SELECTIVE MODIFICATION OF A PRIVATE I-A ALLO-STIMULATING DETERMINANT(S) UPON ASSOCIATION OF ANTIGEN WITH AN ANTIGEN-PRESENTING CELL

By KENNETH L. ROCK and BARUJ BENACERRAF

From the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

Thymus-derived inducer lymphocytes are selected to respond to foreign antigens only when presented in the context of autologous, Class II, MHC[1] molecules (1–3). This specific restriction and the considerable allelic polymorphism of Ia molecules have the inescapable consequence that responsiveness to thymus-dependent antigens is under the control of the genes coding for Class II molecules, the original, MHC-linked Ir genes (4). Moreover, Ia molecules not only determine responsiveness to foreign antigens but appear to select the epitope against which the T cell response is directed, a phenomenon that has been termed determinant selection (5, 6).

Two distinct but not necessarily exclusive mechanisms have been proposed to account for the manner in which Ia molecules restrict the specificity of T cells to foreign epitopes. (*a*) A specific association was postulated to occur between Ia molecules and antigen or its processed fragment, on the surface of antigen-presenting cells, previous to interaction with the T cell receptor (7). Such an interaction was deemed necessary for the Ia-antigen complex to be reactive with the T cell receptor and to be responsible for the phenomenon of determinant selection. (*b*) Alternatively, other investigators suggested that Ir gene phenomena are the result of the indirect effect of Class II MHC molecules on the T cell repertoire during differentiation in the thymus (8). Evidence has been presented in selected systems supporting this model (9, 10). On the other hand, analysis of the determinant selection of related cytochrome antigens by Schwartz and his colleagues (11, 12) has presented indirect but compelling evidence for a physical interaction between antigen and an Ia molecule. In addition, studies in our laboratory have examined the effect of structurally related, Ir gene–controlled antigens on the activation of antigen-specific, MHC-restricted T cells (13, 14). We observed that the antigen L-glutamic acid^{50}-L-tyrosine^{50} (GT) exerted a highly selective competitive inhibition for presentation of the related copolymer L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT). This competition occurred at the

This work was supported by grants AI-14732 and 1-R01-AI-CA-20248-01 from The National Institutes of Health.

[1] *Abbreviations used in this paper:* APC, antigen-presenting cells; GA, L-glutamic acid^{60}-L-alanine^{40}; GAT, L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}; GL, L-glutamic acid^{60}-L-lysine^{40}; GLleu, L-glutamic acid^{55}-L-lysine^{35}-L-leucine^{10}; GLØ, L-glutamic acid^{56}-L-lysine^{35}-L-phenylalanine^{9}; GT, L-glutamic acid^{50}-L-tyrosine^{50}; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; IL-2, interleukin 2 (TCGF); MAb, monoclonal antibody; MHC, major histocompatibility complex; MLC, mixed lymphocyte culture.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/84/04/1238/15 $1.00
Volume 159 April 1984 1238–1252

level of the antigen-presenting cell (APC), and was not attributable to an effect on antigen uptake or processing. The ability of GT to compete for GAT presentation was strongly influenced by the MHC allele with which GAT was corecognized. Thus, I-A^d plus GAT but not I-A^b plus GAT presentation was blocked by GT, even when an F_1 MHC heterozygous APC was used. These findings are consistent with a model of specific antigen-Ia association.

We have sought to more directly test this model of antigen presentation. A prediction of the model we proposed is that Ia should be focally altered when antigen is associated, at least at the site of complexing. This alteration might be detected if a probe specific for the putative antigen association site were available. Such an approach has been applied successfully to the study of ligand interactions with specific antibodies or receptors (15–17). Since I-A^b presenting cells appear to lack the GT/GAT association site of I-A^d cells, H-2^b T cells might alloreact to this putative interaction site on Ia^d cells. Therefore, we generated a series of alloreactive, Ia^d-specific H-2^b T cell hybridomas to probe selected determinants on Ia molecules. The analysis of the effect of GAT, GT, and other related antigens on a panel of Ia^d-specific T cell hybridomas is the subject of this report.

Materials and Methods

Mice. C57BL/10, B10.A, and B10.D2 mice, ages 5–8 wk, were purchased from The Jackson Laboratory, Bar Harbor, ME. BALB/c AnN mice, ages 6–10 wk were purchased from Charles River 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 Institute of Laboratory Animal Resources, National Research Council (DHEW publication [NIH] 78-23, revised 1978).

Antigens. GAT was purchased from Vega Fox, Tucson, AZ. GT, L-glutamic $acid^{60}$-L-$lysine^{40}$ (GL), L-glutamic $acid^{60}$-L-$alanine^{40}$ (GA), L-glutamic $acid^{56}$-L-$lysine^{35}$-L-$phenylalanine^{9}$ (GLØ), L-glutamic $acid^{55}$-L-$lysine^{35}$-L-$leucine^{10}$ (GLLeu), and chicken ovalbumin were obtained from Miles Laboratories, Inc., Elkhart, IN. Antigens were prepared as previously described (13).

