

ALLOANTISERUM-MEDIATED SUPPRESSION OF HISTOCOMPATIBILITY-LINKED *Ir*-GENE-CONTROLLED IMMUNE RESPONSES

SUPPRESSIVE EFFECTS OF IgG FRAGMENTS DERIVED FROM ALLOANTISERA\*

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Immune response (*Ir*)<sup>1</sup> genes controlling cellular and humoral immunity to specific antigenic determinants are linked to the genes controlling the expression of the major histocompatibility antigens of the species (1). *Ir* genes have been defined functionally but there is little known about the molecular nature of the gene product. In contrast, histocompatibility antigens have been isolated and studied physicochemically but their biologic function remains a mystery. The significance of the linkage between *Ir* and histocompatibility loci, therefore, has not been obvious. In mice the linkage group containing *H-2* and *Ir* loci also contain genes controlling the expression of other lymphocyte membrane antigens (2, 3). This suggests that the *Ir*-gene product may also be a molecule found on lymphocyte membranes. That *Ir*-gene function is indeed expressed in lymphocytes is demonstrated by the ability of immunocompetent responder lymphocytes to confer specific immune responsiveness upon nonresponder animals (4, 5).

The demonstration of linkage between histocompatibility and *Ir* loci does not, in itself, suggest that there is a functional interaction between their gene products. However, several recent studies have suggested that histocompatibility antigens are involved in the development of specific immune responses. Histocompatibility-linked *Ir*-gene-controlled immune responses can be specifically suppressed in vitro by antisera containing antibodies directed at the linked histocompatibility antigens. In guinea pigs, antistrain 2 alloantisera can specifically inhibit responses to the synthetic polypeptide antigens, DNP-copolymer of L-glutamic acid and L-lysine (GL) and copolymer of L-glutamic acid and L-alanine (GA), both of which are controlled by strain 2-linked *Ir* genes, without affecting responses to copolymer of L-glutamic acid and L-tyrosine (GT), a

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<sup>1</sup> Abbreviations used in this paper: GA, copolymer of L-glutamic acid and L-alanine; GL, copolymer of L-glutamic acid and L-lysine; GT, copolymer of L-glutamic acid and L-tyrosine; *Ir*, immune response gene; PLL, poly-L-lysine.

strain 13-associated immune response. Conversely, antistrain 13 alloantisera inhibit GT responses without affecting strain 2-associated immune responses (6, 7). We have shown that the suppression of in vitro responses to GA by antistrain 2 alloantisera is observed only when the responding lymphocytes bear strain 2 histocompatibility antigens (7). Thus, the antibodies responsible for the specific immunosuppression are, in fact, directed at the histocompatibility antigens and not at the *Ir*-gene product itself.

Direct evidence for the involvement of histocompatibility antigens in the development of specific immune responses has been provided by the demonstrations that efficient macrophage-T-cell and T-cell-B-cell cooperation requires that both the interacting cell types share a common histocompatibility antigen (8-10). Furthermore, the stimulation of the *Ir*-gene-controlled immune responses in vitro requires that the macrophage and T cell share the linked histocompatibility antigen (11). These studies have suggested that antigen stimulation of T cells requires an interaction between the histocompatibility regions of the T cell and macrophage. One explanation of alloantiserum-mediated immunosuppression is the masking of the histocompatibility sites with antibody, thereby interfering with the stimulation of lymphocytes by macrophage and bound antigen.

If simply masking histocompatibility antigens is sufficient to suppress antigen stimulation, the intact antibody molecule should not be required. Antibody fragments containing the combining site should be sufficient. To examine this possibility Fab, F(ab)<sub>2</sub>, and Fc fragments were prepared from the immunoglobulin fraction of an antistrain 2 alloantiserum and studied for their ability to inhibit immune responses controlled by strain 2 histocompatibility-linked *Ir* genes. The data indicate that divalent F(ab)<sub>2</sub> fragments are potent suppressors of DNP-GL-stimulated responses. Monovalent Fab fragments, on the other hand, have no specific suppressive effects on strain 2-linked immune responses. Furthermore, the addition of the antiserum could be delayed several hours after the addition of antigen to the cultures and still produce complete suppression of the DNP-GL-stimulated responses. These results suggest that rather than preventing antigenic stimuli from reaching their lymphocyte receptors, alloantisera mediate specific immune suppression via a dynamic process that renders the lymphocyte refractory to the antigenic stimuli.

#### *Materials and Methods*

*Animals.*—The (2 × 13)F<sub>1</sub> guinea pigs used in these experiments resulted from interstrain breeding between strain 2 and strain 13 guinea pigs. The parental strains, maintained in our colony, are derived from breeding pairs obtained from the National Institutes of Health Bethesda, Md.

