ALLOANTISERUM-INDUCED INHIBITION OF IMMUNE RESPONSE GENE PRODUCT FUNCTION

II. Genetic Analysis of Target Antigens

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Alloantisera prepared in strain 13 guinea pigs by immunization with strain 2 lymphoid cells markedly inhibit the activation of $(2 \times 13)F_1$ peritoneal exudate lymphocytes by the 2,4-dinitrophenyl (DNP) derivative of a copolymer of L-glutamic acid and L-lysine $(GL)^1$ (1). Responses to DNP-GL and to the closely related antigen, DNP-poly-L-lysine (DNP-PLL), depend upon the presence of an immune response (Ir) gene linked to the major histocompatibility complex (MHC) controlling the strain 2 alloantigen(s) (2). This finding, with appropriate specificity controls, has led us to propose that alloantisera inhibit antigen recognition by thymus-dependent (T) lymphocytes through interference with the activity of Ir gene products (1).

In the first paper (3) of this series, we examined the effect of absorption of the 13 anti-2 serum with different populations of immunocompetent cells. We were unable to demonstrate a clear dissociation between the ability of a cell population to remove the cytotoxic activity of the anti-2 serum for lymphocytes and the ability of that population to remove the capacity of the anti-2 serum to inhibit T-cell proliferation in response to DNP-GL. We concluded that the 13 anti-2 serum contains antibodies directed against cell surface structures present on both thymus-derived (T) lymphocytes and bone marrow-derived (B) lymphocytes, because L_2C leukemia cells, a pure population of malignant B lymphoid cells, were able to remove both the cytotoxic capacity and the T-cell inhibitory capacity from the anti-2 serum. Indeed, the relevant antigens appear to be present in larger amount on B cells than on T cells. It is possible that the anti-2 serum contains different populations of antibodies, some of which are reactive with strain 2 histocompatibility (H) antigen(s) and other which are

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[1] *Abbreviations used in this paper:* CFA, complete Freund's adjuvant; GA, a copolymer of L-glutamic acid and L-alanine; GL, a copolymer of L-glutamic acid and L-lysine; GT, a copolymer of L-glutamic acid and L-tyrosine; H, histocompatibility; Ir, immune response; MHC, major histocompatibility complex; NGPS, normal guinea pig serum; PEC, peritoneal exudate cells; PELs, peritoneal exudate lymphocytes; PHA, phytohemagglutinin; PLL, poly-L-lysine; PPD, purified protein derivative of tuberculin; SI, stimulation index.

reactive with the products of the Ir genes. According to this interpretation both the products of the Ir genes and the relevant alloantigen would be expressed on the surface of B lymphocytes.

In order to further study whether the inhibitory antibodies within the allo-antisera are directed against H antigens or against the products of the Ir genes, it would be advantageous to analyze the ability of alloantisera to inhibit T-cell proliferation to an antigen, the response to which is under the control of a specific Ir gene, in the absence of the H antigen which usually occurs in association with that Ir gene. Unfortunately, in progeny of crosses of $(2 \times 13)F_1$ and parental animals, no recombinant chromosomes in which the DNP-GL Ir gene has been separated from the H gene to which it is normally linked have yet been identified. An alternate approach to this goal is to utilize outbred animals in which relationships between H and Ir genes exist which differ from those obtaining in inbred populations. Table I reviews the previously reported (2, 4, 7) associations between responsiveness to PLL, responsiveness to a copolymer of L-glutamic acid and L-alanine (GA) and possession of strain 2 and 13 H specificities. Inbred strain 2 animals response to both PLL and GA, while inbred strain 13 animals respond to neither of these antigens; $(2 \times 13)F_1$ animals respond to both PLL and GA. Some $(2 \times 13)F_1 \times 13$ backcross progeny respond to GA and to PLL; their cells can be lysed by an anti-2 serum in the presence of complement. On the other hand, the GA nonresponder back-cross animals also fail to respond to PLL and their cells cannot be lysed by an anti-2 serum. Thus, in inbred strain 2 animals, the GA Ir gene is linked to the PLL Ir gene and to the gene(s) controlling strain 2 H antigen(s). However, this relationship is not observed in all outbred animals. Thus, outbred Hartley guinea pigs can be divided into four main groups on the basis of their responses to GA, PLL, and the ability of anti-2 or anti-13 sera to lyse their lymphoid cells. Group I outbred animals respond to both PLL and GA and their cells are lysed by an anti-2 serum, but not by an anti-13 serum; these animals therefore resemble inbred strain 2 animals. Group II outbreds respond to

TABLE I

Relationship between Histocompatibility Type and Responsiveness to PLL and GA

Strain	Phenotype	PLL response	GA respones
Inbred 2	$2^+/2^+$	$+$	$+$
Inbred 13	$13^+/13^+$	$-$	$-$
$(2 \times 13)F_1$	$2^+/13^+$	$+$	$+$
$(2 \times 13)F_1 \times 13$	$\left\{\begin{array}{l} 2^+/13^+ \\ 13^+/13^+ \end{array}\right.$	$+$ $-$	$+$ $-$
Outbred I	$2^+/13^-$	$+$	$+$
Outbred II	$2^-/13^+$	$-$	$-$
Outbred III	$2^+/13^+$	$+$	$+$
Outbred IV	$2^-/13^+$	$-$	$+$

neither PLL nor GA and their cells are lysed by an anti-13, but not by an anti-2 serum. The members of this group thus resemble inbred strain 13 animals. Group III animals respond to both PLL and GA and their cells are lysed both by an anti-2 and by an anti-13 serum. These animals thus resemble $(2 \times 13)F_1$ animals. A 4th group of Hartley animals responds to GA, but not PLL, and their cells are lysed by an anti-13 but not by an anti-2 serum. This last group contains the proposed recombinant chromosome.

