

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

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(Received for publication 12 July 1973)

Recent studies indicate that histocompatibility-linked immune response (Ir)¹ genes, are expressed on thymus-derived (T) lymphocytes. In the mouse, the genetically controlled difference in the IgG antibody response between high and low responders immunized with branched-chain amino acid polymers is lost in thymus-deprived mice (1). In guinea pigs, the initiation of functions thought to be regulated by T lymphocytes (delayed cutaneous hypersensitivity, antigen-induced lymphocyte proliferation in vitro, and carrier function) requires the presence of the appropriate Ir gene (2). Thus, guinea pigs that lack the poly-L-lysine (PLL) gene (nonresponders) fail to develop either delayed hypersensitivity or other cellular immune phenomena upon immunization with 2,4-dinitrophenyl (DNP)-poly-L-lysine. Nonetheless, these animals are capable of recognizing DNP-PLL as a hapten and produce antibodies to DNP-PLL when this molecule is coupled to an immunogenic protein carrier (3). In addition, recent data indicate that the number of specific antigen-binding bone marrow-derived (B) lymphocytes does not differ between responders and nonresponders (4-6). Schlossman (7) has also recently shown that the antibody clones and the variable region gene pool for antibodies to α -DNP-deca-L-lysine, an antigen the response to which is controlled by the PLL gene, appear to be identical in responder and non-responder animals. Thus, the weight of experimental evidence suggests that the main functional role of the Ir gene product is in the process of antigen recognition by the T lymphocyte.

However, an alternative explanation to these studies is that the Ir genes might be expressed in a cell that controls and regulates T-cell activation, i.e., the macrophage. A number of attempts have been made to resolve this issue by using cell transfer studies (8, 9). When spleen and lymph node cells were transferred from (2 × 13)F₁ animals to lethally irradiated strain 13 guinea pigs that had been reconstituted with syngeneic bone marrow, a large percentage of the recipients developed the ability to respond to antigens controlled by the PLL gene; lethally irradiated strain 13 guinea

¹ *Abbreviations used in this paper:* DNP, 2,4-dinitrophenyl; GL, a copolymer of L-glutamic acid and L-lysine; GT, a copolymer of L-glutamic acid and L-tyrosine; Ir, immune response; LNL, lymph node lymphocytes; 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.

pigs reconstituted with $(2 \times 13)F_1$ bone marrow also had a successful transfer of PLL gene function, but in a much smaller percentage of cases. The fact that F_1 lymph node and spleen cells were considerably more successful in transferring PLL gene function than F_1 bone marrow cells (a rich source of monocyte precursors) suggests that the cell in which the PLL Ir gene is expressed is a lymphocyte rather than a monocyte or macrophage. However, it should be noted that the spleen and lymph node cell populations used in these studies were contaminated with 5% of cells that stained with neutral red and were presumably monocytes or macrophages. Therefore, the question of whether the Ir gene might be expressed in the macrophage is difficult to resolve by cell transfer studies of this type.

Another approach to the understanding of the function of the product of the Ir gene and the cell type in which it is expressed is the inhibition in vitro of functions mediated by T lymphocytes. Previous studies have demonstrated that alloantisera that are directed against histocompatibility antigens can inhibit the activation of T lymphocytes by antigens, the response to which is linked to the presence of histocompatibility types against which the alloantisera are directed (10). Thus, when cells from $(2 \times 13)F_1$ guinea pigs immune to DNP-GL (an antigen the response to which is controlled by a 2-linked Ir gene) and to GT (an antigen the response to which is controlled by a 13-linked Ir gene) are cultured in vitro, the anti-2 serum inhibited the proliferative response to DNP-GL, but not GT, and the anti-13 serum inhibited the proliferative response to GT, but did not affect the DNP-GL response. Because the alloantisera had no effect during the initial incubation with antigen, but inhibited proliferation when present during the subsequent 3-day culture period, it was assumed that the alloantisera exerted their inhibitory effect by blocking the recognition of antigen by the T lymphocyte rather than by blocking the initial uptake of antigen by macrophages. It was concluded from those studies that alloantisera block a lymphocyte surface structure coded for by the Ir genes and that this product plays a role in the mechanism of antigen recognition by the T lymphocyte.