*Antigens.*—Poly-α-(L-glutamic acid [50%], L-alanine [50%]), GA, mol wt 45,000, was custom synthesized by the Pilot Chemical Division of New England Nuclear, Boston, Mass. Poly-α-(L-glutamic acid [50%], L-tyrosine[50%]), GT, mol wt 14,500, was obtained from Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill. Poly-α-(L-glutamic acid [60%],

L-lysine(40%), GL, mol wt 90,000, was obtained from the Pilot Chemical Division of New England Nuclear. DNP<sub>26</sub>-GL was prepared by reacting GL with 2,4-dinitrofluorobenzene (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) as described previously (12). The subscript indicates that number of moles of DNP bound per mole of GL.

*Antigen-Stimulated DNA Synthesis In Vitro.*—Lymphocytes were obtained from oil-induced peritoneal exudates from (2 × 13)F<sub>1</sub> guinea pigs that had previously been immunized with GT and DNP-GL, or with GA and DNP-GL. The lymphocytes were cultured in the presence of antigen with alloantisera or fragments of the IgG fraction of the alloantisera added as indicated with each experiment. The methodology used in immunizing the animals, determining their responder status, isolating a lymphocyte-rich fraction from oil-induced peritoneal exudates, the culture conditions, and the procedure for assaying for tritiated thymidine (<sup>3</sup>H]TdR) incorporation are described in detail elsewhere.<sup>2</sup> In cultures to which alloantisera or their immunoglobulin fragments were added, the data, expressed as % Suppression, is calculated as follows:

$$\% \text{ Suppression} = 100 \left( 1 - \frac{\text{Ag-stimulated cpm in presence of antiserum}}{\text{Ag-stimulated cpm in absence of antiserum}} \right)$$

*Preparation of Immunoglobulin Fragments.*—Strain 13 antistrain 2 alloantisera were produced by immunizing strain 13 guinea pigs with lymphoid cells from strain 2 animals as described previously (13). IgG was obtained from the 13 anti-2 sera by fractionation on DEAE-cellulose (14). Papain hydrolysis of the IgG was carried out according to the method of Porter (15). The guinea pig IgG (120 mg) was incubated with 1.2 mg of twice crystallized papain (Worthington Biochemical Corp., Freehold, N. J.), in a 0.1 M sodium phosphate buffer, pH 7.0, containing 0.01 M cysteine, and 0.002 M EDTA. After incubation for 16 h at 37°C the reaction was stopped by dialysis against water at 4°C. The Fab and Fc fragments were separated by chromatography on DEAE-cellulose according to the method of Edelman et al. (16). The dialyzed papain digest was fractionated on a 1.5 × 20-cm column of DEAE-cellulose and eluted with a gradient of 0.005–0.5 M sodium phosphate, pH 7.8.

F(ab)<sub>2</sub> fragments of the IgG from the 13 anti-2 alloantisera were prepared by pepsin digestion (17). The guinea pig IgG (120 mg) was incubated with 1.2 mg of twice crystallized pepsin (Sigma Chemical Co., St. Louis, Mo.) in 2.5 ml of 0.1 M sodium acetate buffer, pH 4.5. After 18 h at 37°C the precipitate was removed by centrifugation, the pH raised to 8 with sodium hydroxide, and sodium sulfate (25 g/100 ml) was added dropwise at room temperature with stirring to a final concentration of 18 g/100 ml. The resulting precipitate was separated by centrifugation, dissolved in water, and then dialyzed against phosphate-buffered saline (.01 M sodium phosphate, 0.15 M sodium chloride, pH 7.2). The Fab, Fc, and F(ab)<sub>2</sub> fragments thus obtained were brought to a protein concentration in the range of 5–10 mg/ml by pressure filtration with an Amicon ultrafiltration cell equipped with a UM-10 filter (Amicon, Corp. Lexington, Mass.).

<sup>51</sup>Cr-Release Cytotoxicity Assay.—The cytotoxicity of the Fab, Fc, and F(ab)<sub>2</sub> fragments and the ability of those fragments to block the cytotoxicity of the intact 13 anti-2 alloantisera were assessed using the <sup>51</sup>Cr-release cytotoxicity assay described previously (18). For determination of the cytotoxicity of each of the fragments, one million <sup>51</sup>Cr-labeled lymphocytes were incubated with each fragment in the quantities indicated in a 1:6 dilution of reconstituted lyophilized guinea pig serum (as a source of complement) in a total vol of 0.3 ml. The same incubation mixture containing, in addition, a 1/300 dilution of antistrain 2 serum was used to assess the inhibitory effects of the fragments on the cytotoxicity induced by the intact 13 anti-2 alloantisera. The spontaneous release of <sup>51</sup>Cr from lymphocytes was assessed with incubation mixtures devoid of antisera and Ig fragments. The data are expressed as % Cytotoxicity (which is equivalent to the % chromium released) calculated as follows:

% Cytotoxicity = 100

$$\times \frac{\text{cpm released by fragments and/or antiserum—cpm spontaneously released}}{\text{cpm released by frozen-thawed cells—cpm spontaneously released}}$$