In the present study, we have used this group of $GA^+PLL^-2^-13^+$ animals in an attempt to demonstrate whether the anti-2 serum can inhibit the function of an Ir gene which is normally linked to strain 2 H genes, when this gene occurs in an animal lacking strain 2 H genes. We also report the results of attempts to prepare an alloantiserum capable of inhibiting the GA response and devoid of anti-2 activity by immunizing strain 13 animals with cells from these outbred animals.

Materials and Methods

Animals.—Inbred strain 2 and strain 13 guinea pigs were obtained from the Division of Research Services, National Institutes of Health, Bethesda, Md. $(2 \times 13)F_1$ animals were obobtained by mating strain 2 with strain 13 animals in our own colony. Outbred Hartley guinea pigs were obtained from Camm Research Institute Inc., Wayne, N. J.

Antigens.—Poly-L-lysine·HBr (PLL, mol wt 110,000), a copolymer of L-glutamic acid (60%), and L-lysine (40%) (GL, mol wt 115,000) and a copolymer of L-glutamic acid (60%), and L-alanine (40%) (GA, mol wt 43,000), were obtained from the Pilot Chemical Division of the New England Nuclear Corp., Boston, Mass. DNP_{21}-PLL and DNP_3-GL were prepared as previously described (8). The subscript refers to the number of DNP groups/molecule. Purified protein derivative of tuberculin (PPD) was purchased from Connaught Medical Research Laboratories, Willowdale, Ontario, Canada. Phytohemagglutinin (PHA) was obtained from Welcome Research Laboratories, Beckenham, England.

Immunization of Guinea Pigs.—Solutions of each antigen were emulsified with an equal volume of complete Freund's adjuvant containing 0.5 mg of Mycobacterium butyricum/ml (CFA, Difco Laboratories, Detroit, Mich.). Animals were immunized simultaneously with either 100 µg of DNP-GL or 100 µg DNP-PLL and 500 µg of GA; each antigen was administered in one front footpad and one rear footpad.

Skin Tests.—3 wk after immunization the animals were injected intradermally with 50 µg of DNP-GL or GA, or 10 µg DNP-PLL in 0.1 ml of normal saline. Animals immunized with adjuvant alone were similarly skin tested to evaluate the degree of nonspecific irritation produced by these polymers. The injection sites were examined after 24 h; erythema and induration greater than 8 mm in diameter was considered a positive skin test.

Preparation of Alloantisera.—A strain 13 antistrain 2 serum was prepared as previously described (1). Strain 13 anti-$GA^+PLL^+2^+13^+$ Hartley and strain 13 anti-$GA^+PLL^-2^-13^+$ Hartley sera were prepared by immunizing strain 13 animals with a CFA emulsion of 100×10^6 lymph node and spleen cells from the appropriate Hartley guinea pig. 2 and 4 wk after primary immunization, the strain 13 animals were boosted with 100×10^6 lymph node and spleen cells from a phenotypically similar animal. 6–8 wk after the initial immunization, the strain 13 animals were exsanguinated and the sera heat-inactivated at 56°C for 40 min and sterilized by Millipore filtration. Absorption studies of the 13 anti-2 serum were performed as described in the accompanying report (3).

Cytotoxicity Testing.—The alloantisera were tested against ^{51}Cr-labeled lymph node cells as previously described (9).

Cell Collection and Technique of Brief Antigen Exposure.—The techniques for the collection of the peritoneal exudate cells (PEC) and the methods for the purification of the peritoneal exudate lymphocytes (PELs) have been described previously (10). When inbred or $(2 \times 13)F_1$ animals were used, the cells from two animals were pooled; when cells from outbred animals were used, the cells from each animal were processed individually.

The technique of brief antigen exposure of the PEC population is described in the accompanying report (3). The final concentrations in the incubation medium of the antigens used in this study were as follows: DNP-GL 100 $\mu g/ml$; DNP-PLL, 1 $\mu g/ml$; GA, 5 mg/ml; PPD, 100 $\mu g/ml$ and PHA, 10$\mu g/ml$.

In Vitro Assay of Antigen-Induced DNA Synthesis.—The methods used in the first paper of this series (3) are identical to the ones used here. When studies were performed on individual outbred animals, the macrophages from that animal were combined only with the lymphocytes from that same animal. The results are expressed as either total cpm per culture, as the difference between control and antigen stimulated cultures (Δ cpm), or as the stimulation index (SI), the ratio of cpm incorporated in the presence of antigen to cpm incorporated in the absence of antigen.