T Cell Hybridomas. T cell blasts for construction of alloreactive hybridomas were derived from bulk mixed lymphocyte cultures (MLC). Briefly, 2.5–5 × 10^6 nylon/wool nonadherent C57BL/10 T cells were stimulated with an equal number of 1,660 rad X-irradiated erythrocyte-free BALB/c splenocytes as previously described except in 2-ml macrocultures (18). Responding T cell blasts were recovered on Ficoll-Hypaque and fused at 4:1 ratio to the hypoxanthine-guanine phosphoribosyltransferase (HGPRT)-negative T cell lymphoma, BW5147 as previously described (19). Hybrid growth was selected by culture in hypoxanthine, aminopterin, and thymidine. In all cases hybrids were seeded at limiting dilution and arose at a clonal frequency. They were subsequently expanded in macrowells and tested for function as described below. All hybrids illustrated in this report, except RF19.19, were further cloned by limiting dilution. Hybrids were plated at 0.3 cells/well with 5 × 10^5 X-irradiated erythrocyte-free spleen cells in microtiter plates. Positive wells were picked, expanded, and tested for function.

Monclonal Antibodies. Monoclonal antibody (MAb)–containing culture supernatants were obtained from: (*a*) The hybridomas MKD6 (20) (αI-A^d, kindly made available by Dr. J. Kappler and Dr. P. Marrack, National Jewish Hospital, Denver, CO) and (*b*) 14.4.4.S (21) (αIa^7, kindly made available by Dr. D. Sachs, National Institutes of Health, Bethesda, MD). MAb were purified from culture supernatant by affinity chromatography on protein A Sepharose.

Cell Culture. Hybridomas were tested in vitro using a modification of the method of Kappler and Marrack, as previously described (13, 19, 20). Briefly, 5–10 × 10^4 T cell

hybrids were cultured with or without 1,660 rad X-irradiated splenocytes as a source of antigen-presenting cells (APC), in the presence or absence of antigen in a final volume of 200 μl. The precise strain of origin and number of APC as well as antigen concentration is detailed in the respective experimental protocols. In some experiments, the in vitro passaged I-A^d–positive B lymphoblastoid line, A20, was used as a source of clonal accessory cells (13, 22). Cultures were incubated at 37°C for 18–24 h, at which time 100 μl of culture supernatant was removed and exposed to 8,000 rad gamma irradiation. T cell hybridoma activation in these cultures was assessed by measuring the presence and amount of the T cell lymphokine interleukin 2 (IL-2) in the culture supernatant, with an IL-2–addicted T cell line. 5×10^3 HT-2 cells (23), (kindly provided by J. Kappler and P. Marrack) were added to test supernatants and incubated at 37°C for 20–24 h with 1 μCi [^{3}H]thymidine added over the last 5 h. Cells were harvested onto glass fiber filter strips with the aid of a semiautomated harvesting device (PHD Harvester, Cambridge Technology, Cambridge, MA). Incorporation of label into DNA was determined by liquid scintillation counting. In all cases, the concentration of IL-2 tested was limiting (data not shown). Data is expressed as the arithmetic mean counts per minute (CPM) of triplicate cultures plus or minus the standard error of the mean (SEM). Antigen pulsing of spleen cells and A20 cells was performed as previously described (13).

Results

Characteristics of Alloreactive T Cell Hybridomas. Series of T cell hybridomas were derived from C57BL/10 (H-2^b) T cell blasts stimulated with allogeneic BALB/c (H-2^d) splenocytes and fused to the lymphoma, BW5147. Individual hybrid clones were screened for reactivity to BALB/c specificities by assaying their production of interleukin 2, upon coculture with X-irradiated allogeneic stimulator cells in vitro. Numerous alloreactive hybrids were obtained as illustrated in Tables I and II. As shown in Table II, these cells are activated to produce IL-2 upon stimulation with H-2^d–bearing cells (B10.D2). The specificity of this interaction was further defined by using a series of stimulator cells from H-2 recombinant inbred mice. As can be seen, activation of the hybrids is H-2 restricted, as H-2^b(B10) and H-2^a(B10.A) stimulators fail to generate a response. Individual clone specificity was mapped to the K/I-A or I-E regions by the presence or absence of a response to H-2^{g2}(B10.GD). The former specificity was further defined as I-A^d, since the response could be blocked by a monoclonal αI-A^d antibody. This blocking is highly specific, as shown by an identical amount of αI-E^d fails to block (this reagent will block I-E^d–specific hybrids, data not

TABLE I
Origin and Specificity of T Cell Hybridomas

Hybrid	Origin	Specificity	GAT inhibition	Reference
RF9.140	B/C α GAT	I-A^d + GAT	−	13
RF19.19	B10 α B/C	I-A^d	−	this paper
RF19.24	B10 α B/C	I-A^d	+	14
RF19.52	B10 α B/C	I-A^d	−	14
RF26.1	B10 α B/C	I-A^d	−	this paper
RF26.12	B10 α B/C	I-E^d	−	this paper
RF26.98	B10 α B/C	I-A^d	+	this paper
RF26.134	B10 α B/C	I-A^d	+	this paper

TABLE II
Mapping of Alloreactive T Cell Specificity

Hybrid	APC	H-2	MAb	CPM ± SEM
RF26.134	—	—	—	811 ± 124
	B10	$H\text{-}2^b$		776 ± 307
	B10.D2	$H\text{-}2^d$	—	19,134 ± 2,131
	B10.GD	$H\text{-}2^{g2}$		34,764 ± 585
	B10.A	$H\text{-}2^a$		525 ± 106
	B10.D2	$H\text{-}2^d$	α I-A^d	375 ± 39
	B10.D2	$H\text{-}2^d$	α I-E^d	21,305 ± 398
RF26.1	—	—	—	860 ± 22
	B10.D2	$H\text{-}2^d$	—	24,331 ± 1,612
	B10.GD	$H\text{-}2^{g2}$	—	30,551 ± 874
	B10.A	$H\text{-}2^a$	—	754 ± 21
RF26.12	—	—	—	931 ± 49
	B10.D2	$H\text{-}2^d$	—	21,085 ± *
	B10.GD	$H\text{-}2^{g2}$	—	1,419 ± 56
	B10.A	$H\text{-}2^a$	—	563 ± 90