## RESULTS

Strain 13 antistrain 2 alloantisera produced in inbred strain 13 guinea pigs immunized with the lymphoid cells from strain 2 animals are cytotoxic in the presence of complement to guinea pig lymphocytes bearing strain 2 histocompatibility antigens. The Ig fragments derived from enzymatic digestion of the antistrain 2 alloantisera, however, are not cytotoxic. As shown in Table I, neither the Fab, Fc, nor F(ab)'<sub>2</sub> fragments were cytotoxic to (2 × 13)F<sub>1</sub> lymphocytes as measured in a <sup>51</sup>Cr-release assay. The Fab and F(ab)'<sub>2</sub> fragments, however, maintained their antigenic specificity as demonstrated by their ability to inhibit the cytotoxicity of the intact 13 anti-2 alloantiserum. This serum at a 1/300 dilution, when tested against lymphocytes from a (2 × 13)F<sub>1</sub> guinea pig produced 24% cytotoxicity. The Fab fragments at 0.9 mg/ml or the F(ab)'<sub>2</sub>

TABLE I  
*Specific Inhibition of Strain 13 Antistrain 2 Alloantiserum-Mediated Cytotoxicity to (2 × 13)F<sub>1</sub> Lymphocytes by Immunoglobulin Fragments Derived from that Alloantiserum*

Antiserum*	Ig fragment†	% Cytotoxicity‡
—	Fab	2
—	Fc	2
—	F(ab)' <sub>2</sub>	0
NGPS	—	0
13 Anti-2	—	24
2 Anti-13	—	16
13 Anti-2	Fab	3
13 Anti-2	Fc	22
13 Anti-2	F(ab)' <sub>2</sub>	3
2 Anti-13	Fab	16
2 Anti-13	Fc	18
2 Anti-13	F(ab)' <sub>2</sub>	18

\* The normal guinea pig serum (NGPS) and the strain 13 antistrain 2 (13 anti-2) and strain 2 antistrain 13 (2 anti-13) alloantisera were used in the cytotoxicity assay at a 1/300 final dilution.

† Fab, Fc, and F(ab)'<sub>2</sub> fragments derived from strain 13 antistrain 2 alloantisera, were prepared as described in the Materials and Methods section. The final concentration of the fragments in the cytotoxicity assay was: Fab, 0.9 mg/ml; Fc, 0.8 mg/ml; and F(ab)'<sub>2</sub>, 0.6 mg/ml.

‡ The <sup>51</sup>Cr-release cytotoxicity assay and the calculation of % cytotoxicity are described in the Materials and Methods section. The data presented represent the mean of duplicate assays in two experiments each using lymphocytes from a different animal.

fragments at 0.6 mg/ml concentration completely inhibited this cytotoxicity. The cytotoxicity of the 13 anti-2 serum was unaffected by the addition of Fc fragments at a concentration of 0.8 mg/ml. The cytotoxicity of a 1/300 dilution of a strain 2 antistrain 13 serum was not inhibited by any of the Ig fragments derived from the 13 anti-2 serum, thus demonstrating the specificity of the Fab and F(ab)<sub>2</sub> fragments for strain 2 histocompatibility antigens.

At concentrations greater than 0.5 mg/ml, the Fab and F(ab)<sub>2</sub> fragments were similar in their effective inhibition of the cytotoxicity of a 1/300 dilution of the intact 13 anti-2 serum. At lower concentrations, however, there was considerable difference in their effectiveness as inhibitors. As shown in Fig. 1, essentially complete suppression (5% cytotoxicity) was achieved with the F(ab)<sub>2</sub> fragments at concentrations as low as 0.06 mg/ml. The Fab fragments, on the other hand, required a concentration of 0.45 mg/ml to achieve the same degree of suppression. Partial inhibition of the 13 anti-2 serum from 24% to 15% cytotoxicity required a 0.18 mg/ml concentration of the Fab fragments and a 0.012 mg/ml concentration of the F(ab)<sub>2</sub> fragments. Thus, the divalent F(ab)<sub>2</sub> fragments were substantially more effective as inhibitors of the cytotoxicity of the intact antibody than were the monovalent Fab fragments.

Although both the Fab and F(ab)<sub>2</sub> fragments derived from the antistrain 2 alloantisera have antigen-combining sites specific for strain 2 histocompatibility antigens, only the F(ab)<sub>2</sub> fragments retained the capacity to suppress strain 2-associated immune responses *in vitro*. The effects of the Fab, Fc, and F(ab)<sub>2</sub> fragments on antigen-stimulated [<sup>3</sup>H]TdR incorporation was assessed in cultures