In the accompanying paper (11), we demonstrated that the activation of F_1 lymphocytes by parental macrophages pulsed with purified protein derivative (PPD), an antigen the response to which is not known to be under unigenic control, could be completely abolished by alloantisera when they were directed against histocompatibility determinants present on both the macrophage and the T lymphocyte. When the alloantisera were directed against determinants present either on the T cell alone, or on the macrophage alone, little inhibition of T-lymphocyte proliferation was seen. These studies suggested that the inhibition of PPD stimulation produced by alloantisera is mediated by blocking macrophage-lymphocyte interaction.

The questions posed in the present report are: First, can macrophages obtained from a parental animal that lacks an Ir gene activate F_1 T-lymphocyte proliferation to an antigen the response to which is controlled by that gene? Second, do the alloantisera exert their inhibitory effect by blocking macrophage-T lymphocyte interaction in a nonspecific manner, do they specifically block the product of the Ir genes, or are they capable of blocking both functions?

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. (2×13)F₁ animals were obtained by mating strain 2 and 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, Boston, Mass. DNP₃-GL was prepared by the reaction of 2,4-dinitrofluorobenzene with GL (12). The subscript refers to the average number of DNP groups per molecule. A copolymer of L-glutamic acid (50%) and L-tyrosine (50%) (GT), mol wt 22,600, was obtained from Miles Laboratories, Inc., Kankakee, Ill. Purified protein derivative of tuberculin (PPD) was purchased from Connaught Medical Research Laboratories, Willowdale, 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 containing 0.5 mg of *Mycobacterium tuberculosis* H₃₇RA/ml (Difco Laboratories, Detroit, Mich.). Strain 2 animals were immunized with 100 μ g of DNP-GL divided equally among the four footpads. Strain 13 animals were immunized with 500 μ g of GT divided among the four footpads. F₁ animals were immunized simultaneously with 100 μ g of DNP-GL and 500 μ g of GT; each antigen was administered in one front footpad and one rear footpad.

Preparation of Alloantisera.—A strain 13 anti-strain 2 serum and a strain 2 anti-strain 13 serum were prepared as previously described (10). They were sterilized by Millipore filtration (Millipore Corp., Bedford, Mass.) and heat inactivated at 56°C for 45 min before use.

Cell Collection and Purification.—Peritoneal exudate cells (PEC) and peritoneal exudate lymphocytes (PELs) were induced and purified as described in the accompanying paper (11). Lymph node lymphocytes (LNLs) were prepared and purified as previously described (13).

Technique of Brief Antigen Exposure.—In all the experiments described in this paper, the PEC population, which is composed of 75–85% macrophages, is used as the source of macrophages. PEC at a concentration of 15×10^6 /ml in the presence of 30 μ g/ml of mitomycin C were allowed to equilibrate at 37°C. Antigen was then added and the cell mixtures were maintained at 37°C for 60 min. The final concentration of antigen or mitogen used in the incubation medium was 1 μ g/ml DNP-GL, 100 μ g/ml GT, 100 μ g/ml PPD, or 10 μ 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 macrophages at a concentration of 1×10^6 /ml were mixed with LNLs or PELs at a concentration of 2×10^6 /ml. 0.2 ml aliquots of these mixtures were cultured in round bottom microtiter plates (Cooke Engineering Co., Alexandria, Va.) in medium RPMI-1640 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with penicillin (100 μ /ml), streptomycin (100 μ g/ml), L-glutamine (300 μ g/ml), and either 10% normal guinea pig serum (NGPS) or 10% alloantisera. The amount of [³H]thymidine incorporated into cellular DNA was assayed as in the accompanying report (11).

RESULTS

Pulsed Macrophages from the "Nonresponder" Parent Fail to Activate F₁ LNL Proliferation.—In the accompanying report (11), we demonstrated that parental macrophages pulsed with PPD were able to activate F₁ T-lymphocyte proliferation although the magnitude of stimulation was less than that produced by the activation of F₁ T cells by F₁ macrophages. In order to evaluate whether macrophages from animals lacking a given Ir gene could efficiently

present an antigen (the response to which is controlled by that gene) to lymphocytes from F_1 animals possessing that gene, strain 2, strain 13, or F_1 macrophages were pulsed with DNP-GL, GT, or PPD, washed, and then added to column-purified lymph node cells obtained from F_1 animals immune to both DNP-GL and GT. The difference between the control and antigen-stimulated cultures (Δ cpm per culture) is shown in Fig. 1. Strain 2 macrophages pulsed with DNP-GL (the response to which is controlled by an Ir gene present in strain 2, but absent in strain 13 animals) initiate F_1 T-cell proliferation (Δ cpm = 4,500), while strain 2 macrophages pulsed with GT (the response to which is controlled by an Ir gene present in strain 13 animals but absent in strain