RESULTS

Inhibition of the Proliferative Response of Cells from $(2 \times 13)F_1$ Animals to GA.—Before the effect of the 13 anti-2 serum on the GA response in the outbred population could be analyzed, it was necessary to demonstrate that the in vitro proliferative response to GA of cells from $(2 \times 13)F_1$ animals can be specifically inhibited by the anti-2 serum. F_1 animals were simultaneously immunized with DNP-GL and GA and exudate lymphocytes from those animals cultured with macrophages which had been pulsed with either DNP-GL or GA. It should be noted that high concentrations (5 mg/ml) of GA during the macrophage pulse step were required in order to obtain significant lymphocyte activation. Table II shows the results of two typical experiments using cells from F_1 animals. In these experiments the in vitro proliferative responses to both DNP-GL and GA were completely inhibited by the anti-2 serum. In experiment 2, moderate suppression of the PPD and PHA responses was also observed indicating a decreased capacity of this lot of antiserum to support cell

TABLE II

Inhibition of Proliferation by Lymphocytes from F_1 (2×13) Animals Immune to DNP-GL and GA

Stimulant	Serum			
	Exp. 1		Exp. 2	
	NGPS	13 Anti-2	NGPS	13 Anti-2
0	236*	180	2,072	1,723
DNP-GL	11,715	198	11,115	942
GA	2,344	299	10,175	1,903
PPD	9,478	9,164	44,506	59,484
PHA	25,878	29,685	33,539	66,773

* Results are expressed as cpm per tube; each value is the mean of three determinations.

growth in culture. This study demonstrates that specific inhibition of F_1 T-cell proliferation by the anti-2 serum is not confined to antigens controlled by the PLL gene, but can also be extended to the in vitro proliferative response to another antigen the response to which is controlled by a 2-linked Ir gene.

Analysis of PLL and GA Responses in Outbred Animals.—Approximately 150 Hartley guinea pigs were immunized with both DNP-PLL and GA, skin tested with these antigens to determine their responder status, and typed with both anti-2 and anti-13 sera in a ^{51}Cr release assay. This particular population could be divided into three groups of animals. One-third of the guinea pigs were GA$^+$PLL$^+$2$^+$13$^+$; one-third were GA$^+$PLL$^-$2$^-$13$^+$, and one-third were GA$^-$PLL$^-$2$^-$13$^+$. In this population 2$^+$13$^-$ animals were not identified although animals of this phenotype are seen in other outbred populations (Table I). We did not identify any GA$^-$PLL$^+$ animals although a small number of animals of this type have been identified previously (6).

Inhibition of the Response to GA of T Lymphocytes from GA$^+$PLL$^+$ and GA$^+$PLL$^-$ Hartley Animals.—The PELs of GA responder Hartley guinea pigs of both types (GA$^+$PLL$^+$2$^+$, GA$^+$PLL$^-$2$^-$) were cultured in the presence of antigen pulsed macrophages either in normal guinea pig serum (NGPS) or in anti-2 serum. Table III illustrates the results with cells from GA$^+$PLL$^+$2$^+$ animals. Table III A shows the detailed results in four experiments each employing cells from a different individual animal of this type. In experiments 1–3, marked and specific suppression of the response to both DNP-PLL and GA was observed; in experiment 4 the absolute number of cpm incorporated in response to GA was decreased in the presence of the anti-2 serum; however, when the results are expressed as the stimulation index, the SI is higher in the presence of the anti-2 serum then in the presence of normal serum. Table III B summarizes the results in these four and nine other individual experiments using cells from GA$^+$PLL$^+$2$^+$ Hartley animals. The results are expressed as the SI in NGPS compared to the SI in anti-2 serum. In 9 out of 13 cases, marked

TABLE III A

Inhibition of Proliferation by Lymphocytes from Ga$^+$ PLL$^+$2$^+$ Hartley Guinea Pigs

Stimulant	Serum							
	Animal 1		Animal 2		Animal 3		Animal 4	
	NGPS	13 Anti-2	NGPS	13 Anti-2	NGPS	13 Anti 2	NGPS	13 Anti-2
0	2,989*	2,250	733	542	2,482	1,369	4,097	805
DNP-PLL	37,218	6,284	13,824	592	67,436	2,601	21,213	1,549
GA	24,258	4,098	3,046	545	11,693	4,104	16,537	10,380
PPD	29,261	20,118	8,032	5,300	50,094	34,884	58,115	27,103
PHA	49,555	57,534	9,499	7,494	53,369	36,667	47,069	34,570

* Results are expressed as cpm per tube; each value is the mean of three determinations.

TABLE III B

Suppression of Proliferative Response to GA by Cells from GA$^+$PLL$^+$2$^+$ Hartley Animals

Animal	Serum		Suppression*
	NGPS	13 Anti-2	
			%
1	8.1‡	1.8	89
2	4.1	1.0	100
3	4.7	2.9	49
4	4.0	12.8	—
5	15.4	1.5	97
6	6.9	1.1	98
7	12.2	5.9	56
8	6.6	12.2	—
9	2.0	1.0	100
10	3.2	4.2	—
11	5.3	2.6	63
12	13.9	15.5	—
13	2.5	2.1	27

* % Suppression $= \left[1 - \dfrac{(\text{SI in anti-2 serum-1})}{(\text{SI in NGPS-1})} \right] \times 100.$

‡ SI.

suppression of the in vitro proliferative response to GA was observed in the presence of the anti-2 serum. In four cases, the GA response was either unchanged or enhanced in the presence of the anti-2 serum. In each of these cases, the response to DNP-PLL was completely inhibited by the alloantiserum.

