ALLOANTISERUM-INDUCED INHIBITION OF IMMUNE RESPONSE GENE PRODUCT FUNCTION

I. CELLULAR DISTRIBUTION OF TARGET ANTIGENS

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The capacity of individual animals to mount specific immune responses to many antigens is under precise genetic control. One group of genes controlling responsiveness to individual antigens are the histocompatibility-linked immune response (Ir) genes (1). As implied by their name, these genes are linked to the major histocompatibility complex (MHC) of the species. Considerable experimental evidence indicates that IR gene products play a role in antigen recognition by thymus-derived (T) lymphocytes (2). We have previously demonstrated (3) that alloantisera prepared by reciprocal immunization of strain 2 and strain 13 guinea pigs specifically block the activation of T lymphocytes from immune guinea pigs by antigens, the response to which is controlled by Ir genes. Thus, when the lymphocytes from (2 X 13)F1 guinea pigs were cultured in vitro, anti-2 serum inhibited the response to a 2,4-dinitrophenyl derivative of the copolymer of L-glutamic acid and L-lysine (DNP-GL, an antigen, the response to which is controlled by a 2-linked Ir gene), while anti-13 serum inhibited the response to a copolymer of L-glutamic acid and L-tyrosine (GT, an antigen, the response to which is controlled by a 13-linked Ir gene).

A number of possible mechanisms were suggested by which alloantisera might block T-lymphocyte antigen recognition. First, the Ir genes might be identical with the genes controlling strain 2 or 13 histocompatibility (H) specificities. It was felt that this explanation was unlikely because studies in the mouse (4) have demonstrated that Ir genes are separate from D and K region genes, which control the major serologically identifiable H antigens in this species. Second, the Ir gene product could be physically related to H antigen on the cell surface and allo-antisera might exert their inhibitory function by reacting with H antigen and sterically interfering with the function of

1 Abbreviations used in this paper: CFA, complete Freund's adjuvant; 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; PEC, peritoneal exudate cells; PELs, peritoneal exudate lymphocytes; PHA, phytohemagglutinin; PPD, purified protein derivative of tuberculin.

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the Ir gene product. Thirdly, in addition to anti-H antibodies, the alloantisera may contain antibodies directly reactive with and capable of directly blocking the specific Ir gene products. Such antibodies would facilitate the isolation and characterization of the Ir gene product and increase our understanding of the function of Ir genes in T-lymphocyte antigen recognition.

In these papers, we will present further studies on the mechanism and specificity of alloantisera-induced inhibition of T-lymphocyte function. In the first paper, we will examine the effects of absorption of the alloantisera with different populations of immunocompetent cells and attempt to determine whether the inhibitory antibody is directed against antigens solely on T lymphocytes, or, alternatively, against antigens also present on bone marrow-derived (B) lymphocytes. We will also compare the effects of the alloantisera with a number of anti-immunoglobulin reagents on the inhibition of the in vitro lymphocyte proliferative response to antigen.

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 X 13)F1 animals were obtained by mating strain 2 with strain 13 animals in our own colony.

Antigens.—A copolymer of L-glutamic acid (60%) and L-lysine (40%) (GL) with an average mol wt of 115,000 was obtained from the Pilot Chemical Division of New England Nuclear Corp., Boston, Mass. DNP-GL was prepared by the reaction of 2,4-dinitrofluorobenzene with GL (5). The subscript refers to the average number of DNP groups/molecule. A copolymer of L-glutamic acid (50%) and L-tyrosine (50%) (GT), mol wt 22,600, was obtained from Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill. Purified protein derivative of tuberculin (PPD) was purchased from Connaught Medical Research Laboratories, Ontario, Canada. Phytohemagglutinin (PHA) was obtained from Wellcome Research Laboratories, Beckenham, England.

Immunization of Guinea Pigs.—Solutions of each antigen in 0.15 M phosphate buffer, pH 7.4 containing 0.15 M NaCl were emulsified with an equal volume of complete Freund's adjuvant (CFA) containing 0.5 mg of Mycobacterium butyricum/ml (Difco Laboratories, Detroit, Mich.). F1 animals were immunized simultaneously with 100 #g of DNP-GL and 500 #g of GT; each antigen was administered in one front footpad and one rear footpad.

Preparation of Alloantisera.—A strain 13 antistrain 2 serum and a strain 2 antistrain 13 serum were prepared by cross-immunizing strain 2 and strain 13 animals with a CFA emulsion of a homogenate of lymph node and spleen cells. The animals were boosted 2 and 4 wk after primary immunization by an intradermal injection of a homogenate of lymph node and spleen cells. The sera used in these studies were obtained 8–10 wk after initial immunization. They were sterilized by Millipore filtration and heat inactivated at 56°C for 45 min before use.

L2C Leukemia Cells.—The L2C leukemia cells were originally a gift of Dr. L. Kaplow, Yale University, and have been maintained by serial passage in strain 2 animals in our colony. Leukemia cells were obtained from the peripheral blood of moribund animals with peripheral white blood cell counts in excess of 200,000/mm$^3$ by gelatin sedimentation as previously described (6).