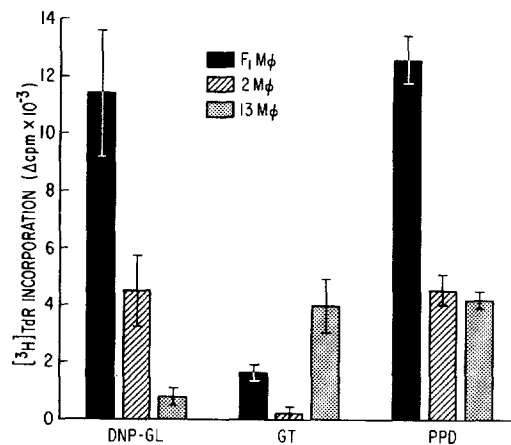


FIG. 1. Stimulation of $(2 \times 13)F_1$ LNLs by parental or $(2 \times 13)F_1$ macrophages that have been pulsed with DNP-GL, GT, or PPD. Results are expressed as the difference between the control and antigen-stimulated cultures (Δ cpm per culture). Each bar represents the arithmetic mean of three experiments \pm 1 SEM.

2) fail to initiate significant F_1 T-cell proliferation (Δ cpm = 170). Conversely, strain 13 macrophages pulsed with GT activate F_1 T-cell proliferation (Δ cpm = 3,970), while strain 13 macrophages pulsed with DNP-GL fail to activate F_1 cells (Δ cpm = 780). The stimulation of F_1 LNLs by either strain 2 or strain 13 macrophages pulsed with PPD, although substantial, was again significantly less than that seen with F_1 macrophages pulsed with PPD.

Pulsed Macrophages from the Nonresponder Parent Activate F_1 PEL Proliferation.—The results of the above study suggest that when parental macrophages are pulsed with an antigen, the response to which is controlled by an Ir gene not present in that parent, these macrophages are incapable of initiating F_1 T-cell proliferation to this antigen. This result suggests that a defect in the non-responder animal might be at the level of the macrophage. However, one problem with such an interpretation is that the magnitude of stimulation of F_1 LNL produced by either DNP-GL or GT-pulsed macrophages is relatively

small and we may therefore be missing a small degree of stimulation produced by antigen-pulsed macrophages from the nonresponder parent. In order to evaluate this possibility, we repeated the study using the highly reactive PEL population obtained from F_1 animals immune to both DNP-GL and GT as the indicator cells. Strain 2, strain 13, or F_1 macrophages were pulsed with DNP-GL, GT, PPD, or PHA, washed, and then added to F_1 PELs. F_1 macrophages pulsed with any of the antigens or with PHA activate F_1 T-cell proliferation (Fig. 2). Parental macrophages pulsed with an antigen controlled by an Ir gene present in that parent activate F_1 lymphocyte DNA synthesis to an equal or greater extent than F_1 macrophages pulsed with the same an-

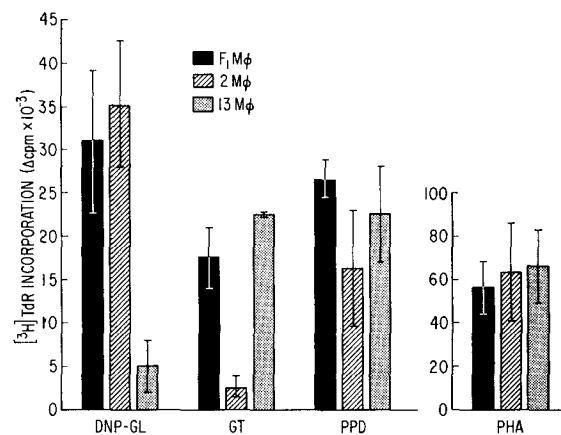


FIG. 2. Stimulation of $(2 \times 13)F_1$ PELs by parental or $(2 \times 13)F_1$ macrophages that have been pulsed with DNP-GL, GT, PPD, or PHA. Results are expressed as Δ cpm per culture. Each bar represents the arithmetic mean of three experiments \pm 1 SEM.

tigen. When PELs are used as the responder population, parental macrophages pulsed with an antigen, the response to which is under control of an Ir gene not present in that parent, do initiate significant DNA synthesis. However, the magnitude of stimulation produced by antigen-pulsed macrophages from the nonresponder parent is considerably less than the stimulation produced by antigen-pulsed responder macrophages. Thus, strain 2 macrophages pulsed with DNP-GL initiate a response of 35,000 cpm, while strain 13 macrophages pulsed with DNP-GL initiate a response of only 5,000 cpm. Strain 13 macrophages pulsed with GT initiate a response of 22,600 cpm, while strain 2 macrophages pulsed with GT initiate a response of 2,700 cpm.