Table IV illustrates the effect of the anti-2 serum on the in vitro proliferative response to GA of cells from GA$^+$PLL$^-$2$^-$ animals. Table IV A gives the detailed results observed in three typical experiments. No evidence of proliferation is seen in response to DNP-PLL in vitro; this confirms the unresponsiveness of these animals to DNP-PLL as demonstrated by skin tests. In experiments 1 and 2 no suppression of the GA response was observed while in experiments 3 moderate suppression (60%) of the GA response was seen. Table IV B summarizes the effect of the anti-2 serum on the GA response of cells from these three and six other GA$^+$PLL$^-$2$^-$ animals. In seven out of nine cases no evidence of suppression of the response to GA by the anti-2 serum was seen. In two cases, the anti-2 serum produced moderate suppression of the GA response. We conclude from this study that the anti-2 serum is much more capable of suppressing the GA response of cells from GA$^+$PLL$^+$2$^+$ Hartley animals than cells from GA$^+$ PLL$^-$2$^-$ Hartley animals. The observation that the response to GA of cells from some GA$^+$2$^+$ animals was not inhibited is probably explained by the likelihood that such animals are heterozygous GA$^+$2$^+$, GA$^+$2$^-$. Thus, the ability of the anti-2 serum to inhibit the GA response appears to depend, in the main, on the presence of strain 2 H antigen on the responder cells.

TABLE IV A

Inhibition of Proliferation by Cells from $GA^+PLL^-2^-$ Hartley Guinea Pigs

Stimulant	Serum					
	Animal 1		Animal 2		Animal 2	
	NGPS	13 Anti-2	NGPS	13 Anti-2	NGPS	13 Anti-2
O	1,910*	2,038	845	810	1,145	1,300
DNP-PLL	1,676	1,757	1,256	1,040	1,462	1,637
GA	14,703	14,766	40,489	51,740	22,057	9,947
PPD	19,335	17,599	135,679	144,295	31,138	29,803
PHA	16,249	13,077	96,314	147,452	—	—

* Results are expressed as cpm per tube; each value is the mean of three determinations.

TABLE IV B

Suppression of Proliferative Response to GA by Cells from $GA^+PLL^-2^-$ Animals

Animal	NGPS	13 Anti-2	Suppression*
			%
1	7.7‡	7.2	—
2	47.9	63.9	—
3	19.3	7.7	63
4	6.6	10.9	—
5	3.1	4.8	—
6	3.1	3.1	—
7	11.4	18.9	—
8	31.6	19.5	40
9	7.8	8.2	—

* For calculation see Table III B.
‡ SI.

Effect of 13 Anti-$GA^+PLL^+2^+$ and 13 Anti-$GA^+PLL^-2^-$ Sera on the GA Response of Cells from F_1 (2 × 13) Animals.—The results of the previous study suggested that suppression of the proliferative response to GA is primarily mediated by antibodies directed against strain 2 H antigen rather than by antibodies specific for the GA Ir gene product. Another approach to the study of the same problem is to prepare an alloantiserum which might be anticipated to be specific for the GA Ir gene product and to be free of antibodies to strain 2 H antigens. To accomplish this, strain 13 animals were immunized with the lymph node and spleen cells of $GA^+PLL^-2^-13^+$ Hartley animals. As a control, another group of strain 13 animals were immunized with the lymph node and spleen cells of $GA^+PLL^+2^+13^+$ Hartley animals. In the former case, the resultant antisera would lack anti-strain 2 antibodies whereas such antibodies should be present in sera obtained from the latter immunization. The cytotoxic activities of these two sera are shown in Table V and compared with the standard anti-2 and anti-13 sera. The 13 anti-$GA^+PLL^+2^+$ serum contains significant

TABLE V

Cytotoxic Activity of Alloantisera

Antiserum	Target lymphocytes			
	Inbred 2	Inbred 13	$GA^+PLL^{+}2^+13^+$ outbred	$GA^+PLL^-2^-13^+$ outbred
13 anti-2	44*	0	17	0
2 anti-13	0	31	23	31
13 anti-$GA^+PLL^{+}2^+13^+$	58	0	79	65
13 anti-$GA^+PLL^-2^-13^+$	0	0	75	73

* $\%$ ^{51}Cr release from labeled lymph node cells by a 1/5 dilution of antiserum.

cytotoxic activity for strain 2 lymph node cells. The 13 anti-$GA^+PLL^-2^-$ serum is devoid of anti-2 activity. Both of these sera exhibit high levels of cytotoxic activity for cells from animals of the immunizing type and also demonstrate almost equal cytotoxic activity for cells from outbred animals of the opposite phenotype. Thus, the 13 anti-$GA^+PLL^{+}2^+$ sera is able to lyse both $GA^+PLL^{+}2^+$ lymph node cells and $GA^+PLL^-2^-$ lymph node cells.

The ability of these sera to inhibit the proliferation of T cells derived from (2 × 13)F_1 animals which have been doubly immunized with DNP-GL and GA is examined in Fig. 1. The response of the F_1 T cells to DNP-GL and GA was completely inhibited by the 13 anti-2 serum and by the 13 anti-$GA^+PLL^{+}2^+$ serum; these sera also produced moderate suppression of the PPD response. In contrast, the 13-anti $GA^+PLL^-2^-$ serum was without inhibitory effect for the DNP-GL, GA or PPD responses. Here again, the inhibitory capacity of the alloantiserum appears to be related to its anti-strain 2 activity. There is no evidence to suggest that the 13 anti-$GA^+PLL^-2^-$ serum contains antibodies capable of inhibiting the GA response.

