder animals. This behavior is therefore not limited to antigens to which the animal is genetically nonresponsive. Additionally, the present results clearly show that the antigen competition is highly selective. Using GLϕ and GAT with their respective inhibitors, we have shown specific and reciprocal inhibition even with the same clonal APC. Finally, the current report reveals a high degree of complexity to this phenomenon, where there are multiple independent inhibitors for the same antigen.

The finding that the presentation of GLϕ to two different T cell hybrids was blocked by two distinct inhibitors, respectively, was surprising. The mechanism accounting for this observation is not defined by our data. This result could arise

if the same antigen associated with more than one site on the APC, or if a single site allowed more than one association. The possibility that this site could be involved with antigen processing has to be considered; however, as discussed above, our current data strongly favor a site distal to a processing step. Alternatively, if the inhibitors are complexing with the Ia molecule, they might disrupt Ia determinants (histotopes) rather than antigen association (desetopes) (19). The finding that the specificity of the inhibitors correlated with the fine specificity of the T cell hybridomas is reminiscent of the phenomenon of determinant selection (5, 6). However, only two GLϕ-specific hybrids have been analyzed and this may or may not be generalizable. Additional, hybrid clones need to be studied. It is interesting that there is a dissociation of responsiveness to the various GL copolymers controlled by the different Ir gene alleles. Thus, H-2^d allows responsiveness to GLϕ, GLleu, and GLT, while several other haplotypes that respond to GLϕ fail to respond to the related copolymers (20, 21). Given the current results, it is possible that these patterns reflect differences in antigen association. We conclude that there is a specific and functionally relevant association of the antigen GLϕ with H-2^d-bearing accessory cells. The methods outlined in this report may allow further investigations of the issues raised above.

Summary

Our previous studies have defined a highly specific competitive inhibition between a pair of structurally related antigens (GT and GAT) for antigen presentation by accessory cells. The present report investigates this phenomenon in a second antigenic system, which is controlled by a distinct Ir gene product. Two GLϕ-specific, I-E^d-restricted, interleukin 2–producing T cell hybridomas were constructed. The antigenic fine specificity of these two hybrid clones was distinct. One hybrid reacted solely with GLϕ while the second cross-reacted with GLleu and GLT. These latter two copolymers, as well as the antigen GL, were found to inhibit the GLϕ response of the non–cross-reactive hybrid. The structurally related antigen Gϕ was not inhibitory for this clone's response. The cross-reactive GLϕ hybrid could also be inhibited, but, in this case, Gϕ and not GL caused the inhibition. Reciprocal inhibitions could be demonstrated between these and other hybrids (e.g., GAT responsive), indicating a very high degree of specificity to the inhibition. The inhibition caused by the various copolymers was reversible by increasing the concentration of GLϕ. This effect was localized to the antigen-presenting cell and not the T cell hybridoma. Functionally, this competition did not appear to be for antigen uptake or general antigen processing. These findings generalize the phenomenon of antigen competition to a second antigen system in the context of a second Ia molecule. The possible mechanisms accounting for the complex pattern of specificities in this system are discussed.

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References

1. Rosenthal, A. S., and E. M. Shevach. 1973. The function of macrophages in antigen recognition by guinea pig T lymphocytes. I. Requirement for histocompatibility macrophages and lymphocytes. *J. Exp. Med.* 138:1194.
2. Shevach, E. M., and A. S. Rosenthal. 1973. Function of macrophages in antigen recognition by guinea pig T lymphocytes. II. Role of the macrophage in the regulation of genetic control of the immune response. *J. Exp. Med.* 138:1213.
3. Benacerraf, B., and R. N. Germain. 1978. The immune response genes of the major histocompatibility complex. *Immunol. Rev.* 38:70.
4. Schwartz, R. H., A. Yano, and W. E. Paul. 1978. Interaction between antigen-presenting cells and primed T lymphocytes. *Immunol. Rev.* 40:153.
5. Rosenthal, A. S. 1978. Determinant selection and macrophage function between antigen-presenting cells and primed T lymphocytes. *Immunol. Rev.* 40:136.
6. Benacerraf, B. 1978. A hypothesis to relate the specificity of T lymphocytes and the activity of I region-specific Ir genes in macrophages and B lymphocytes. *J. Immunol.* 120:1809.
7. Marrack, P. C., R. Shimonkevitz, D. Hannum, K. Haskins, and J. Kappler. 1983. The major histocompatibility complex-restricted antigen receptor on T cells. IV. An antiidiotypic antibody predicts both antigen and I-specificity. *J. Exp. Med.* 158:1635.
8. Werdelin, O. 1982. Chemically related antigens compete for presentation by accessory cells to T cells. *J. Immunol.* 129:1883.
9. Rock, K. L., and B. Benacerraf. 1983. Inhibition of antigen-specific T lymphocyte activation by structurally related Ir gene-controlled polymers. Evidence of specific competition for accessory cell antigen presentation. *J. Exp. Med.* 157:1618.
10. Rock, K. L., and B. Benacerraf. 1983. MHC-restricted T cell activation: analysis with T cell hybridomas. *Immunol. Rev.* 76:29.
11. Rock, K. L., and B. Benacerraf. 1984. Selective modification of a private Ia allostimulating determinant upon antigen association with an antigen-presenting cell. *J. Exp. Med.* 159:1238.
12. Rock, K. L., and B. Benacerraf. 1983. The role of Ia molecules in the activation of T lymphocytes. III. Antigen-specific, Ia-restricted interleukin 2-producing T cell hybridomas with detectable affinity for the restricting I-A molecule. *J. Exp. Med.* 157:359.
13. Kim, K. J., C. Kanellopoulos-Langvin, R. M. Merwin, D. H. Sachs, and R. Asofsky. 1979. Establishment and characterization of BALB/c lymphoma lines with B cell properties. *J. Immunol.* 122:549.
14. Kappler, J. W., B. Skidmore, J. White, and P. Marrack. 1981. Antigen inducible, H-2 restricted, interleukin 2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. *J. Exp. Med.* 153:1198.
15. Ozato, K., M. Mayer, and D. H. Sachs. 1980. Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. *J. Immunol.* 124:533.
16. Rock, K. L., M. C. Barnes, R. N. Germain, and B. Benacerraf. 1983. The role of Ia molecules in the activation of T lymphocytes. II. Ia-restricted recognition of allo K/D antigens is required for class I MHC-stimulated mixed lymphocyte responses. *J. Immunol.* 130:457.
17. Dorf, M. E., and B. Benacerraf. 1975. Complementation of H-2-linked Ir genes in the mouse. *Proc. Natl. Acad. Sci. USA.* 72:3671.
18. Shimonkevitz, R., J. Kappler, P. Marrack, and H. Grey. 1983. Antigen recognition by H-2-restricted T cells. I. Cell-free antigen processing. *J. Exp. Med.* 158:303.
19. Heber-Katz, E., D. Hansburg, and R. H. Schwartz. 1983. The Ia molecule of antigen-

presenting cells plays a critical role in immune response gene regulation of T cell activation. *J. Mol. Cell. Immunol.* 1:3.

20. Dorf, M. E., M. B. Twigg, and B. Benacerraf. 1976. Genetic control of the immune response to the random linear terpolymer of L-glutamic acid, L-lysine, and L-leucine (GLleu) by complementing Ir genes. *Eur. J. Immunol.* 6:552.
21. Dorf, M. E., P. H. Maurer, C. F. Merryman, and B. Benacerraf. 1976. Inclusion group systems and *cis-trans* effects in responses controlled by the two complementing Ir-GLO genes. *J. Exp. Med.* 143:889.