Cytotoxicity Testing.—The 13 anti-2 serum was tested in a $^{31}$Cr release assay as previously described (7). In brief, 50 X 10$^6$ lymph node cells or L2C leukemia cells were incubated in 1 ml of Hanks' balanced salt solution containing 10% heat-inactivated fetal calf serum (Industrial Biological Laboratories, Rockville, Md.) and 100 μCi $^{31}$Cr (spec act 185 μCi/μg, Amersham Searle, Des Plaines, Ill.) for 30-60 min at 37°C. These cells were then washed three
times and resuspended in media at a concentration of $10 \times 10^6 / \text{ml}$. An aliquot (0.1 ml) of this suspension was then added to 10 x 75-mm glass tubes, followed by 0.1 ml of the appropriate dilution of antibody and 0.1 ml of a 1:2 dilution of lyophilized guinea pig serum (Grand Island Biological Co., Grand Island, N. Y.) as a source of complement (C). After a 30 min incubation at 37°C, the cells were sedimented by centrifugation at 1,500 rpm and the radioactivity in 0.1 ml of the supernate was measured. The amount of radioactivity in the sample was compared with the radioactivity present in an equal volume from tubes which contained cells in medium plus C only and with that present in tubes which were frozen and thawed four times. The percent maximum $^{51}$Cr release was calculated as follows:

$$\text{percent maximum } ^{51}\text{Cr release} = \frac{\text{radioactivity released by antiserum dilution} - \text{radioactivity released in absence of antiserum}}{\text{radioactivity released by frozen thawed cells} - \text{radioactivity released in absence of antiserum}} \times 100.$$

_Absorption of Antibodies._—In the majority of studies 1 x $10^6$ cells were used to absorb the equivalent of 1 ml of undiluted antiserum; the sera were absorbed with an equal volume of the absorbing cell suspension for 2 h at 4°C. The absorbed antiserum was then spun at 15,000 rpm for 30 min and sterilized by Millipore filtration.

_Anti-Immunoglobulin Reagents._—A number of different anti-guinea pig immunoglobulin reagents were used. Rabbits were immunized with either 2 mg of guinea pig gamma globulin (Fraction II, Miles Laboratories) or 2 mg of a Fab fragment of guinea pig $\gamma_2$ globulin (a generous gift of Dr. V. Nussenzweig, New York University School of Medicine). Two separate lots of the anti-Fab serum were prepared. The antiguainea pig Ig reagent was reactive with $\gamma_1$, $\gamma_2$, and $\gamma_M$ heavy chains and with Fab fragment as determined by Ouchterlony analysis; the two anti-Fab reagents were reactive only with the Fab fragments. A rabbit antiserum specific for $\gamma_2$ heavy chains was a gift of Dr. J. Davie of the Washington University School of Medicine; the method of antibody purification and the specificity of this antibody have been previously described (8). All of the anti-immunoglobulin reagents had at least 1–2 mg of antibody protein/ml of serum. Before use in cell cultures, the anti-immunoglobulin reagents were fractionated by ammonium sulfate precipitation and dialyzed against culture media.

_Cell Collection and Purification._—The techniques for the collection of peritoneal exudate cells (PEC) have been described previously (9). In brief, animals were injected intraperitoneally with 25 ml of sterile mineral oil (Marcol 52, Humble Oil & Refining Co., Houston, Texas). 3–4 days later the exudate cells were harvested by lavaging the peritoneal cavity with 150 ml of Hanks' balanced salt solution. The resultant PEC population is composed of about 75% macrophages, 10% neutrophils, and 15% lymphocytes. Peritoneal exudate lymphocytes, a population of highly enriched antigen-reactive T lymphocytes were obtained by passing the PEC over adherence columns. After purification the resultant population is composed of 80–90% lymphocytes and 10–20% macrophages.

_Technique of Brief Antigen Exposure._—In all of the experiments described in this report, the unfractionated PEC population was used as the source of macrophages. PEC at a concentration of $15 \times 10^6 / \text{ml}$ in the presence of 30 $\mu$g/ml of mitomycin C (Nutritional Biochemicals, Cleveland, Ohio) were allowed to equilibrate at 37°C for 60 min. Mitomycin C was employed in order to inhibit DNA synthesis by the small number of lymphocytes present in the PEC population. The final concentration of antigen or mitogen used in the incubation medium was 100 $\mu$g/ml DNP-GL, 100 $\mu$g/ml GT, 100 $\mu$g/ml PPD, or 10 $\mu$g/ml PHA. At the end of the exposure period the cell suspensions were washed four times with media.

_In Vitro Assay of Antigen-Induced DNA Synthesis._—Antigen-pulsed PEC at a concentration of $1 \times 10^6 / \text{ml}$ were mixed with exudate lymphocytes at a concentration of $2 \times 10^4 / \text{ml}$. 0.2 ml aliquots of these mixtures were cultured in round-bottom microtiter plates (Cooke Laboratory Products, Cooke Engineering Co., Arlington, Va.) in medium RPMI-1640 (GIB-
CO) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (300 μg/ml), and 10% serum. When alloantisera were added to the cell cultures equal parts of alloantisera and normal guinea pig serum were used. In those experiments where the anti-immunoglobulin reagents were added to the cultures, guinea pig serum was omitted and cell growth was supported by the addition of 10% fetal calf serum. The final concentration of anti-immunoglobulin present in each culture represented a 1:2 dilution of the original antiserum. The cultures were maintained at 37°C for 72 h in a humid atmosphere of 5% CO₂ in air. 18 h before harvesting, 1.0 μCi of tritiated thymidine ([³H]thymidine, 6.7 Ci/mM, New England Nuclear Corp.) was added to each culture. The amount of [³H]thymidine-incorporated into cellular DNA was measured with the aid of a semiautomated microharvesting device (10). Radioactivity was counted in a Beckman liquid scintillation counter (Beckman Instruments, Fullerton, Calif.) and expressed as either total cpm/culture or as the difference between control and antigen stimulated cultures (Δ cpm/culture). In some experiments because of the variable capacity of different sera to support growth in culture, the results were normalized and expressed as the fraction (Fx) of the PHA response:

\[ Fx \text{ PHA Response} = \frac{\text{cpm with antigen} - \text{cpm with no antigen}}{\text{cpm with PHA} - \text{cpm with no antigen}}. \]