Effect of 13 Anti-$GA^+PLL^{+}2^+$ and 13 Anti-$GA^+PLL^-2^-$ Sera on the GA Response of Cells from Outbred Animals.—Since both the 13 anti-$GA^+PLL^{+}2^+$ and the 13 anti-$GA^+PLL^-2^-$ sera exhibited significant cytotoxic activity toward cells from animals of the immunizing type, it was of interest to examine the effect of these sera on the proliferative response of outbred cells to either GA or DNP-PLL. As expected (Fig. 2 A) the 13 anti-$GA^+PLL^{+}2^+$ serum produced suppression of both the GA and DNP-PLL responses of cells from $GA^+PLL^{+}2^+$ animals. However, neither the 13 anti-$GA^+PLL^{+}2^+$ nor the 13 anti-$GA^+PLL^-2^-$ sera had any effect on the in vitro proliferative responses of cells from $GA^+PLL^-2^-$ animals (Fig. 2 B). This is particularly interesting in view of the fact that both of these sera demonstrate high levels of complement dependent cytotoxic activity for $GA^+PLL^-2^-$ cells (Table V). This result suggests that the ability of an alloantiserum to inhibit T-cell proliferation is critically dependent on the precise nature of the antigen to which the sera are directed.

Effect of Absorption of the Anit-2 Serum with $GA^+PLL^+2^+$ or $GA^+PLL^-2^-$ Cells.—One possible explanation for some of the results observed in the above studies is that the anti-2 serum is composed of antibodies directed to strain 2 H antigen and of antibodies specific for the products of the GA Ir gene and that both populations of antibodies are necessary for the inhibition of the proliferative response to GA. According to this interpretation, the failure of the anti-2 serum to inhibit the GA response of cells from $GA^+PLL^-2^-$ animals is not due to an absence of anti-GA Ir gene product antibodies but rather to lack
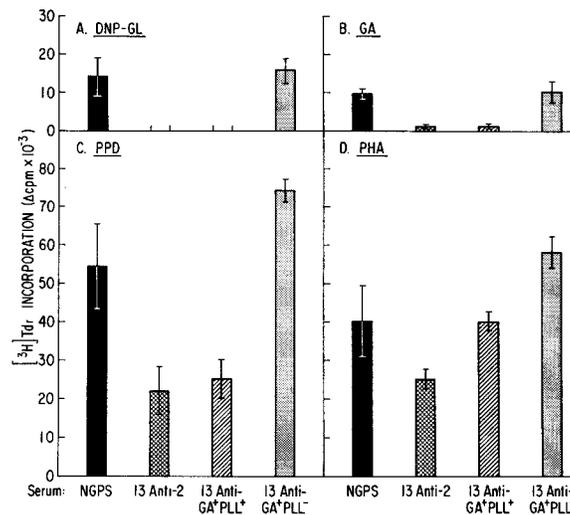
FIG. 1. (2 × 13)F_1 lymphocytes were cultured with DNP-GL, GA, PPD, or PHA pulsed macrophages in the presence of normal guinea pig serum, 13 anti-2 serum, 13 anti-GA^+PLL^+-2^+13^+ serum, or 13 anti-$GA^+PLL^-2^-13^+$ serum. Results are expressed as Δcpm/culture. Each bar represents the arithmetic mean of three experiments ±1 SEM.

of anti-strain 2 antibodies, the concerted action of which is required for inhibition. In order to rule out this possibility, aliquots of the 13 anti-2 serum were absorbed with either $GA^+PLL^+2^+$ lymphocytes or with $GA^+PLL^-2^-$ lymphocytes. The inhibitory activity of the absorbed antisera was tested on (2 × 13)F_1 lymphocytes from an animal immune to both DNP-GL and GA (Fig. 3). Absorption of the anti-2 serum with $GA^+PLL^-2^-$ cells did not diminish the inhibitory capacity of the anti-2 serum for either the GA or DNP-GL responses, while absorption of the anti-2 serum with $GA^+PLL^+2^+$ cells markedly decreased the inhibitory activity of the antisera for both the DNP-GL and GA responses (Fig. 3 *A* and 3 *B*). This study lends further support to the concept that the inhibitory activity of the anti-2 serum is directed toward strain 2 H specificities and not toward the product of the GA Ir gene.
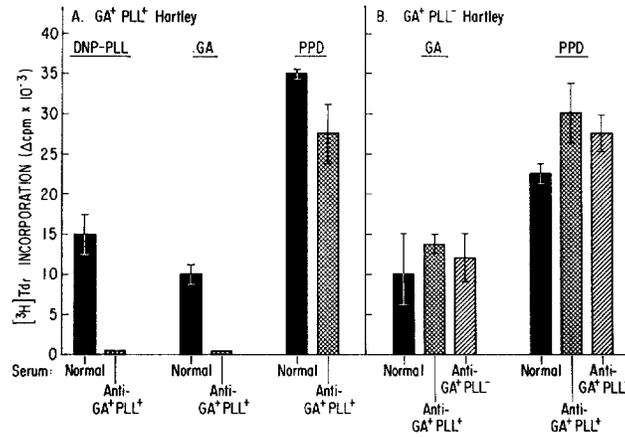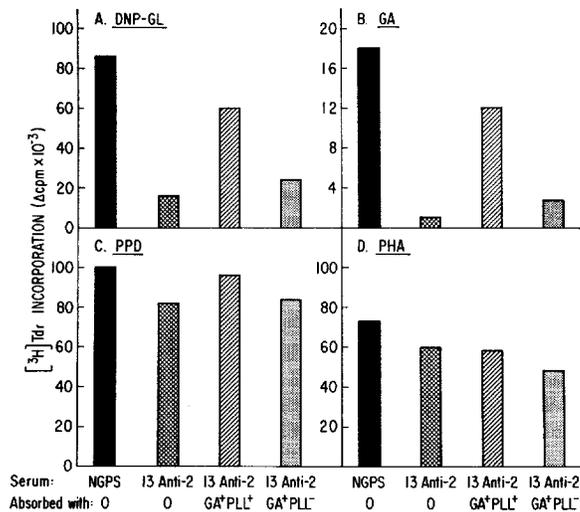
FIG. 2. (A) Lymphocytes from GA+PLL+2+13+ Hartley animals were cultured with DNP-PLL, GA, or PPD pulsed macrophages in the presence of NGPS or 13 anti-GA+PLL+-2+13+ serum. (B) Lymphocytes from GA+PLL-2-13+ Hartley animals were cultured with GA or PPD pulsed macrophages in the presence of normal serum, 13 anti-GA+PLL+2+13+ serum, or 13 anti-GA+PLL-2-13+ serum. Results are expressed as Δcpm/culture. Each bar represents arithmetic mean of four experiments ± 1 SEM.