Microcultures were prepared with 10^5 hybrids with or without 10^6 X-irradiated spleen cells from the indicated strains. Where indicated, 25 μg/ml of purified α I-A^d (MKD6) or α I-E^d (14.4.4.S) was included. Cultures were incubated for 24 h and 100 μl of supernatant removed, X-irradiated, and assayed for IL-2 content.
* Duplicate culture.

shown) and by reciprocal blocking experiments with MHC heterozygous (F_1) cells (data not shown). These hybrids recognize $H\text{-}2^d$ on at least three distinct genetic backgrounds, including that of syngeneic C57BL/10 (Table II and data not shown). Therefore, individual hybrid clones are either I-A^d or I-E^d-reactive.

Effect of Antigen on the Activation of Alloreactive Hybrids. We next assessed what effect a foreign antigen had, if any, upon the activation of these allo Ia–specific hybridomas. We chose to examine the effects of the amino acid copolymer GAT, as it is a simple antigen under Ir gene control that appears to specifically associate with an antigen-presenting cell in an Ia-restricted manner (13). Seven alloreactive hybrids were identified in our first fusion. Five were sufficiently stable to allow testing in the presence or absence of GAT. One of these five was markedly inhibited when GAT was included in culture. A representative experiment is illustrated in Fig. 1. As can be seen, the activation of RF19.24 by BALB/c stimulators is markedly diminished in the presence of 500 μg/ml of GAT. In contrast, the response of two other identically constructed BALB/c reactive hybrids is unaffected by GAT. As shown, these two hybrids, RF19.19 and RF19.52, give equivalent responses as compared to RF19.24, and are not GAT inhibited even under limiting stimulatory conditions.

To confirm this observation, as well as to determine the MHC specificity and approximate frequency of this phenomenon, we performed five additional fusions. In total we examined 497 hybrids with the following results, which are summarized in Table III. 77 alloreactive hybrids were identified. Of these hybrids, three (including RF19.24) were GAT inhibited; four additional hybrids were tentatively phenotyped as GAT inhibitable but were too unstable to allow definitive characterization. The ability to be inhibited did not reflect the strength of the hybrids response, as most weakly responding hybrids were not GAT

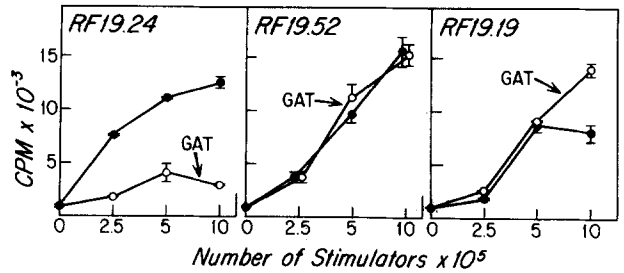

FIGURE 1. Effect of GAT on the activation of respresentative I-A^d–specific alloreactive T cell hybridomas. Microcultures were prepared with 10^5 hybrid cells with or without the indicated number of X-irradiated splenic stimulator cells in the presence (○) or absence (●) of 500 μg/ml of GAT. The data was obtained from a quantitative IL-2 assay on hybridoma microculture supernatants as described in the Materials and Methods. The three hybrids are of B10 α BALB/c origin.

TABLE III

Approximate Frequency and MHC Specificity of GAT-Inhibitable, Alloreactive T Cell Hybridomas

	No.	Percent of total	MHC Specificity§	
			%I-A	%I-E
Fusions	6			
Total hybrids examined	497			
Alloreactive hybrids	77	16		
Alloreactive hybrids examined* for GAT inhibition	73	95	79	21
GAT-Inhibitable hybrids‡	7 (3)	10 (4)	12 (5)	0

* The initial fusion was first screened for alloreactive hybrids and positive clones were further characterized. Four positive clones were neither mapped nor tested for GAT inhibition. All subsequent fusions were analyzed for MHC specificity and GAT inhibition in the initial screen. Since many clones are unstable and did not permit retesting, the numbers in each category should be considered as approximations.

‡ Three stable clones were isolated that were GAT inhibitable. Four additional hybrids that gave >50% inhibition, however, had single or limited testing due to instability. Numbers in parentheses indicate the three well-characterized hybrids.

§ I-A and I-E specificity was assigned on the basis of a positive or negative response with H-2g2, respectively.

inhibited (data not shown). All of the GAT-inhibitable alloreactive hybrids were I-A^d specific. This is of interest as the immune response to GAT is governed and restricted by the I-A molecule (24, 25). The initial suggestion of a correlation of this phenomenon with the recognition of I-A$^{l.g}$ has not been observed with this more extensive analysis (14). However, restriction digests of this allele has so far indicated identical gene structure with I-A^d (K. Klein, K. L. Rock, and J. Seidman, unpublished results).