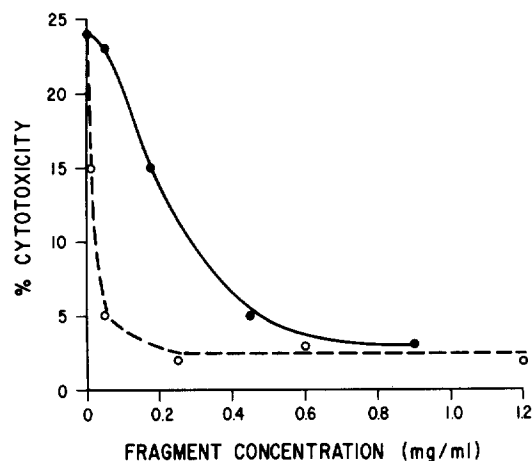


FIG. 1. Comparative effect of Fab (●—●) and F(ab)<sub>2</sub> (○—○) fragments derived from strain 13 antistrain 2 alloantisera as inhibitors of the complement-dependent cytotoxicity mediated by the intact 13 anti-2 serum. The fragments were added at the final concentrations indicated. The 13 anti-2 serum was present at a final dilution of 1/300. Lymphocytes from strain 2 guinea pigs were the target cells.

of lymphocytes obtained from  $(2 \times 13)F_1$  guinea pigs that had been immunized to both DNP-GL and GT (Table II). The  $F(ab)'_2$  fragments at a final in vitro concentration of 0.12 mg/ml completely suppressed the response to DNP-GL which is controlled by a strain 2 histocompatibility-linked *Ir* gene. The response to GT, which is controlled by a strain 13 histocompatibility-linked *Ir* gene, was not suppressed by the  $F(ab)'_2$  fragments at that concentration. There was no specific suppression of DNP-GL-stimulated  $[^3H]TdR$  incorporation by the Fab fragments at a concentration of 0.45 mg/ml. At this concentration, however, it slightly inhibited both the DNP-GL and GT responses (14% and 21%, respectively). The Fc fragments had no effect on either DNP-GL or GT-stimulated  $[^3H]TdR$  incorporation.

The  $F(ab)'_2$  fragments are potent suppressors of strain 2 histocompatibility-linked immune responses in vitro. The suppression of DNP-GL-stimulated  $[^3H]TdR$  incorporation is observed at concentrations of  $F(ab)'_2$  less than 5  $\mu g/ml$ . Table III presents a summary of the results obtained in several experiments in which  $F(ab)'_2$  fragments at varying concentrations were added to DNP-GL and GT-stimulated cultures of sensitized lymphocytes from  $(2 \times 13)F_1$  guinea pigs. At  $F(ab)'_2$  concentrations of 0.06 mg/ml and higher, the suppression of DNP-GL-stimulated  $[^3H]TdR$  incorporation was virtually complete. At .024 mg/ml the  $F(ab)'_2$  fragments still demonstrated considerable immunosuppressive capacity (76% suppression). After a further fivefold dilution of the  $F(ab)'_2$  fragments (.0048 mg/ml), however, there was a decrease in suppression to 23%. At all concentrations tested, the suppression was highly spe-

TABLE II  
*The Effect of Fab, Fc, and  $F(ab)'_2$  Fragments Derived from Antistrain 2 Sera on Antigen-Stimulated  $[^3H]TdR$  Incorporation in Vitro\**

Antigen	Ig fragment	$[^3H]TdR$ incorporation $\S$
	mg/ml	
—	—	606
DNP-GL	—	8,996
DNP-GL	$F(ab)'_2$ (0.12) $\dagger$	671
DNP-GL	Fab (0.45)	7,830
DNP-GL	Fc (0.32)	8,787
GT	—	3,904
GT	$F(ab)'_2$ (0.12)	4,275
GT	F(ab) (0.45)	3,222
GT	Fc (0.32)	3,815

\* Lymphocytes were obtained from individual  $(2 \times 13)F_1$  guinea pigs immunized to both DNP-GL and GT.

$\dagger$  The quantities in parentheses indicate the final in vitro concentration in mg/ml of each fragment.

$\S$  The data are expressed as the mean cpm of triplicate cultures.

TABLE III

*The Concentration of F(ab)'<sub>2</sub> Fragments Derived from Antistrain 2 Sera Specifically Suppressing DNP-GL-Stimulated Immune Responses in Vitro\**

F(ab)' <sub>2</sub> concentration†	% Suppression‡	
	DNP-GL	GT
<i>mg/ml</i>	mean (S.E.)	mean (S.E.)
0.12	100 (0.25)	5 (0.75)
0.06	95 (2)	0 (0)
0.024	76 (9)	0 (0)
0.0048	23 (7)	0 (0)

\* Lymphocytes were obtained from individual (2 × 13)F<sub>1</sub> guinea pigs immunized to both DNP-GL and GT.

† F(ab)'<sub>2</sub> fragments derived from strain 13 antistrain 2 alloantisera were added at the final in vitro concentrations in mg/ml indicated.

‡ The data are expressed as the mean % suppression (calculated as described in the Materials and Methods section) with the standard error in the parentheses obtained in four experiments, each using the lymphocytes from a different guinea pig.

cific for the strain 2-associated DNP-GL responses. The F(ab)'<sub>2</sub> fragments had no measurable effect on responses to GT.