FIG. 3. (2 × 13)F1 lymphocytes were cultured with DNP-GL, GA, PPD, or PHA pulsed macrophages in the presence of normal guinea pig serum, 13 anti-2 serum, 13 anti-2 serum which had been absorbed with lymphocytes from a GA+PLL+2+ Hartley animal, or 13 anti-2 serum which had been absorbed with lymphocytes from a GA+PLL-2- Hartley animal. Results are expressed as Δcpm/culture.

DISCUSSION

In this report we have examined whether alloantisera raised against lymphoid cells in which a given H gene is linked to a given Ir gene can block the function of the product of that Ir gene when the latter occurs without the product of the H gene to which it is normally linked. Ideally, this experiment should be performed using cells from an animal whose H region derives from a recombination event during an observed breeding and in which adequate documentation of parental genotypes exists. We have not yet identified a recombination event separating the strain 2 H gene(s) and the DNP-GL Ir gene or one separating the strain 13 H gene(s) and the Ir gene controlling responsiveness to the copolymer of L-glutamic acid and L-tyrosine (GT). This is not surprising in view of the low recombination rates in mouse and human MHC and the relatively small number of backcross progeny we have studied. We therefore chose a situation in which an Ir gene (the GA gene), which is linked to strain 2 H genes in inbred animals, is frequently present in the outbred Hartley guinea pig population in the absence of detectable strain 2 H specificities.

Initially, we demonstrated that the anti-2 serum was able to specifically inhibit the proliferative response to GA of T lymphocytes from immunized $(2 \times 13)F_1$ animals. Thus, the ability of 13 anti-2 alloantisera to inhibit the activation of T lymphocytes by antigens the response to which is linked to the presence of strain 2 H types is not confined to antigens the response to which is controlled by the PLL gene (e.g. DNP-PLL and DNP-GL). We then examined the ability of the anti-2 serum to inhibit the GA response of cells derived from outbred animals that manifested both delayed hypersensitivity and antibody production when immunized with GA, but lacked both the PLL Ir gene and the gene(s) controlling strain 2 H specificities. As a control, we also tested the effect of the anti-2 serum on the cells from outbred animals that were GA^+, but which were also PLL^+ and 2^+. It was assumed that the GA Ir gene in most of these latter animals is linked to both the PLL Ir gene and the gene controlling strain 2 H specificities. It should also be noted that the PLL Ir gene is very closely linked to the gene(s) controlling strain 2 H antigens in the outbred population because 2^-PLL^+ and 2^+PLL^- animals have not yet been identified in our colonies (4).[2] Consequently, we also examined the effect of the anti-2 serum on the DNP-PLL response on cells from the $GA^+PLL^+2^+$ animals. The results of these experiments demonstrated that, in the majority of cases, the anti-2 serum was capable of inhibiting the in vitro proliferative response to GA of T cells derived from animals that were $GA^+PLL^+2^+$, but the serum had little, if any, effect on the GA response of T cells from $GA^+PLL^-2^-$ animals. A similar finding has been presented by Bluestein using cells from GA^+2^+ and GA^+2^- random bred guinea pigs (11). It thus appears that the inhibition of the GA response by the anti-2 serum is primarily mediated via antibodies directed toward strain 2 H antigens rather than antibodies specific for the product of

the GA Ir gene. In four instances (out of 13 experiments) involving cells from experimental animals of the $GA^+PLL^+2^+$ outbred group, the anti-2 serum failed to suppress the GA response, but completely inhibited the response of the cells to DNP-PLL. One possible explanation for this result is that these animals possessed two different chromosomes bearing the GA Ir gene. On one chromosome, the GA Ir gene would be linked to both the PLL Ir gene and strain 2 H antigen, while on the other chromosome, the GA Ir gene would be linked either to strain 13 H genes or to unknown H genes. Recent linkage tests[2] have indicated the existence of a $GA^+GT^+13^+2^-$ chromosome in outbred animals. It is likely that the inhibition by anti-2 serum of the GA response of cells derived from a GA^+2^+, GA^+2^- animal would not be complete due to the contribution of the products of the GA^+2^- chromosome. The response to DNP-PLL, depending exclusively on products of the $GA^+PLL^+2^+$ chromosome, would be anticipated to be completely inhibited, as it was.