RESULTS

The Relationship of the Ability of Alloantisera to Inhibit T-Lymphocyte Proliferation and to Lyse B Lymphocytes.—In this and the companion paper (11), the anti-H activity of a serum is defined by its capacity to lyse lymphoid cells of appropriate phenotype in the presence of C. The capacity of a serum to block Ir gene product function is measured by inhibition of the stimulation of DNA synthesis by immune F₁ lymphocytes by antigens, the response to which is controlled by that Ir gene. Initially, we wished to compare the relative effect of dilution of the alloantiserum on these two assays. The population of lymphocytes used as targets in the cytotoxicity assay were L₂C leukemia cells. These leukemia cells are of strain 2 origin and bear large amounts of strain 2 H antigens. Furthermore, L₂C cells are of B-cell derivation as evidenced by the presence of surface immunoglobulin and the receptor for the third component of C (6). The top half of Fig. 1 illustrates the cytotoxic activity of the 13 anti-2 serum for L₂C leukemia cells at a number of different antibody dilutions. Even at a dilution of 1:120, 15% ⁵¹Cr release is observed from L₂C cells. The lower half of Fig. 1 illustrates the inhibitory activity of this same anti-2 serum on the proliferation of F₁ lymphocytes to DNP-GL and to PPD. Complete suppression of the DNP-GL response is observed at a 1:10 and 1:20 antiserum dilution. As reported previously (3), only minimal suppression of the response to PPD is observed under these conditions. At a 1:120 dilution of the antiserum inhibition of the DNP-GL response is no longer seen. We conclude from this experiment that the ability of the 13 anti-2 serum to inhibit the response to DNP-GL in F₁ animals is roughly related to its cytotoxic activity for the B-lymphocyte leukemia, L₂C, although the inhibitory activity of the antiserum for T-lymphocyte proliferation tends to be lost at lower dilutions of antiserum than is the cytotoxic activity for L₂C leukemia cells.
Absorption of the Alloantisera with "Immune" or "Nonimmune Cells."—Our initial studies on the inhibition of T-cell function with alloantisera were performed with alloantisera which had been prepared by cross-immunizing strain 2 and strain 13 animals with lymph node and spleen cells; in these studies the lymph node cells used were obtained from animals immunized with CFA. Although the resultant antisera were effective inhibitors of T-cell function, one possible explanation for their activity is that they are directed not toward H antigen but rather toward unique antigenic determinants (idiotypes) of receptors with specificity for DNP-GL. If the inhibitory activity of the alloantisera is based on such "anti-idiotype" activity, then one would anticipate that the cells of an animal immune to DNP-GL would be much more effective than the cells of an animal immunized to another antigen in absorbing the inhibitory activity. To evaluate this possibility a 13 anti-2 serum prepared against lymph node and spleen cells from a CFA immune animal was absorbed with either $0.8 \times 10^8$ or $2.0 \times 10^8$ lymph node and spleen cells from a strain 2 animal that had received CFA alone or with similar numbers of lymph node and spleen cells from an animal that had received DNP-GL and CFA. The top half of Fig. 2 illustrates the residual cytotoxic activity of these absorbed sera for strain 2 lymph node cells. No significant difference is noted in the ability of the DNP-GL or CFA cells to remove the cytotoxic activity of the anti-2 serum. In similar fashion (Fig. 2, bottom) no significant difference is noted in the ability of these two cell populations to remove the inhibitory activity from the anti-2 serum; indeed, in the experiment illustrated the CFA cells were slightly more...
effective than the DNP-GL cells. It therefore is unlikely that the anti-2 serum is directed against unique antigenic determinants of a receptor specific for DNP-GL, which is represented in a clonal fashion on the population of DNP-GL immune cells.

Absorption of the Anti-2 Serum with Different Populations of Lymphoid Cells.—We next determined the cellular distribution of the antigens against which the inhibitory antibodies of the anti-2 sera are directed. Our approach was to absorb the anti-2 serum with lymphoid cells from a variety of sources and to measure the diminution in cytotoxic titer and in inhibitory activity. Initial attempts to absorb inhibitory activity with several nonlymphoid tissues revealed that the relevant antigen is either absent from these tissues or present at too low a density for us to detect by these procedures. Among lymphoid cell populations we found a general correlation in the efficiency of absorption of cytotoxic activity and of inhibitory activity (Fig. 3). As we have previously reported (7), absorption with thymocytes removed little of the cytotoxic activity of the anti-2 serum while absorption with an equivalent number of L4C cells completely removes cytotoxic activity (Fig. 3, top). Peritoneal exudate lymphocytes (PELs) which are mainly T lymphocytes are also ineffective in absorption of cytotoxic activity; lymph node lymphocytes, a mixed population of T and B lymphocytes, and unpurified PECs, a mixture of macrophages, polymorphonuclear cells and lymphocytes, partially remove cytotoxic
activity. The unabsorbed anti-2 serum (Fig. 3, bottom) completely inhibits the response of F1 PELs to DNP-GL and also causes moderate inhibition of the PPD response. The inhibitory activity of the anti-2 serum is only slightly diminished by absorption with thymocytes or PELs; absorption with lymph node cells or exudate cells produces a somewhat greater decrease in the inhibitory activity of the anti-2 serum. On the other hand, absorption with L4C cells produces the greatest decrease in the inhibitory capacity of the antiserum. Nevertheless, significant inhibitory activity is still present in the antiserum even though there is no detectable cytotoxic activity. The results indicate that the tissue distribution of the antigens against which the cytotoxic activity and the inhibitory activity of the anti-2 sera are directed is generally similar.