Effective suppression of DNP-GL-stimulated [<sup>3</sup>H]TdR incorporation does not require that the alloantiserum be present at the time of the initiation of the cultures; i.e., when antigen is added to the lymphocytes. The presence of a 1/150 dilution of the 13 anti-2 serum, at the inception of the cultures completely suppressed the DNP-GL-stimulated [<sup>3</sup>H]TdR incorporation by lymphocytes obtained from strain 2 guinea pigs immunized to that antigen (Fig. 2). No significant loss of suppression (90% mean suppression) was noted when addition of the 13 anti-2 serum was delayed until 3 h after initiation of the cultures. An average suppression of 45% was still observed after waiting 6 h before addition of the alloantiserum. After a 24 h delay, however, the 13 anti-2 serum only slightly suppressed the response to DNP-GL (mean 15%). When added 48 h after initiation of the cultures (24 h before terminating the cultures), the alloantiserum did not affect DNP-GL-stimulated [<sup>3</sup>H]TdR incorporation.

A comparison of the suppressive effects of strain 13 antistrain 2 alloantisera on two different strain 2 histocompatibility-associated immune responses is shown in Table IV. When added to cultures of lymphocytes obtained from strain 2 guinea pigs immunized to both DNP-GL and GA, the 13 anti-2 sera suppressed the response to DNP-GL to a significantly greater degree than it did the GA response. At a 1/150 dilution, the 13 anti-2 serum completely suppressed DNP-GL-stimulated [<sup>3</sup>H]TdR incorporation (95% mean suppression). The response to GA in cultures of lymphocytes obtained from the same animals was suppressed only 40% on the average by that dilution of the alloantiserum.

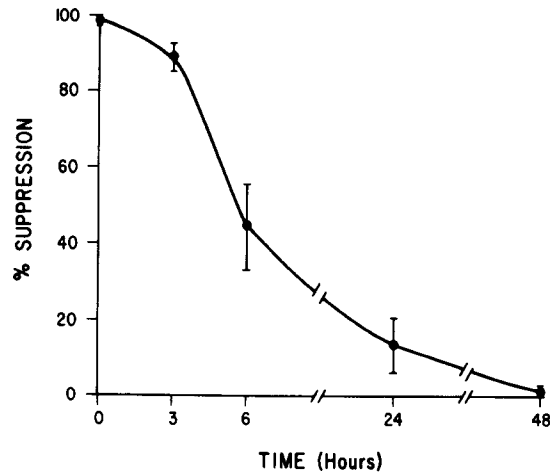


FIG. 2. The effect of delayed addition of strain 13 antistrain 2 alloantiserum on the suppression of DNP-GL-stimulated [ $^3$ H]TdR incorporation in vitro. The % suppression induced by a 1/150 final dilution of the 13 anti-2 serum added at the times indicated after initiation of the DNP-GL-stimulated cultures, was calculated as described in the Materials and Methods section. Each point represents the mean of four separate experiments, each using lymphocytes from individual strain 2 guinea pigs immunized to DNP-GL. The brackets incorporate the mean  $\pm$  the SE.

TABLE IV  
*Comparative Suppression of DNP-GL and GA-Stimulated Immune Responses in Vitro by Strain 13 Antistrain 2 Alloantisera\**

Antigen	13 Anti-2 serum dilution	% Suppression (mean (S.E.))
GA	1/150	40 $\pm$ 7
DNP-GL	1/150	95 $\pm$ 5

\* Lymphocytes were obtained from individual strain 2 guinea pigs immunized to both DNP-GL and GA.

Strain 13 antistrain 2 alloantisera were added at the final in vitro dilution indicated.

The data are expressed as the mean % suppression (calculated as described in the Materials and Methods section) and standard error obtained from five experiments using the lymphocytes from five animals. The difference observed between the two groups is statistically significant ( $P < .01$ ) as calculated by the Student's  $t$  test.

#### DISCUSSION

Alloantisera obtained from guinea pigs immunized with allogeneic lymphocytes specifically suppress immune responses controlled by  $I\tau$  genes that are linked to the allogeneic histocompatibility antigens (6, 7). The antihistocompatibility antibodies contained in the alloantisera are responsible for the observed immunosuppression (7, 19). The experiments described in this report were aimed at elucidating the mechanisms through which antibodies directed at histocompatibility antigens alter the responsiveness of lymphocytes to antigenic