Antigen Specificity of Inhibition. The results presented above demonstrates that GAT selectively interferes with the activation of a small number of allo-I-A–reactive hybrids. To determine the antigen specificity of this phenomenon, we tested a panel of structurally related and distinct antigens for their effects upon these hybrids. As can be seen in Table IV, high concentrations of the copolymers GA, GL, GLØ, and GLLeu fail to cause inhibition of RF26.98. Similarly, the protein antigen OVA is not inhibitory (data not shown). The only additional

TABLE IV
Effect of Related Amino Acid Copolymers on Alloreactive T Cell Hybridomas

Expt.	Hybrid	Stimulator	μg/ml Ag	CPM ± SEM
1	RF26.98	—	—	734 ± 72
		H-2^d	—	11,154 ± 3,130
		H-2^d	500 GAT	3,902 ± 497
		H-2^d	GA	15,425 ± 1,424
		H-2^d	GLØ	16,976 ± 2,037
		H-2^d	GL	9,687 ± 804
	RF26.1	—	—	328 ± 39
		H-2^d	—	18,033 ± 537
		H-2^d	500 GAT	18,680 ± 528
2	RF26.98	—	—	701 ± *
		H-2^d	—	21,662 ± *
		H-2^d	500 GAT	2,183 ± *
		H-2^d	GLLeu	17,010 ± *
		H-2^d	200 GT	3,183 ± 311
	RF26.134	—	—	1,131 ± 146
		H-2^d	—	21,064 ± 983
		H-2^d	500 GAT	4,367 ± 297
		H-2^d	GLLeu	15,983 ± 1,060
		H-2^d	200 GT	1,189 ± 89
	RF19.52	—	—	875 ± 23
		H-2^d	—	12,617 ± 789
		H-2^d	500 GAT	18,943 ± 295
		H-2^d	GLLeu	10,184 ± 391
		H-2^d	200 GT	13,458 ± 919

Cultures were prepared as described in Table II.
* Duplicate culture.

inhibitory antigen we have identified is the copolymer GT (Table IV). This is of interest since we have previously shown that GT and GAT, but not the other control antigens, associate at the same site on an APC. As further shown in Table IV, the GT inhibitory effect is selective for the GAT-sensitive, but not GAT-insensitive alloreactive hybrids. All three GAT-inhibitable hybrids have shown identical findings with these antigens (data not shown).

Comparison of GAT Presentation with Allo-I-A Inhibition. We have previously described the construction of T cell hybridomas with specificity for GAT in association with I-A^d (13, 25). These hybridomas were used to monitor the productive association of GAT with the I-A^d–bearing cells used to stimulate the alloreactive hybrids. As illustrated in Fig. 2, increasing amounts of GAT result in a dose-dependent selective inhibition of the activation of RF26.98 and RF26.134. As demonstrated above, another I-A^d–specific hybrid, RF19.52, is not inhibited by GAT. When the ability of these same cultures to present GAT plus I-A^d is measured with RF9.140, a GAT-specific I-A^d–restricted hybrid, a parallel but inversely related effect is seen over the identical range of antigen concentration. Thus, as increasing amounts of GAT are titrated in cultures there is a graded loss of a particular I-A^d–stimulated response, while there is a parallel appearance of an I-A^d plus GAT response.

Localization of the GAT/GT Inhibitory Effect. The inhibitory effect of GAT on

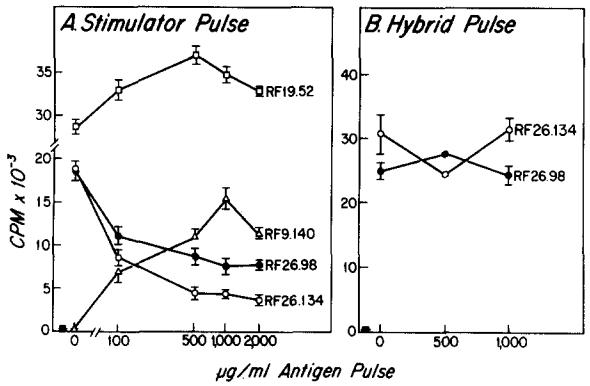

FIGURE 2. The relationship between GAT presentation and allo-I-A^d stimulation in the presence of GAT. Microcultures were prepared as described in Fig. 1 except that 5×10^4 RF9.140 hybrid cells were used and the amount of GAT in culture was varied with constant BALB/c stimulator cell numbers (10^6), as indicated. Data is expressed on a logarithmic plot. The hatched bar indicates the range of background values. The RF9.140 hybrid (△) is a BALB/c α I-A^d plus GAT specific hybrid all others are B10 α I-A^d: □, RF19.52; ▲, RF26.134; ●, RF26.98.

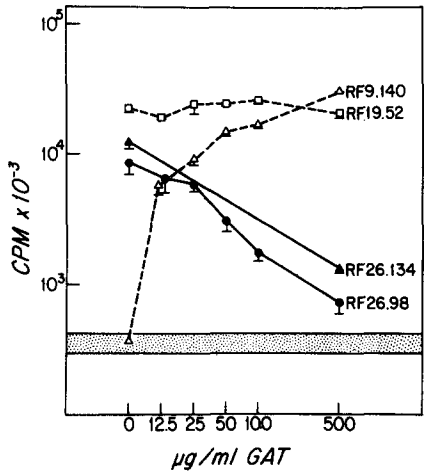

FIGURE 3. Cellular locus of GAT inhibition: (*A*) 10^7 X-irradiated BALB/c stimulator cells/ml or (*B*) 10^6 hybrids/ml were exposed to the indicated concentration of GAT for 3 h at 37°C, washed, and tested for functional activity with 10^6 irradiated stimulator cells, as described in Fig. 1. The results in panel *A* and *B* are from the same experiment. The increased response seen in panel *B* may reflect an effect of preculture in fresh media, although this has not been formally studied. All hybrids are B10 α I-A^d, except for RF9.140 which is BALB/c α I-A^d plus GAT: □, RF19.52; △, RF9.140; ●, RF26.98; ○, RF26.134; ■, hybrids without stimulator cells.