Further Studies on Absorption of the Anti-2 Serum with L4C Cells.—The results of the above study demonstrated that neither immature T cells (thymocytes) nor mature T cells (exudate lymphocytes), in the numbers used, were capable of selectively removing the inhibitory activity of the anti-2 serum on F1 T-cell proliferation. On the other hand, absorption of the anti-2 serum with a pure population of B cells, L4C leukemia cells, resulted in an antiserum which was totally devoid of cytotoxic activity toward either lymph node cells or L4C cells yet retained some ability to inhibit the proliferation of F1 T cells. In view of our initial demonstration that the cytotoxic titers of the sera were somewhat higher than their inhibitory titers, this observation suggests that following absorption of the anti-2 serum with L4C cells a population of inhibitory antibodies is obtained which lack anti-H activity, as demonstrated by
their lack of cytotoxicity. In order to evaluate this possibility, several lots of anti-2 serum were absorbed with L2C cells and their residual cytotoxic activity for L2C cells and their residual T-cell inhibitory activity measured. Three representative experiments are illustrated in Table I. In Exp. 1 and 2 all of the cytotoxic activity of the anti-2 serum was removed and the absorbed antiserum retained considerable, but not complete, inhibitory activity for T-cell proliferation. In Exp. 2, a control is included in which the unabsorbed anti-2 serum is diluted to the point (1:160) where it just lacks cytotoxic activity for L2C cells. This serum had very little inhibitory activity, in contrast to the absorbed anti-2 serum which retained substantial inhibitory activity. However, the absorbed antiserum in Exp. 3 was totally devoid of both cytotoxic and T-cell inhibitory activity. These contrasting results were observed in a number of other experiments performed with different lots of the anti-2 serum prepared in similar fashion. Inhibition of the DNP-GL response by some lots of the absorbed antisera was almost complete, while other lots produced only marginal inhibition of the DNP-GL response.

In two experiments involving sera which retained inhibitory activity after a single absorption with 10⁶ L2C cells, the absorption procedure was repeated

| Exp. No. | Antigen | Serum | NGPS* | 13 Anti-2 (1/20) | 13 Anti-2 (1/160) | 13 Anti-2 absorbed with L2C (1/20) |
|----------|---------|-------|-------|----------------|------------------|----------------------------------|
| 1        | 0       | DNP-GL | 709‡ | 427           | 384              |                                  |
|          |         | PPD    | 9,522| 451           | 2,411            |                                  |
|          |         |        | 15,781| 10,178       | 16,594           |                                  |
|          |         | % ⁵¹Cr release | 0% | 62%           | 2%               |                                  |
| 2        | 0       | DNP-GL | 282  | 537           | 1,207            |                                  |
|          |         | PPD    | 21,394| 854          | 2,022            |                                  |
|          |         |        | 7,353 | 9,064        | 7,210            |                                  |
|          |         | % ⁵¹Cr release | 0% | 56%           | 0%               | 0%                              |
| 3        | 0       | DNP-GL | 643  | 259           | 416              |                                  |
|          |         | PPD    | 31,948| 335          | 31,000           |                                  |
|          |         |        | 55,890| 65,107       | 75,805           |                                  |
|          |         | % ⁵¹Cr release | 0% | 78%           | 0%               | 0%                              |

* NGPS, normal guinea pigs serum.
† Results are expressed as cpm/tube; each value is the mean of three determinations.
‡ % ⁵¹Cr release in the presence of C from labeled L2C cells by a given antiserum dilution.
Before the initial absorption both lots of the anti-2 serum (no. 220 and no. 495) demonstrated significant cytotoxic activity for strain 2 lymph node cells and produced complete inhibition of the proliferative response to DNP-GL by F1 PELs. Following one absorption with L2C cells the cytotoxic activity was reduced to ~5% and considerable, although not complete, inhibition of the DNP-GL response was still observed. After a second absorption with L2C cells, both lots of antisera were completely devoid of cytotoxic activity and the degree of inhibition of the DNP-GL response was considerably diminished. Indeed, although the absolute number of counts observed after stimulation with DNP-GL in the presence of the absorbed antisera (21,000 cpm with no. 220 and 20,000 cpm with no. 492) was still less than the number of counts seen in the presence of normal serum (30,000 cpm) it is unlikely that this apparent inhibition of the DNP-GL response was specific because an equal suppression of the PHA response was seen. It is therefore likely that the observed suppression of the DNP-GL response is secondary to the reduced capacity of these serially absorbed antisera to support the growth of cells in culture. We conclude from these studies that large numbers of L2C leukemia cells are capable of completely removing the inhibitory activity of the anti-2 sera on T-cell activation. The fact that cytotoxic activity can often be removed by smaller number of L2C cells than are required to completely absorb inhibitory activity could be explained in two general ways: (a) The antigens against which the respective antibodies are directed, although having generally similar tissue distribution are different and are present in somewhat different densities on these cells. (b)...
The antibodies mediating cytotoxicity and blocking are directed at the same antigenic determinants, but it is easier to absorb the cytotoxic antibodies perhaps because of their molecular class or affinities.