Another approach to resolving the problem of whether the inhibitory activity of the anti-2 serum is due to antibodies which are directly reactive with the products of the Ir gene is to produce antisera which, for genetic reasons, could contain antibodies to the products of an Ir gene and yet be devoid of antibodies reactive with the H antigen to which that Ir gene is normally linked. Lymphocytes from the Hartley animals that are $GA^+PLL^-2^-$ are always lysed by an anti-13 serum and therefore bear at least some of the strain 13 H antigen specificities. It therefore seemed reasonable to immunize strain 13 animals with cells from these $GA^+PLL^-2^-13^+$ Hartley animals. The goal of this immunization procedure was the preparation of an antiserum which would suppress the GA response of a strain 2 or a $(2 \times 13)F_1$ animal and be free of antibodies reactive with strain 2 (or strain 13) H antigens. Here again, as a control, strain 13 animals were immunized with cells from $GA^+PLL^+2^+13^-$ animals. In this case, the putative antiserum to the GA Ir gene product would also contain antibodies reactive with strain 2 H antigens. The resultant antisera were tested with the lymphocytes from $(2 \times 13)F_1$ animals immune to both DNP-GL and GA. The alloantiserum which contained antibodies reactive with strain 2 H antigens (the 13 anti-$GA^+PLL^+2^+13^+$ serum) was capable of specifically inhibiting the response of F_1 lymphocytes to GA and to DNP-GL. On the other hand, the alloantiserum devoid of anti-2 activity (the 13 anti-$GA^+PLL^-2^-13^+$ serum) was also devoid of inhibitory activity for either the GA or the DNP-GL responses. This further supports the hypothesis that the alloantibodies reactive with strain 2 H antigens are responsible for the inhibition of the in vitro proliferative response to GA which is caused by anti-2 sera. However, it should be noted that no data have been presented to demonstrate that the GA Ir gene which is linked to the PLL gene and to strain 2 H specificities is the same GA gene found in the $GA^+PLL^-2^-13^+$ animals. Indeed, Bluestein has presented

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[2] Shevach, E. M., L. Lee, S. Pickeral, and I. Green. Manuscript in preparation.

evidence which raises the possibility that the GA genes in these animals may be different (11). Despite this reservation, it appears likely to us that the inhibitory activity of the alloantisera is due to antibodies specific for products of the strain 2 and strain 13 (2/13) H antigen genes rather than to antibodies specific for the products of the Ir genes themselves. The mechanism of alloantiserum induced suppression of Ir gene function would then be by steric interference with the Ir gene product on the cell surface rather than by direct binding to it. This conclusion implies that the products of both the H genes and the Ir genes are physically related on the cell surface. The implications of such a relationship will be discussed below.

If anti-H antibodies are capable of indirectly inhibiting Ir gene function, why did the 13 anti-GA$^+$PLL$^-$2$^-$13$^+$ serum fail to inhibit the proliferative response to either GA or PPD of cells from Hartley animals of the GA$^+$PLL$^-$2$^-$13$^+$ phenotype even though this serum exhibited significant complement dependent cytotoxic activity as measured by ^{51}Cr release from these same cells? Although the 13 anti-GA$^+$PLL$^-$2$^-$13$^+$ serum does not contain antibodies reactive with 2 or 13 H specificities, it should contain antibodies to H antigens of unknown types which are present in the outbred population. One possible explanation for the failure of the 13 anti-GA$^+$PLL$^-$2$^-$13$^+$ serum to suppress the GA response of cells from GA$^+$PLL$^-$2$^-$13$^+$ animals is that the GA Ir gene in these animals is linked to the gene controlling strain 13 H specificities. Anti-13 activity is lacking in this serum as it is produced in strain 13 animals. Indeed, as noted above, we have recently demonstrated[2] that the GA Ir gene in GA$^+$PLL$^-$2$^-$13$^+$ animals is linked to the GT gene and very likely to the strain 13 H antigen gene. One might then predict that the GA response of the GA$^+$PLL$^-$2$^-$13$^+$ animals should be inhibited by anti-13 sera. However, preliminary studies have shown that the anti-13 serum has no effect on the response of lymphocytes from GA$^+$PLL$^-$2$^-$13$^+$ animals.