stimulation. The data show that the divalent  $F(ab)'_2$  fragments prepared by pepsin hydrolysis of the Ig fraction of strain 13 antistrain 2 alloantisera effectively suppress DNP-GL-stimulation [ $^3H$ ]TdR incorporation. In addition, the  $F(ab)'_2$ -mediated immunosuppression, like that of the intact antibody, is specific for immune responses controlled by strain 2 histocompatibility-linked *Ir* genes. These fragments do not suppress the response to GT, which is controlled by a strain 13 histocompatibility-linked *Ir* gene. On a mole for mole basis, the  $F(ab)'_2$ -mediated immunosuppression is as efficient as the intact antibody. We have shown,<sup>2</sup> that a 1/375 dilution of the 13 anti-2 serum produced 86% suppression of DNP-GL-stimulated [ $^3H$ ]TdR incorporation. Assuming an Ig concentration of 20 mg/ml in the alloantiserum and a mol wt of 150,000 for the guinea pig IgG, the 1/375 dilution represents an IgG concentration of  $3.5 \times 10^{-4}$   $\mu$ mol/ml. The suppression decreased to 25% at a concentration of  $7 \times 10^{-5}$   $\mu$ mol/ml. A similar degree of suppression of DNP-GL-stimulated [ $^3H$ ]TdR incorporation (76%) was achieved with the  $F(ab)'_2$  fragments at a concentration of  $2.4 \times 10^{-4}$   $\mu$ mol/ml (.024 mg/ml, 100,000 mol wt); and at  $4.8 \times 10^{-5}$   $\mu$ mol/ml of  $F(ab)'_2$  there was 23% suppression. Thus, elimination of most of the Fc portion of the alloantibodies did not alter their effectiveness in suppressing antigen stimulation.

The monovalent Fab fragments of the 13 anti-2 serum, retained their antibody-combining site, as shown by their ability to inhibit the complement-dependent cytotoxicity mediated by the intact antibody; but, in contrast to the  $F(ab)'_2$  fragments, they did not suppress antigen stimulation in vitro. The data indicates that this difference between the Fab and  $F(ab)'_2$  fragments cannot be attributed to the higher binding efficiency of the divalent fragment. The  $F(ab)'_2$  fragments do have a higher avidity for the strain 2 histocompatibility antigens than do the monovalent Fab fragments. At 0.45 mg/ml, the Fab fragments almost completely inhibits alloantiserum-mediated cytotoxicity; the same degree of inhibition requires only 0.06 mg/ml of  $F(ab)'_2$ . However, even at 0.45 mg/ml the Fab fragments don't significantly suppress responses to DNP-GL, while concentrations of  $F(ab)'_2$  as low as .005 mg/ml are effective. Thus, although concentrations of the Fab and  $F(ab)'_2$  fragments were chosen that completed equally with the intact alloantibodies for the binding to histocompatibility antigens, only the divalent molecule suppressed antigen stimulation.

The suppression of antigen-stimulated [ $^3H$ ]TdR incorporation could result either from preventing the antigenic stimuli from reaching the appropriate sites on the sensitized lymphocyte membrane or by interfering with the response of the lymphocyte to the stimuli. There are at least two ways in which the antigenic stimulus could be kept from the cell. (a) The antibody could block access

<sup>2</sup> Bluestein, H. G. 1974. Alloantiserum-mediated suppression of histocompatibility-linked *Ir*-gene-controlled immune responses. Histocompatibility antigen requirement. Manuscript submitted for publication.

to the antigen receptor by directly combining with it or by attaching to a nearby site on the membrane and sterically masking it. Direct attachment of the antibody to the receptor is unlikely. The antistrain 2 alloantisera do not suppress GA responses in lymphocytes from responder guinea pigs who, as the result of a crossover, inherited the ability to respond to GA but did not inherit the genes coding for the strain 2 histocompatibility antigens (7, 19). Furthermore, if the suppression were due simply to the combining of antibody to the receptor, the monovalent Fab fragment should effectively suppress, and it does not. Steric hindrance also seems to be an unlikely mechanism.  $F(ab)'_2$  fragments lack almost all of the Fc portion of the molecule, which is the portion that would be expected to produce the steric inhibition, and yet they are equally as effective as the intact antibody in suppressing antigen stimulation. (b) Antihistocompatibility antibodies could suppress antigen stimulation by masking the histocompatibility antigens; thereby, preventing cell-to-cell interaction. In guinea pigs, optimal *in vitro* lymphocyte stimulation requires that the antigen be presented on syngeneic macrophages, suggesting that macrophage-T-cell interaction is facilitated through matched histocompatibility antigens (11). However, masking of the histocompatibility sites is not sufficient to suppress immune responses, since the Fab fragments, as shown by their inhibition of antibody-mediated cytotoxicity, effectively mask the histocompatibility antigens, but do not suppress antigen stimulation.

The effective suppression of antigen-stimulated [ $^3H$ ]TdR incorporation, when addition of the alloantisera is delayed for several hours after initiation of the cultures, also indicates that the suppression is not due to preventing antigenic stimuli from reaching their receptors on the lymphocyte membrane. It is likely that interaction of lymphocytes with antigen laden macrophages has already occurred within that time period. There is significant uptake of antigen by macrophages after exposures as brief as 5 min (20). In addition, *in vitro* lymphocyte proliferation has been demonstrated when lymphoid populations have been exposed to antigen for only 10 min (21). Our results suggest, therefore, that alloantiserum-mediated suppression results from interference with the response of the lymphocyte to the antigenic stimuli.