The Effect of Anti-Immunoglobulin Sera on the In Vitro Proliferative Response To Antigen.—We have previously demonstrated (3) that absorption of the alloantisera with gammaglobulin of the opposite strain failed to reduce their inhibitory effect on in vitro T-cell proliferation. We concluded from this study that it was unlikely that the inhibition of T-cell function produced by the alloantisera was secondary to contaminating anti-immunoglobulin-allotype antibodies; however, the results of this study do not allow a conclusion to be drawn about the possible role of immunoglobulin as the specific T-cell receptor. Indeed, we suggested that one possible role for the Ir gene product would be to function as an auxiliary receptor to the prime antigen-binding receptor. According to this interpretation the alloantisera would inhibit T-cell function by interfering either directly or indirectly with this auxiliary receptor. In such a model, immunoglobulin would be the most likely candidate for the “prime” antigen-binding receptor of T cells. Thus, it is of interest to examine the effect of anti-immunoglobulin sera on the in vitro proliferative response to antigen under the identical experimental conditions where the inhibitory function of the alloantisera can be demonstrated. F1 PELs were cultured in the presence of macrophages which had been pulsed with DNP-GL, GT, PPD, or PHA. Under these conditions the absolute concentration of antigen in the culture is limiting. This should increase the likelihood that inhibition would occur. A number of different anti-immunoglobulin sera were used: (a) a polyvalent anti-GP immunoglobulin preparation which reacted with \( \gamma_1, \gamma_2, \gamma_M \) heavy chains and with Fab pieces; (b) an anti-\( \gamma_2 \) reagent which was specific for \( \gamma_2 \) heavy chains; (c) two anti-Fab reagents which were specific for Fab determinants. All of the anti-immunoglobulin sera were devoid of any inhibitory effect (Fig. 5) and, in addition, failed to stimulate DNA synthesis in the absence of antigen. Although slight deviations were observed between the total number of counts incorporated in the presence of normal rabbit serum and the number of counts incorporated in the presence of the various anti-immunoglobulin sera, the degree of depression of the antigen response was no greater than the depression of the response to PHA. For example, anti-Fab 1 suppressed the response to all three antigens by about 20%, but also suppressed the PHA response to the same degree. Thus, the observed inhibition is probably secondary to variability in the ability of the different sera to support cell growth in vitro. Under the same conditions the anti-2 serum produced specific suppression of the DNP-GL response (Fig. 5 A) and did not affect the GT, PPD, or PHA responses.

**DISCUSSION**

We have previously demonstrated that alloantisera can specifically block the activation of guinea pig T lymphocytes by antigens, the response to which is
linked to the presence of H specificities against which the alloantisera are directed (3). On the basis of this, we concluded that Ir genes produce a cell surface-associated product and that this product plays a role in antigen recognition by the T lymphocyte. Although the manner by which alloantisera inhibit the process of antigenic recognition is not as yet known, one possible explanation is that alloantisera contain antibodies which are directly reactive with the products of the Ir genes. In the present report we have further defined the mechanisms by which alloantisera inhibit antigen-induced lymphocyte proliferation in vitro by examining the effect of absorption of the 13 anti-2 serum with different populations of lymphoid cells.

In the initial experiments reported in this paper we tested the possibility that the inhibitory activity of the alloantisera was due to the presence of antibodies specific for the unique antigenic determinants, or idiotypes, of clonally distributed T-lymphocyte receptors. If this were the case then cells obtained from animals immunized to DNP-GL would be anticipated to be much more efficient than cells from animals immunized to a noncross-reactive antigen in absorbing the antibodies which inhibit in vitro responsiveness to DNP-GL. This expectation is based on the assumption that the clone of T cells specific for
DNP-GL would expand as a result of immunization. In fact, the cell populations were equivalent in their absorptive capacity. Furthermore, in experiments not reported here, it was found that alloantisera produced by immunizing strain 13 guinea pigs with lymphocytes from strain 2 guinea pigs immunized to DNP-GL were no more inhibitory of the in vitro response to DNP-GL than alloantisera raised against lymphocytes from strain 2 guinea pigs not immunized to DNP-GL. It is therefore highly unlikely that the inhibition of the response to DNP-GL of F1 lymphocytes is mediated by a population of antibodies directed at the idiotypic determinants of clonally distributed T-lymphocyte receptors specific for DNP-GL.

As outlined in the introduction, the inhibitory activity of the alloantisera could derive from antibodies directed at the Ir gene product itself or from antibodies directed at linked surface structures, such as "conventional" H antigens. A decision on this crucial point is, in fact, quite difficult as no method of identifying Ir gene products other than function now exists. Two types of approaches have been employed by us in an effort to resolve this issue. In the first, we sought to determine the tissue distribution of the antigen(s) against which the inhibitory antibodies are directed and to compare it with the tissue distribution of the antigen(s) against which the cytotoxic activity of the alloantisera are directed. In the second, we studied the inhibitory activity of the alloantisera against cells from animals possessing a given Ir gene but lacking the histocompatibility genes to which that Ir gene is normally linked. The results of the latter studies are presented in a companion paper (11).

In the analysis of tissue distribution we assumed that the cytotoxic activity of the alloantisera principally reflects antibodies specific for the "serologically-defined" antigens of the guinea pig H complex. As discussed below, this assumption clearly oversimplifies the complex situation now known to obtain from studies of the H-2 complex of mice. We have previously shown that strain 2 antigens definable by cytotoxicity with anti-2 sera are present in much larger amounts on B lymphocytes than on T lymphocytes (7). The strain 2 antigen is clearly present on the T-cell surface as it can be detected by indirect immunofluorescence3 and, under some circumstances, by direct cytotoxicity.3 Studies to be presented separately have shown that these antigens are also easily detectable on peritoneal macrophages but that they are either absent from or present in very low density on normal and malignant fibroblasts, and a hepatoma.3 In the studies presented here we have extended our previous analysis of the lymphoid distribution of the antigens against which the cytotoxic activity of 13 anti-2 sera is directed. Furthermore, we have shown that the tissue distribution of the antigens against which the inhibitory activity of the 13 anti-2 sera are directed is quite similar. Thus, populations of immature T lymphocytes

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3 Shevach, E. M., Z. Ben-Sasson, F. Finkelman, and I. Green. Manuscript in preparation.