Another possible explanation for the failure of some potent cytotoxic alloantisera to inhibit the in vitro proliferative response to antigen is that the MHC in the guinea pig is bipartite in structure and thus similar to the MHC of both mice and man. If this explanation is valid, one might postulate that the Ir region would be very closely linked to the 2/13 locus and the products of these loci linked on the cell surface. Alloantibodies directed at determinants of a putative second, less closely linked, MHC locus might be devoid of inhibitory activity for T-lymphocyte functions. Furthermore, Sato and de Weck have identified a major H antigen (B) which is shared by strain 2 and strain 13 animals (12). Studies to define the relationship between the 2/13 locus and the ABCD locus of Sato and de Weck are now in progress. Alternatively, as outlined in the preceeding paper (3), the antigens recognized by the anti-2 and anti-13 sera may be the product of MHC genes which are not formally equivalent to the genes controlling the major serologic specificities in the mouse (the D and K genes) but rather to genes mapping within the Ir region of mice

(13, 14). According to this thesis, the cytolytic antibodies in the anti-$GA^+PLL^-2^-13^+$ sera would be directed not at products of these critical Ir-related H genes but rather at products of other genes in the MHC. Antisera directed at these other MHC antigens would have limited inhibitory activity because of the less close linkage of their genes to Ir genes. A final possibility is that the genes controlling the antigens against which these 13 anti-$GA^+PLL^-2^-13^+$ sera are directed are not linked to the MHC and thus their failure to inhibit might be due to a complete lack of linkage. Studies to evaluate these possibilities are now in progress.

The model we have proposed for the mechanism whereby alloantisera mediate their suppressive effects on Ir gene product function, namely indirect blockade of the Ir gene products by antibodies directly reactive with H antigens, requires phenotypic linkage of the products of independent but closely linked genes. Such linkage of membrane markers has interesting implications in the context of a fluid mosaic model of the lymphocyte surface. In this model, individual protein molecules are capable of free diffusion in the plane of the membrane (15). Indeed, recent studies of ligand-induced redistribution of surface markers such as immunoglobulin (16), H antigens (17) and receptors for concanavalin A (18) are consistent with this thesis. In such a fluid-mosaic model, the maintenance of a stable relationship between two determinants would suggest a physical link between these moieties in order to avoid their separation in the course of diffusion. As a specific blockade of the DNP-GL response of F_1 cells by anti-2 sera and of the GT response by anti-13 sera implies such a stable relation, we suggest that on the surface of an individual immunocompetent T cell, the Ir gene product exists bound covalently or noncovalently, to the strain 2 (or 13) H antigen. An interesting analogy to the genotype and phenotype of immunoglobulin H chains may then be drawn. Thus, the genes specifying variable (V_H) and constant (C_H) regions of immunoglobulin heavy chains are independent but linked in the genome. These independent, linked genes produce a single polypeptide chain. One might postulate that the genes for the 2/13 antigens are analogous to C_H genes and that the Ir genes are analogs of the V_H genes. Thus, all T lymphocytes would be anticipated to bear a 2/13-Ir complex. The 2/13 antigen would be the same on all T cells while an individual Ir gene product would have a clonal distribution. Moreover, a possible function of this 2/13 antigen "constant region" would be to mediate macrophage T-cell and T-cell-B-cell interaction. Ir gene products or closely related MHC-encoded structures have recently been shown to be of importance for both these interactions (19, 20).

In this model, it would be logical to postulate that the 2/13-Ir complex might be "immunoglobulin-like" in that it had the main structural features of an immunoglobulin and that it derived from the primordial genes from which classical immunoglobulin genes also evolved. In view of the independent evolution of these genes, it would be very unlikely that the 2/13-Ir complex would be serologically related to contemporary immunoglobulins. On the other hand,

one can envisage an antigen recognition molecule consisting of an 2/13-Ir complex which had no structural or genetic relationship to immunoglobulin and constituted an entirely different class of substances. The resolution of this problem and further progress in the understanding of T-lymphocyte antigen-recognition mechanisms will depend on further functional studies of defined alloantisera and chemical analysis of the MHC gene products.

SUMMARY

It has been previously demonstrated that alloantisera can specifically block the activation of T lymphocytes by antigens, the response to which is linked to the presence of histocompatibility (H) types against which the alloantisera are directed. Thus, strain 13 anti-2 serum can inhibit the activation of $(2 \times 13)F_1$ T lymphocytes by a DNP derivative of a copolymer of L-glutamic acid and L-lysine (DNP-GL), an antigen the response to which is controlled by a 2-linked Ir gene. It was proposed that alloantisera can inhibit T-lymphocyte antigen recognition through interference with the activity of immune response (Ir) gene products. In order to further study whether the inhibitory antibodies within the alloantisera are directed against H antigens or against the products of the Ir genes, we have examined whether the anti-2 serum can inhibit the function of an Ir gene (the L-glutamic acid and L-alanine [GA] gene), which is normally linked to strain 2 H genes when this gene occurs in an outbred animal lacking strain 2 H genes. In the majority of cases, the anti-2 serum was capable of inhibiting the in vitro proliferative response to GA of T cells derived from animals that were GA^+2^+, but the serum had little if any effect on the GA response of T cells from GA^+2^- animals. Furthermore, an antiserum prepared in strain 13 animals against the lymphoid cells of a GA^+2^- outbred animal was devoid of inhibitory activity on the GA response of cells from a $(2 \times 13)F_1$, while an antiserum prepared in strain 13 animals against the lymphoid cells of a GA^+2^+ outbred animal was capable of specifically inhibiting the response to GA. It thus appears that the inhibition of the GA response by the anti-2 serum is primarily mediated via antibodies directed toward strain 2 H antigens rather than antibodies specific for the product of the GA Ir gene. The mechanism of alloantiserum induced suppression of Ir gene function would then be by steric interference with the Ir gene product on the cell surface, rather than by direct binding to it. This conclusion implies that the products of both the H genes and the Ir genes are physically related on the cell surface. The implications of such a relationship in terms of the fluid-mosaic model of the lymphocyte surface are discussed.

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