Alloantisera added 24 h after initiation of the antigen-stimulated cultures caused only slight suppression of the proliferative response. The hot-pulsed "suicide" experiments of Dutton and Mishell (22) demonstrated that the onset of the DNA synthetic response of sensitized lymphocytes occurs in the first 24 h after secondary antigenic stimulation. Thus, once a key step in antigen stimulation has occurred, the response cannot readily be suppressed by antihistocompatibility antibody.

The histocompatibility antigens of both parental strains appear to be expressed on the lymphocytes of hybrid  $F_1$  animals. Using an indirect immunofluorescent technique we have found that both the antistrain 2 and antistrain 13 sera bind to over 90% of the lymphocytes purified from peritoneal exudates in-

duced in  $(2 \times 13)F_1$  guinea pigs (R. Coss and H. G. Bluestein, unpublished data). It is improbable that the majority of the antigen reactive cells are limited to the small percentage of cells that do not bind both antibodies. Although each cell possesses both strain 2 and strain 13 histocompatibility antigens, the addition of antistrain 2 sera to cultures of  $(2 \times 13)F_1$  guinea pig lymphocytes sensitized to both GT and DNP-GL suppresses the strain 2 histocompatibility-associated DNP-GL response but does not affect the response of the same cell population to the strain 13 histocompatibility-associated GT response. This phenomenon suggests that the genetic linkage of histocompatibility and *Ir* loci may have a counterpart in the phenotypic expression of the products of those genes on the lymphocyte membrane. The observed differences in the degree of suppression of two different immune responses controlled by *Ir* genes linked to the same histocompatibility locus supports that view. The GA and the poly-L-lysine (PLL) gene (controlling immune responses to DNP-GL) are both linked to strain 2 histocompatibility antigens (18), yet the response of sensitized strain 2 lymphocytes to DNP-GL is more profoundly suppressed by the 13 anti-2 sera than is their response to GA. This suggests a closer relationship between strain 2 histocompatibility antigens and the PLL-gene product than between strain 2 histocompatibility antigens and the GA-gene product. The PLL gene is very closely linked to the strain 2 histocompatibility locus. No recombinants between these two loci have yet been reported. Recombinant animals have been found, however, that respond to GA and not to PLL and vice versa (23). Among those recombinants, the animals inheriting the PLL gene also inherited the strain 2 histocompatibility locus; while those inheriting the GA gene did not. Thus, the relative closeness of the genetic linkage of the strain 2 histocompatibility locus to the PLL and GA genes is mirrored on the lymphocyte surface by the degree of interaction of antistrain 2 histocompatibility sera with the immune responses controlled by the PLL and GA genes.

The apparent similarities between the functional relationship of histocompatibility antigens to the development of *Ir*-gene-controlled immune responses, and the genetic relationship of the histocompatibility locus to *Ir*-gene loci suggest that the histocompatibility region of the chromosome is copied as a polycistronic message, and, after translation, expressed "en bloc" on the lymphocyte surface. We hypothesize, further, that because of the regionalized representation of histocompatibility antigens and linked *Ir*-gene products, divalent antihistocompatibility antibody or its  $F(ab)'_2$  fragments can produce localized perturbations of the lymphocyte membrane that prevent the lymphocyte from responding to antigenic stimulation in those regions.

#### SUMMARY

Fab, Fc, and  $F(ab)'_2$  fragments were prepared by enzymatic hydrolysis of the IgG fraction of strain 13 antistrain 2 alloantisera. These fragments were not cytotoxic to lymphocytes bearing strain 2 histocompatibility antigens, but the

Fab and F(ab)<sub>2</sub> fragments retained functional combining sites as indicated by their ability to suppress the cytotoxicity mediated by the intact antistrain 2 antibodies. The F(ab)<sub>2</sub> fragments were much more efficient as inhibitors in this system than the Fab fragments. F(ab)<sub>2</sub> at 0.06 mg/ml and 0.45 mg/ml Fab produced comparable degrees of suppression. The F(ab)<sub>2</sub> at 0.06 mg/ml completely suppressed DNP copolymer of L-glutamic acid and L-lysine (GL)-stimulated tritiated thymidine incorporation. The monovalent Fab at 0.45 mg/ml, however, had no significant effect on the in vitro responses to DNP-GL. Addition of the intact alloantisera can be delayed 3 h after initiation of the antigen-stimulated cultures with no loss of suppression. After a delay of 6 h 45% suppression was observed. The requirement for the divalent molecule and the observation that effective suppression of the in vitro responses is still obtained when the alloantiserum is added several hours after initiation of the cultures both suggest that the immunosuppression results from an active process affecting the lymphocyte membrane that renders the cell refractory to the antigenic stimulus.