the activation of some allo I-A–specific cells could reflect an effect on the stimulator cell, on the T cell hybridoma, or both. To identify the locus of inhibition, we performed antigen-pulsing experiments. As shown in Fig. 3*A*, when stimulator cells are prepulsed with GAT, they lose their ability to stimulate the GAT-sensitive hybrids, RF26.98 and RF26.134, in a dose-dependent manner. This effect is selective, as pulsed stimulators are unaffected in their ability

to activate other I-A^d–specific T cell hybrids. Further, along with this selective loss of I-A^d–stimulating capacity, there is the concomitant appearance I-A^d plus GAT stimulatory determinant. To assess the effect of GAT directly on the T cell hybridomas, we performed two experiments. First, T cell hybrids were prepulsed with GAT and then tested for their ability to be activated by I-A^d–bearing stimulators. As can be seen in Fig. 3 *B*, no inhibition is caused by prepulsing the T cell hybrid, even at high GAT concentration. In this same experiment (Fig. 3*A*), pulsing of the stimulator with GAT was sufficient to effect inhibition, as described above. It is possible, nevertheless, that these T cell hybrids are directly affected by GAT, but require activation to manifest sensitivity. To examine this point, we activated these hybrids directly with concanavalin A in the presence or absence of GAT. As can be seen in Table V, GAT fails to inhibit activation of RF19.24 by mitogen under conditions that blocks its activation by I-A^d. The two other "GAT-sensitive" hybrids have been tested with identical results. Thus, the inhibitable hybrids are not uniquely sensitive to GAT directly. Rather, their stimulating target is uniquely affected by GAT. Since identically constructed hybrids with specificity for the same molecule are not inhibited, this effect on the stimulator cell is not nonspecific (e.g. toxicity).

Effects of GAT on Allo-I-A Stimulation by a Clonal APC. To ascertain whether the GAT-inhibitable and -uninhibitable hybrids were activated by the same stimulating cells, we tested their activation by the cloned I-A^d–bearing B lymphoblastoid cell line, A20 (22, 26, 27). As can be seen in Fig. 4, both RF19.24 (GAT inhibitable) and RF19.52 (GAT insensitive) are activated by this clone. Further, in the presence of GAT, the activation of RF19.24 is inhibited. RF19.52 is not affected by GAT even under limiting stimulation. Similarly, GT also selectively blocks the activation of RF19.24. Both allo hybrids are susceptible to inhibition with a monoclonal αI-A^d antibody, which demonstrates that A20 is stimulating these cells via I-A^d as expected and further that RF19.52 is inhibitable with the appropriate reagent. Finally, as GAT is added to culture, A20 cells are capable of activating RF9.140, an I-A^d plus GAT specific hybrid.

To confirm the locus of inhibition with the clonal presenting cell, we prepulsed

TABLE V

Effect of GAT on Mitogen Activation of T Cell Hybridomas

Hybrid	Stimulator	Con A	μg/ml GAT	CPM ± SEM
RF19.24	—	+	—	4,026 ± 129
	—	+	500	4,263 ± 255
	—	+	1,000	4,710 ± 101
	H-2^b	−	—	982 ± 214
	H-2^d	−	—	12,667 ± 495
	H-2^d	−	500	6,071 ± 890
	H-2^d	−	1,000	2,918 ± 170
RF19.52	H-2^b	−	—	854 ± 73
	H-2^d	−	—	15,744 ± 1,347
	H-2^d	−	500	15,557 ± 849
	H-2^d	−	1,000	17,781 ± 1,077

Microcultures were prepared as described in Table II, except for the addition of 10 μg/ml of Con A where indicated.

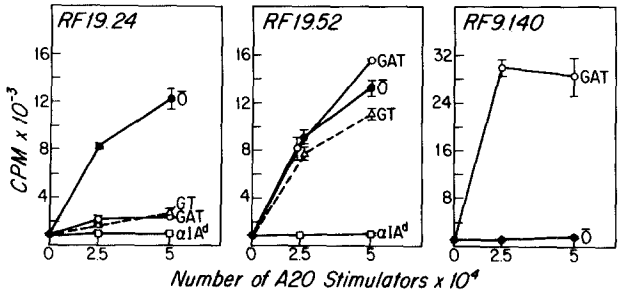

FIGURE 4. Stimulation and antigen inhibition of alloreactive hybrids using a clonal stimulator cell. Cultures were identical to those in Fig. 1, except that the A20 cloned I-A^d-positive cell line was used for stimulation in the presence of absence (●) of 2 mg/ml GAT (○), 0.2 mg/ml GT (△), or 25 μg/ml of α I-A^d (□).

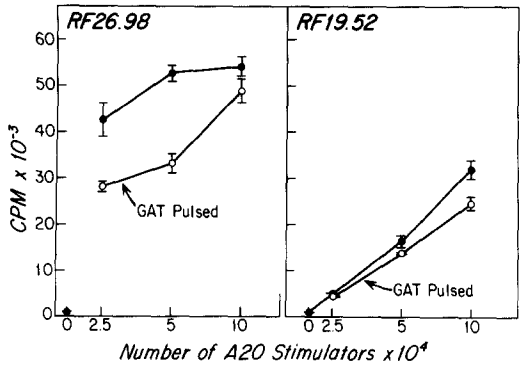

FIGURE 5. Allostimulation by antigen-pulsed A20 cells. Microcultures were prepared as described in Fig. 4, except that the A20 cells were prepulsed in the presence (○) or absence (●) of 2 mg/ml of GAT for 18 h.