3 Dorf, M. E., and B. Benacerraf. Unpublished observations.
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(thymocytes) or of mature T lymphocytes (column-purified peritoneal exudate lymphocytes) are relatively ineffective in absorbing either the cytotoxic or inhibitory activity of these sera. Lymph node lymphocytes, a mixed B-cell-T-cell population, and unfractionated PEC are intermediate in their absorptive activity, and L2C leukemic cells, a malignant B-cell population, are most efficient for absorbing both activities. Thus, the antigens against which the cytotoxic and inhibitory activities are directed appear to be expressed to a greater extent on B lymphocytes than on T lymphocytes and a general correlation exists in the capacity of the various tissues employed to absorb both activities.

More detailed analysis of absorption of inhibitory and cytotoxic activity of 13 anti-2 sera by L2C cells reveals that cytotoxic activity can generally be absorbed by smaller numbers of cells than are required to absorb inhibitory activity, although inhibitory activity can be completely removed when larger numbers of L2C cells are used. Since the cytotoxic activity of the sera used are detectable at lower serum concentrations than is the inhibitory activity, the preferential removal of cytotoxic activity by absorption with L2C cells could be interpreted as evidence that the antigens against which the cytotoxic and inhibitory antibodies are directed are distinct. An equally plausible interpretation of the results is that the specificity of the two types of antibodies are similar, but that the cytotoxic antibodies are preferentially absorbed because they are of higher affinity or different molecular class than some or all of the inhibitory antibodies.

Thus, these results do not allow us to definitively determine whether the inhibitory activity of the alloantisera is due to antibodies specific for Ir gene products or antibodies specific for linked antigens in the MHC. What is strikingly revealed is that the relevant antigen is present in much larger amounts on B lymphocytes than on T lymphocytes. Thus, if the inhibitory alloantibodies are directed at Ir gene products, such products must exist on B cells in higher concentrations than on T cells. Although the bulk of evidence indicates that Ir genes express their principal function in T lymphocytes, recent studies of cell cooperation between macrophages and T lymphocytes (12, 13), and between T lymphocytes and B lymphocytes (14), raise the possibility that Ir gene products, or products of closely linked MHC genes, play a major role in such interactions. Thus, expression of Ir gene products on the surface of B lymphocytes and macrophages, as well as on T lymphocytes, is a distinct possibility. It should be noted that the alloantisera can block T-cell activation by antigen, by interfering with H-determined macrophage-T-cell interaction, as well as by interfering with antigen interaction with Ir gene products on T lymphocytes (12, 13). Indeed, it is possible that the very same antibodies are responsible for both effects.

In the above discussion we have regarded the cytotoxic alloantisera as detecting H antigens and have implicitly assumed that putative antisera to Ir gene
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products would not be cytotoxic. These assumptions were based upon views of the specificity of alloantisera directed against MHC antigens of mice and of men generally held when the present studies were begun (15). At that time it was believed that cytotoxic sera which detected \textit{H}-2 specificities were directed at the products of two genes, the K gene, located at the "left" end of the \textit{H}-2 complex, and the D gene, located at the "right" end of the \textit{H}-2 complex. The \textit{Ir} genes of the mouse had been shown to be distinct from, although linked to, the K and D genes and thus it was believed that cytotoxic antibodies directed at \textit{Ir} gene products would be very difficult to produce.

It is now recognized that this schema was highly over simplified. Thus, antisera identifying MHC antigens which are not the product of K or D genes have been recently reported by at least five laboratories (16–19). In some instances the genes controlling these antigens have been mapped into the region in which most \textit{Ir} genes are located, although no evidence that such antigens are \textit{Ir} gene products has yet been obtained. Interestingly, these antigens, unlike the traditional \textit{H}-2 antigens, are not present on some non-lymphoid tissues and, in at least two cases (18), these antigens have a higher representation on B lymphocytes than on T lymphocytes.

In light of these new findings it is important to reevaluate the situation regarding the guinea pig MHC, and in particular the relation of the \textit{Ir} genes to the genes governing the antigens detected by cytotoxicity with 13 anti-2 and 2 anti-13 sera. Strain 2 and strain 13 guinea pigs are highly inbred and promptly reject reciprocal skin grafts suggesting they differ at the major H region of the species (20). Moreover mixed lymphocyte responses are observed when their cells are cultured together (21) and these animals differ at \textit{Ir} loci, supporting the concept that they differ in the MHC. Finally, backcross analysis reveals a linkage between \textit{Ir} genes, possession of antigens against which cytotoxic alloantisera are directed, and the ability to reject a transplantable guinea pig leukemia (22). Thus, it appears certain that the antigens detected by the 2 anti-13 sera and the 13 anti-2 sera are coded for by MHC genes. Whether these genes are equivalent to the D and K genes of mice and to the LA and Four genes of humans, or alternatively to the more recently described mouse MHC genes is uncertain. The observation that the antigens detected by anti-2 alloantisera are in higher amount on B cells than on T cells and are difficult to detect on many nonlymphoid cells strongly suggests that these antigens are more analogous to the products of the newer non-D, non-K MHC genes of mice. Moreover, Sato and de Weck have described a series of histocompatibility antigens of guinea pigs (A, B, C, and D) and have reported that strain 2 and 13 guinea pigs both share the B antigen (23). Thus, if (2–13) differences are MHC differences coded for by genes analogous to non-D, non-K genes of

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\(^4\) Hammerling, G., and H. O. McDevitt. Unpublished observations.