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#### REFERENCES

1. Benacerraf, B., and H. O. McDevitt. 1972. Histocompatibility-linked immune response genes. *Science (Wash. D. C.)*. **175**:273.
2. Boyse, E. A., L. Flaherty, E. Stockert, and L. J. Old. 1972. Histoincompatibility attributable to genes near H-2 that are not revealed by hemagglutination or cytotoxicity tests. *Transplantation (Baltimore)*. **13**:431.
3. Klein, J., and D. C. Shreffler. 1971. The H-2 model for the major histocompatibility systems. *Transplant. Rev.* **6**:3.
4. Tyan, M. L., H. O. McDevitt, and L. A. Herzenberg. 1969. Genetic control of the antibody response to a synthetic polypeptide: transfer of response with spleen cells or lymphoid precursors. *Transplant. Proc.* **1**:548.
5. Foerster, J., I. Green, J.-P. Lamelin, and B. Benacerraf. 1969. Transfer of responsiveness to hapten conjugates of poly-L-lysine and of a copolymer of L-glutamic acid and L-lysine to lethally irradiated nonresponder guinea pigs by bone marrow or lymph node and spleen cells from responder guinea pigs. *J. Exp. Med.* **130**:1107.
6. Shevach, E. M., W. E. Paul, and I. Green. 1972. Histocompatibility-linked immune response gene function in guinea pigs. Specific inhibition of antigen-induced lymphocyte proliferation by alloantisera. *J. Exp. Med.* **136**:1207.
7. Bluestein, H. G. 1973. Specific suppression of GA responses in lymphocytes from Hartley guinea pigs by an anti-strain 2 alloantiserum. *Fed. Proc.* **32**:985. (Abstr.)
8. Kindred, B., and D. C. Shreffler. 1972. H-2 dependence of cooperation between T and B cells in vivo. *J. Immunol.* **109**:940.
9. Katz, D. H., T. Hamaoka, and B. Benacerraf. 1973. Cell interactions between histocompatible T and B lymphocytes. II. Failure of physiologic cooperative interactions between T and B lymphocytes from allogeneic donor strains in humoral response to hapten-protein conjugates. *J. Exp. Med.* **137**:1405.

10. Rosenthal, A. S., and E. M. Shevach. 1973. Function of macrophages in antigen recognition by guinea pig T lymphocytes. I. Requirement for histocompatible macrophages and lymphocytes. *J. Exp. Med.* **138**:1194.
11. 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.
12. Kantor, F. S., A. Ojeda, and B. Benacerraf. 1963. Studies on artificial antigens. I. Antigenicity of DNP-poly-lysine and DNP copolymers of lysine and glutamic acid in guinea pigs. *J. Exp. Med.* **117**:55.
13. Ellman, L., I. Green, W. J. Martin, and B. Benacerraf. 1970. Linkage between the poly-L-lysine gene and the locus controlling the major histocompatibility antigens in strain 2 guinea pigs. *Proc. Natl. Acad. Sci. U. S. A.* **66**:322.
14. Oettgen, H. F., R. A. Binaghi, and B. Benacerraf. 1965. Hexose content of  $\gamma_1$  and  $\gamma_2$  immunoglobulins. *Proc. Soc. Exp. Biol. Med.* **118**:336.
15. Porter, R. R. 1959. The hydrolysis of rabbit  $\gamma$ -globulin and antibodies with crystalline papain. *Biochem. J.* **73**:119.
16. Edelman, G. M., J. F. Heremans, M. T. Heremans, and H. G. Kunkel. 1960. Immunological studies of human  $\gamma$ -globulin. Relation of the precipitin lines of whole  $\gamma$ -globulin to those of the fragments produced by papain. *J. Exp. Med.* **112**:203.
17. Edelman, G. M., and J. J. Marchalonis. 1967. Methods used in studies of the structure of immunoglobulins. *Methods Immunol. Immunochem.* **1**:405.
18. Bluestein, H. G., L. Ellman, I. Green, and B. Benacerraf. 1971. Specific immune response genes of the guinea pig. III. Linkage of the GA and GT immune response genes to histocompatibility genotypes in inbred guinea pigs. *J. Exp. Med.* **134**:1529.
19. Shevach, E. M., I. Green, and W. E. Paul. 1974. Alloantiserum induced inhibition of immune response gene product function. II. Genetic analysis of target antigens. *J. Exp. Med.* **139**:679.
20. Rosenstreich, D. L., and A. S. Rosenthal. 1973. Peritoneal exudate lymphocyte. II. In vitro lymphocyte proliferation induced by brief exposure to antigen. *J. Immunol.* **110**:934.
21. Kasakura, S. 1969. Blastogenesis of lymphocytes induced by PPD-primed X-irradiated autologous lymphocytes. *J. Immunol.* **103**:1078.
22. Dutton, R. W., and R. I. Mishell. 1967. Cell populations and cell proliferation in the in vitro response of normal mouse spleen to heterologous erythrocytes. Analysis by the hot pulse technique. *J. Exp. Med.* **126**:443.
23. Bluestein, H. G., I. Green, and B. Benacerraf. 1971. Specific immune response genes of the guinea pig. II. Relationship between the poly-L-lysine gene and the genes controlling immune responsiveness to copolymers of L-glutamic acid and L-alanine and L-glutamic acid and L-tyrosine in random-bred Hartley guinea pigs. *J. Exp. Med.* **134**:471.