A20 cells with or without GAT. Our initial results demonstrated an inhibitory effect, however, it was only moderate even at high GAT concentrations (Fig. 5). We considered that this partial effect could arise from this tumor presenting cell rapidly turning over its I-A molecules in culture (K. L. Rock, C. F. Gramm, unpublished observations). We therefore exposed A20 to GAT and subsequently fixed the cells to eliminate metabolic activity, as previously described (14, 28). As can be seen in Fig. 6, GAT-pulsed fixed A20 cells have completely lost their stimulatory capacity for RF26.98. This effect is dependent on GAT pulsing, as nonantigen-exposed fixed A20 cells are stimulatory for RF26.98. This effect is also selective, as fixed GAT-pulsed A20 cells activate RF19.52, as well as the nonantigen exposed control cells. Taken together, those results demonstrate that as an APC interacts with a nominal antigen, it loses a particular I-A allostimulatory determinant(s) at the same time as it gains an I-A^d plus GAT stimulatory capacity.

Discussion

These studies were undertaken to examine the effect of a foreign antigen on Ia molecule structure and/or function upon antigen association with an APC. A

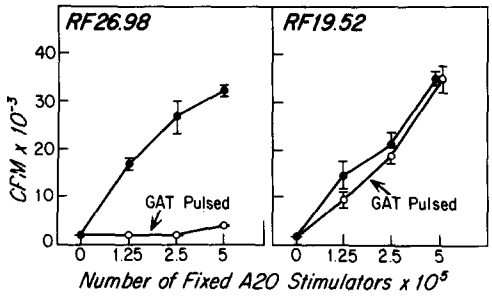

FIGURE 6. Allostimulation by antigen-pulsed and fixed A20 cells. Microcultures were prepared as described in Fig. 5 except after pulsing, all A20 cells were fixed with 1% paraformaldehyde for 10 min at room temperature.

panel of alloreactive Ia-specific T cell hybridomas was generated and used to probe I region determinants on accessory cells in the presence or absence of antigen. The antigen chosen for study was the synthetic amino acid copolymer GAT, as the associational specificity of this Ir gene–controlled antigen has been previously defined in our laboratory with H-2^d–restricted antigen-specific T cell hybridomas (13, 25). An H-2^b strain was used for the derivation of the Ia^d-specific hybrids, since I-A^d presenting cells have a GT/GAT association site that I-A^b APC apparently lack, which would in theory, allow the generation of alloreactive T cells against that portion of the I-A^d molecule. The major findings of this report are as follows: (*a*) GAT blocks the activation of several alloreactive T cell hybrids. (*b*) This effect is antigen-specific, in so far as several structurally related antigens fail to cause inhibition. (*c*) GT, which is known to compete for GAT association with an APC, also blocks these same alloreactive hybrids. (*d*) All inhibited hybrids have specificity for I-A, which is the locus known to control GAT responsiveness. (*e*) The majority of I-A–specific hybrids interacting with the same APC are not affected by GAT. (*f*) Antigen inhibition occurs at the level of the APC. (*g*) A direct relationship exists between GAT-induced loss of an allo–I-A-stimulating determinant and the acquisition of a GAT plus I-A^d determinant.

T cell hybridomas were derived from B10 (H-2^b) T cells stimulated in a primary MLC with BALB/c (H-2^d) stimulators, and were screened for IL-2 production in response to BALB/c cells. Alloreactive T cell hybrids were identified, which as expected, demonstrated a clonal specificity for either I-A^d or I-E^d. The response of these hybrids to I-A^d–bearing stimulators was tested in the presence or absence of the antigen GAT. A small percentage of clones were inhibited by the inclusion of GAT in culture in a dose-dependent manner. This GAT-inhibitable phenotype was observed with hybridomas from independent fusions.

The inhibitory effect of GAT is not attributable to general toxicity, as most identically constructed alloreactive hybrids are not affected by this copolymer. Further, these same culture conditions allow the efficient presentation of GAT to antigen-specific T cells. To further analyze the mechanism of GAT inhibition, we attempted to identify the cellular locus of inhibition by pulsing experiments. GAT pulsing of stimulator cells reproduces the effects described above. Again,

this effect is highly selective as the GAT-pulsed stimulator cells, which fail to activate the relevant hybrids, are still competent to activate other alloreactive hybrids, even those with specificity for the same Ia molecule. The reciprocal experiment of antigen prepulsing the T cell hybrids failed to cause inhibition. Additionally, the continuous presence of GAT in culture does not affect the activation of these hybrids by a mitogen. These experiments strongly argue that GAT inhibition is not attributable to a peculiar sensitivity of some T cell hybrids to GAT. Rather, these results demonstrate that exposure of the stimulator cells to GAT is both necessary and sufficient to account for the results.

The further interpretation of these results is dependent on whether this differential effect of GAT on the presentation of I-A^d by stimulator cells is reflecting a selective effect on the same or different stimulator cells. To address this issue, we examined the ability of a cloned I-A^d-bearing stimulator cell to activate both groups to T cell hybrids. Hybrids of both phenotypes responded to Ia molecules on this clonal stimulator cell. Further, GAT selectively blocked this interaction with the appropriate group of T cell hybrids and this effect could again be localized to the stimulator cell. Therefore, the differential effect of GAT on alloreactive T cell hybrid activation reflects a selective defect in the stimulatory capacity of a single accessory cell population.