\(^5\) Shevach, E. M., and E. Sehgal. Unpublished observations.
mice, the ABCD system of Sato and de Weck may represent the guinea pig analogue of D and/or K. Studies are now in progress to evaluate these possibilities.

It has been suggested that the products of the Ir genes might be the prime antigen-binding receptors of the T lymphocyte. However, it is also possible that the Ir gene product might function as an auxiliary receptor for antigen and act in concert with the prime receptor. According to this latter explanation, the Ir gene product need not be expressed in a clonal manner; in this event, the most likely candidate for the prime antigen-binding receptor of the T lymphocyte would be immunoglobulin. We therefore examined the effect of a number of anti-immunoglobulin reagents, which had specificity for the heavy and/or light chains of guinea pig immunoglobulin, on the in vitro proliferative response both to antigens, the response to which is known to be under genetic control, and to antigens, the response to which is not known to be under unigenic control. Under conditions in which we were able to completely and specifically suppress the response of (2 X 13)F1 T lymphocytes to DNP-GL with anti-2 serum, all of the anti-immunoglobulin reagents were devoid of inhibitory effect on the response of these same F1 cells to DNP-GL, GT, or PPD. A number of comments should be made about the interpretation of these experimental results. First, the population of lymphocytes used in these studies, the column-passaged peritoneal exudate lymphocytes, are composed of 95–100% T lymphocytes (9). Stimulation of T-cell proliferation by the anti-immunoglobulin reagents in the absence of antigen was not observed, and thus it was not necessary to prepare Fab fragments of the anti-Ig sera. Second, in our studies antigen is presented to the T lymphocytes in the form of antigen-pulsed macrophages. The macrophages are exposed to antigen in the presence of normal serum, washed, and then added to the lymphocytes which have been preincubated in the anti-immunoglobulin reagents. Under these conditions the anti-immunoglobulin reagents cannot inhibit the binding of antigen to cytophilic antibody present on immune macrophages as such binding occurs before the introduction of the antisera. These same anti-immunoglobulin reagents, if present during the incubation of antigen and macrophages, are very effective in the inhibition of binding of antigen to cytophilic antibody present on macrophages. Third, it should be noted that the absolute quantity of antigen added to the cultures when antigen pulsed macrophages are used is exceedingly small. It has recently been shown that when low concentrations of antigen are used, macrophage-cytophilic antibody is most important in antigen binding and thus in this situation antigen binding to macrophages is sensitive to blockade by anti-immunoglobulin reagents (24). A previous study (25) of the inhibition of antigen-induced proliferation of human lymphocytes by antilight-chain reagents demonstrated that the degree of inhibition was maximal when the absolute quantity of soluble antigen added to the cultures was limiting. Such reported instances of inhibition of T-lymphocyte activation by anti-immunoglobulin reagents may
be explained by the effect of the anti-immunoglobulin antibody on antigen binding by macrophages rather than on any direct effect on T lymphocytes.

Our failure to inhibit T-cell proliferation with anti-immunoglobulin reagents cannot be interpreted as definitive evidence for the absence of immunoglobulin on the T-cell surface. It is possible that the antisera used failed to recognize determinants shared between serum immunoglobulin and a putative T-cell surface immunoglobulin. Alternatively, different classes of T cells which have different functional roles may have antigen receptors with different chemical characteristics. Thus, the T-cell subset responsible for helper activity in antibody production may bear surface immunoglobulin, while the T cells responsible for DNA synthesis in vitro may recognize antigen through receptors of a different nature. In any case, the latter function is readily inhibitable by alloantisera and not by anti-immunoglobulin. Our data strongly suggest that conventional serum-type immunoglobulin is not important in antigen recognition by the T cells involved in the DNA synthetic response.

SUMMARY

It has been previously shown that alloantisera prepared by reciprocal immunization of strain 2 and strain 13 guinea pigs specifically block the activation of T lymphocytes from immune guinea pigs by antigens, the response to which is controlled by Ir genes. In this report we have examined the effect of absorption of the 13 anti-2 serum with different populations of lymphoid cells. It is unlikely that the inhibitory activity of the anti-2 serum on the proliferation of (2 × 13)F1 lymphocytes to a DNP derivative of a copolymer of L-glutamic and L-lysine (DNP-GL) is due to the presence of antibodies specific for the unique antigenic determinants (idiotypes) of clonally distributed T-lymphocyte receptors. Thus, cells obtained from a normal animal and a DNP-GL immune animal were equivalent in their absorptive capacity. Populations of T lymphocytes were ineffective in absorbing either the cytotoxic or inhibitory activity of the anti-2 serum, while L5178Y leukemia cells, a malignant B-cell population, were most efficient in absorbing both activities. Thus, the antigen(s) against which the cytotoxic and inhibitory activities are directed are present to a greater extent on B lymphocytes than on T lymphocytes. However, these results do not allow us to definitively determine whether the inhibitory activity of the alloantisera is due to antibodies specific for Ir gene products or antibodies specific for linked antigens in the MHC.