Since the capacity of the stimulator cell to present I-A^d is only focally affected, it appears that an allo-stimulatory determinant(s) is selectively lost upon antigen association. The alternative explanation, that apart from specificity, the activation requirements of the hybrids are quantitatively or qualitatively different in a way that is differentially affected by GAT, appears unlikely. First and as noted above, the effect of GAT is on the stimulator cell. Second, the activation of these hybrids by mitogen is unaffected by GAT. Finally, the two groups of hybrids have almost identical dose response curves even when tested with fixed clonal stimulator cells. On the other hand, several observations support the interpretation that inhibition of hybrid activation is due to the selective "modification" of an allo-I-A stimulatory determinant(s) by antigen association. First, the MHC specificity of the inhibitable hybrids is restricted and appropriate for the known genetic control of the GAT Ag. Thus, the immune response gene for GAT is encoded in the I-A subregion (24) and GAT recognition by T cells is absolutely restricted by the I-A molecule (25, 29). All of the inhibitable alloreactive hybrids thus far identified are specific for I-A. Considering the frequency of this inhibitable phenotype, an I-E–specific, GAT-inhibitable hybrid would have been expected if the inhibition were unrelated to MHC specificity. Second, there is a close correlation between the selective loss of allostimulatory activity and the appearance of an I-A^d plus GAT determinant. Thus, loss of a determinant parallels antigen association. Finally, the antigen specificity of this phenomenon matches the known MHC allele-specific association of the copolymers with the APC. We have previously shown that GT, but not other antigens, specifically competes for GAT presentation in association with I-A^d but not I-A^b. Both GAT and GT selectively block the activation of the alloreactive hybrids. Several other related and chemically similar antigens fail to cause inhibition. Again, these results would not have been predicted if the mechanism of inhibition were unrelated to MHC specificity.

Our results support and extend the previous data on antigen-Ia molecule association. Studies on the Ir gene control of T cell responses and the related phenomenon of determinant selection led to the proposal that antigen specifically interacts with an Ia molecule on an APC (7). Alternate mechanisms acting on the T cell repertoire have also been proposed, for which there is some evidence in selected systems (8–10). However, recent elegant studies by Schwartz and colleagues (11, 12) on determinant selection in a clonal system have provided strong indirect evidence for a physical interaction between Ag and Ia. Further, experiments analyzing the phenomenon of antigen competition in the guinea pig (30) and mouse (13) have independently suggested such an interaction. In this latter system, specific antigen competition between GAT and GT has been localized to the APC and appears to occur at a locus distal to antigen uptake and/or processing (13). Further, and as noted above, the particular I-A allelic gene product on the APC determines the specificity of the competition. These results indirectly argued for a specific interaction between GAT/GT and the $I\text{-}A^d$ molecule. The present report extends these observations, using an independent approach. The finding that a panel of alloreactive clones can detect the selective loss of an allo-$I\text{-}A^d$ determinant(s) upon antigen association, strongly argues for a specific antigen-Ia association. Whether this effect represents actual determinant blocking or some other focal change in Ia structure (e.g. a conformational change) is not defined by our data. The alternative possibility that the inhibitable hybrids are corecognizing allo-$I\text{-}A^d$ plus an unknown antigen (e.g. a minor histocompatibility antigen) whose association is competed for by GAT and GT, appears unlikely for several reasons. In vitro antigen-specific primary responses have not been obtainable in our hands and a primary MLR would not be expected to result in such specificities, especially at the frequency at which the inhibitable phenotype is observed. Also, mapping experiments have failed to find a requirement for background genes outside of the MHC I region. Therefore, although such an alternate mechanism is not formally excluded, it is highly unlikely. While our data provides one documented example of apparent specific antigen association, other mechanisms may, in certain instances, affect the nature and specificity of T cell responses to foreign antigen, e.g. by the selective affects of Ia molecules on the T cell repertoire, as proposed by others (8).

The alloreactive hybrids that are GAT/GT-inhibitable are specific for a private I-A determinant. Therefore, these results suggest that the site of antigenic association is genetically polymorphic, at least for the antigens under study. This finding is consistent with studies on determinant selection and the effect of MHC polymorphism on antigen competition (5, 6, 13). As we have previously suggested, if this observation is general, it may serve to explain the dominant role of MHC allelic polymorphism in T cell responses (14).

Summary

A large panel of alloreactive, interleukin 2 (IL-2)–producing T cell hybridomas was constructed from B10 α BALB/c primary mixed lymphocyte cultures (MLC). Functional hybrids had specificity for either $I\text{-}A^d$ or $I\text{-}E^d$. These cells were used to probe determinants on Ia molecules in an attempt to detect molecular association between a nominal antigen and an Ia molecule on an antigen-

presenting cell (APC). The response of a small number of these clones was significantly blocked by the addition of the Ir gene–controlled copolymer L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT) to culture. A comparison of the inhibited and uninhibited hybrids revealed an identical dose response curve. Further, both types of hybrids were activated by the same stimulator cell and frequently recognized the identical Ia molecule on that cell. Nevertheless, the inhibitory effect of GAT was localized to the stimulator cell and not to the T cell hybrids. All of the hybrids whose stimulation was blocked had specificity for the I-A molecule, which is the gene product known to control and restrict responsiveness to GAT. Further, only GT, but not a number of other related antigens, was also specifically inhibitory, which correlates with the known associational specificity of these antigens on an APC. Finally, the same stimulator cell could be shown to coordinately lose an allostimulatory determinant(s), while it was gaining an I-A^d plus GAT determinant(s). The implications of these findings on the nature of antigen-Ia association and on the role of polymorphic Ia determinants are discussed.

The authors wish to acknowledge the outstanding technical assistance of Mary Carol Mellen and Colette F. Gramm. We thank Mrs. Mary Jane Tawa for preparation of this manuscript. We also wish to thank Drs. J. Kappler, P. Marrack, J. Watson, D. Sachs, and R. Asofsky for making available several of the cell lines used in this study.

Received for publication 3 January 1984.

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. Schwartz, R. H., A. Yano, and W. E. Paul. 1978. Interaction between antigen-presenting cells and primed T lymphocytes. *Immunol. Rev.* 40:153.
4. Benacerraf, B., and R. N. Germain. 1978. The immune response genes of the major histocompatibility complex. *Immunol. Rev.* 38:70.
5. Rosenthal, A. S. 1978. Determinant selection and macrophage function between antigen presenting cells and primed T lymphocytes. *Immunol. Rev.* 40:136.
6. Thomas, D. W., S. K. Meltz, and G. D. Wilner. 1979. Nature of lymphocyte recognition of macrophage associated antigen. II. Macrophage determinants of guinea pig T cell response to human fibrinopeptide B. *J. Immunol.* 123:1299.
7. 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.
8. Schwartz, R. H. 1978. A clonal deletion model for Ir gene control of the immune response. *Scand. J. Immunol.* 7:3.
9. Ishi, N., N. Baxevasis, Z. A. Nagy, and J. Klein. 1981. Responder T cells depleted of alloreactive cells react to antigen presented on allogeneic macrophages from nonresponder strains. *J. Exp. Med.* 154:978.
10. Clark, R. B., and E. M. Shevach. 1981. Generation of T cell colonies from responder

strain 2 guinea pigs that recognize the copolymer L-glutamic acid L-lysine in association with nonresponder strain 13 Ia antigens. *J. Exp. Med.* 155:635.

11. Herber-Katz, E., R. H. Schwartz, L. A. Matis, C. Hannum, T. Fairwell, E. Appella, and D. Hansburg. 1982. Contribution of antigen-presenting cell major histocompatibility complex gene products to the specificity of antigen induced T cell activation. *J. Exp. Med.* 155:1086.
12. Hedrick, S. M., L. A. Matis, T. T. Hecht, L. E. Samelson, D. L. Longo, E. Herber-Katz, and R. H. Schwartz. 1982. The fine specificity of antigen and Ia determinant recognition by T cell hybridoma clones specific for pigeon cytochrome *c*. *Cell.* 30:141.
13. 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.
14. Rock, K. L., and B. Benacerraf. 1983. MHC-restricted T cell activation: analysis with T cell hybridomas. *Immunol. Rev.* 76:29.
15. Claflin, J. L., and J. M. Davie. 1975. Specific isolation and characterization of antibody directed to binding site antigenic determinants. *J. Immunol.* 114:70.
16. Flier, J. S., C. R. Kahn, D. B. Jarrett, and J. Roth. 1976. Characterization of antibodies to the insulin receptors. A cause of insulin-resistant diabetes in man. *J. Clin. Invest.* 58:1442.
17. T. Kawamota, J. D. Sato, A. Le, J. Polikoff, G. H. Sato, and J. Mendelsohn. 1983. Growth stimulation of A431 cells by epidermal growth factor: identification of high affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. *Proc. Natl. Acad. Sci. USA.* 80:1337.
18. 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.
19. Rock, K. L. 1982. The role of Ia molecules in the activation of T lymphocytes. I. The activation of an IL-1 dependent IL-2 producing T cell hybridoma by Con A requires an interaction, which is not H-2 restricted with an Ia bearing accessory cell. *J. Immunol.* 129:1360.
20. 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.
21. Ozato, K., N. Mayer, and D. H. Sachs. 1980. Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. *J. Immunol.* 124:533.
22. 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.
23. Watson, J. 1979. Continuous proliferation of murine antigen-specific helper T lymphocytes in culture. *J. Exp. Med.* 150:1510.
24. Dorf, M. E., J. M. D. Plate, J. H. Stimpfling, and B. Benacerraf. 1975. Characterization of immune response and mixed lymphocyte reactions in selected intra-H-2 recombinant strains. *J. Immunol.* 1145:602.
25. 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.
26. Chesnut, R. W., and H. M. Grey. 1981. Studies on the capacity of B cells to serve as antigen presenting cells. *J. Immunol.* 126:1075.
27. Glimcher, L. H., K. J. Kim, I. Green, and W. E. Paul. 1982. Ia antigen bearing B

cell tumor lines can present protein antigen and alloantigen in a major histocompatibility complex- restricted fashion to antigen-reactive T cells. *J. Exp. Med.* 155:445.

28. Ziegler, K., and E. R. Unanue. 1981. Identification of a macrophage antigen-processing event required for I-region restricted antigen presenting to T lymphocytes. *J. Immunol.* 127:1869.
29. Schwartz, R. H., A. Yano, and W. E. Paul. 1978. Interaction between antigen-presenting cells and primed T lymphocytes. *Immunol. Rev.* 40:153.
30. Werdelin, O. 1982. Chemically related antigens compete for presentation by accessory cells to T cells. *J. Immunol.* 129:1883.