We also examined the effect of a number of anti-immunoglobulin reagents which had specificity for the heavy and/or light chains of guinea pig immunoglobulin on the in vitro lymphocyte proliferative response to antigen. Under conditions in which we were able to completely and specifically suppress the response of (2 × 13)F1 lymphocytes to DNP-GL with anti-2 serum, the anti-immunoglobulin reagents were devoid of inhibitory effect on the response of these same F1 cells to DNP-GL, a copolymer of L-glutamic and L-tyrosine.
(GT), or purified protein derivative of tuberculin (PPD). These results strongly suggest that conventional serum-type immunoglobulin is not important in antigen recognition by the T cells involved in the DNA synthetic response.

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REFERENCES
1. McDevitt, H. O., and B. Benacerraf. 1969. Genetic control of specific immune responses. *Adv. Immunol.* 11:31.
2. Benacerraf, B., and H. O. McDevitt. 1972. Histocompatibility-linked immune response genes. *Science (Wash. D. C.)* 175:273.
3. 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.
4. McDevitt, H. O., B. D. Deak, D. C. Shreffler, J. Klein, J. H. Stimpfling, and G. D. Snell. 1972. Genetic control of the immune response. Mapping of the Ir-I locus. *J. Exp. Med.* 135:1239.
5. Kantor, F. S., A. Ojeda, and B. Benacerraf. 1963. Studies on artificial antigens. I. Antigenicity of DNP-poly-L-lysine and DNP-copolymer of lysine and glutamic acid in guinea pigs. *J. Exp. Med.* 177:55.
6. Shevach, E. M., L. Ellman, J. M. Davie, and I. Green. 1972. LeC guinea pig lymphatic leukemia: a “B” cell leukemia. *Blood J. Hematol.* 39:1.
7. Shevach, E. M., D. L. Rosenstreich, and I. Green. 1973. The distribution of histocompatibility antigens on T and B cells in the guinea pig. *Transplantation.* 16:126.
8. Davie, J. M., and W. E. Paul. 1971. Receptors on immunocompetent cells. II. Specificity and nature of receptors on dinitrophenylated guinea pig albumin-125I-binding lymphocytes. *J. Exp. Med.* 134:495.
9. Rosenstreich, D. L., J. T. Blake, and A. S. Rosenthal. 1971. The peritoneal exudate lymphocyte. I. Differences in antigen responsiveness between peritoneal exudate and lymph node lymphocytes from immunized guinea pigs. *J. Exp. Med.* 134:1170.
10. Harrison, M. R., G. B. Thurman, and G. M. Thomas. 1973. A simple and versatile microculture harvesting device for processing radioactive label incorporated into and/or released from cells in microculture. *J. Immunol. Methods.* In press.
11. Shevach, E. M., I. Green, and W. E. Paul. 1973. Alloantiserum-induced inhibition of immune response gene product function. II. Genetic analysis of target antigens. *J. Exp. Med.* 139:679.
12. Rosenthal, A. S., and E. M. Shevach. 1973. The function of macrophages in antigen recognition by guinea pig T lymphocytes. I. Requirement for histocompatible macrophages and lymphocytes. *J. Exp. Med.* 138:1194.
13. Shevach, E. M., and A. S. Rosenthal. 1973. The function of macrophages in antigen recognition of guinea pig T lymphocytes. II. Role of the macrophage in the regulation of genetic control of the immune response. *J. Exp. Med.* 138:1213.
14. Katz, D. H., T. Hamaoka, and B. Benacerraf. 1973. Cell interactions between T and B lymphocytes from allogeneic donor strains in humoral response to hapten protein conjugates. *J. Exp. Med.* 137:1405.
15. Bach, F. H., M. B. Widmer, M. Segall, M. L. Bach, and J. Klein. 1972. Genetic and immunological complexity of major histocompatibility regions. *Science* (Wash. D. C.). 176:1024.

16. Hauptfeld, V. D., D. Klein, and J. Klein. 1973. Serological identification of an Ir-region antigen. *Science* (Wash. D. C.) 181:167.

17. Götze, D., R. A. Reisfeld, and J. Klein. 1973. Serologic evidence for antigens controlled by the Ir region in mice. *J. Exp. Med.* 138:1003.

18. Sachs, D. H., and J. L. Cone. 1973. A mouse “B” cell alloantigen determined by gene(s) linked to the major histocompatibility complex. *J. Exp. Med.* 138:1289.

19. David, C. S., D. C. Shreffler, and J. A. Frelinger. 1973. New lymphocyte antigen system (Lna) controlled by the Ir region of the mouse H-2 complex. *Proc. Natl. Acad. Sci. U. S. A.* 70:2509.

20. Bauer, J. A. 1960. Genetics of skin transplantation and an estimate of the number of histocompatibility genes in inbred guinea pigs. *Ann. N. Y. Acad. Sci.* 87:78.

21. Ellman, L., I. Green, W. J. Martin, and B. Benacerraf. 1970. Linkage between the polyl-lysine gene and the locus controlling the major histocompatibility antigens in strain 2 guinea pigs. *Proc. Natl. Acad. Sci. U. S. A.* 66:322.

22. Ellman, L., I. Green, and B. Benacerraf. 1971. The PLL gene and histocompatibility genotype in inbred and random-bred guinea pigs. *J. Immunol.* 107:382.

23. Sato, W., and A. L. deWeck. 1972. Leukocyte typing in guinea pigs. *Z. Immunforsch., Allerg. Klin. Immunol.* 144:549.

24. Cohen, B. E., A. S. Rosenthal, and W. E. Paul. 1973. Antigen-macrophage interaction. II. Relative roles of cytophilic antibody and other membrane sites. *J. Immunol.* 111:820.

25. Greaves, M. F. 1970. Biological effects of anti-immunoglobulins. Evidence for immunoglobulin receptors on T and B lymphocytes. *Transplant. Rev.* 5:45.