The Stimulation of F_1 PELs by Nonresponder Macrophages Is Not Due to Antigen Carryover.—The results of the study using PELs from F_1 animals as the responder population suggest that when parental macrophages are pulsed with an antigen, the response to which is under control of an Ir gene not present in that parent, these macrophages are capable of initiating F_1 T lympho-

cyte proliferation although markedly less efficiently than macrophages obtained from the parent that possesses the appropriate Ir gene. However, one problem in the interpretation of this study is that the PEL population is contaminated with a considerable number of F₁ macrophages that remain after adherence column purification; it is possible that the macrophages obtained from the nonresponder parent might merely act as passive vehicles for the antigen that is subsequently bound by the residual F₁ macrophages that then stimulate the F₁ T cells. This explanation is rather unlikely in the case of macrophages pulsed with GT since stimulation can be induced with macrophages that have been pulsed with 100 µg/ml of GT although concentrations of GT of <10 µg/ml, even present continuously, fail to stimulate responses. Macrophages pulsed with GT retain no more than 1.0% of the added GT so that the concentration of antigen carried into the culture should be insufficient to produce stimulation (B. E. Cohen and W. E. Paul, unpublished observation).

When DNP-GL is used in continuous culture exceedingly low concentrations (10⁻⁴ – 10⁻⁵ µg/ml) of antigen may produce some degree of stimulation. There is a greater possibility in this case that the stimulation of F₁ PELs by strain 13 macrophages that have been pulsed with DNP-GL could be the result of antigen that has been carried over by the strain 13 macrophages and subsequently bound by the residual F₁ macrophages, even though the strain 13 macrophages were pulsed with only 1 µg/ml of DNP-GL. In order to evaluate this possibility, strain 13 macrophages were pulsed with either 0.1 µg/ml or 1 µg/ml of DNP-GL, washed, and added to either immune strain 2 or F₁ PELs. As can be seen in Table I, strain 13 macrophages pulsed with DNP-GL were incapable of activating strain 2 lymphocyte proliferation, but did initiate significant stimulation of F₁ lymphocyte proliferation. If the stimulation of F₁ T cells by DNP-GL-pulsed strain 13 macrophages had been the result of antigen carryover with subsequent uptake by the F₁ macrophages, stimulation of strain 2 PELs should also have been observed because both the strain 2 and F₁ PEL populations were contaminated with equivalent numbers of syngeneic macrophages. Thus, strain 13 macrophages pulsed with DNP-GL are capable of activating F₁ lymphocyte proliferation, albeit inefficiently

TABLE I
Activation of Strain 2 or F₁ Lymphocyte Proliferation by DNP-GL-Pulsed Strain 13 Macrophages

Macrophage-associated antigen*	Strain 2 PELs	F ₁ PELs
0	4,902‡	1,891
DNP-GL 0.1 µg/ml	4,467	5,154
DNP-GL 1.0 µg/ml	5,761	7,568

* Strain 13 macrophages were pulsed with 0, 0.1 µg/ml, or 1.0 µg/ml of DNP-GL, washed, and then added to either immune strain 2 or (2 × 13)F₁ PELs.

‡ Results are expressed as counts per minute per culture (cpm); each value is the mean of three determinations.

when compared with pulsed strain 2 or F₁ macrophages, but are incapable of activating strain 2 lymphocyte proliferation.

Alloantisera Do Not Inhibit the Uptake of Antigen By Macrophages.—Another approach to the understanding of the regulation of T cell activation by antigen-pulsed macrophages is to inhibit that activation with alloantisera. F₁ macrophages were pulsed with DNP-GL, GT, or PPD either in the presence of NGPS, 13 anti-2 serum, or 2 anti-13 serum, washed, and then added to immune F₁ PELs. No significant difference (Fig. 3) is noted in the activation of F₁ PELs by macrophages pulsed with any of the antigens in either NGPS or

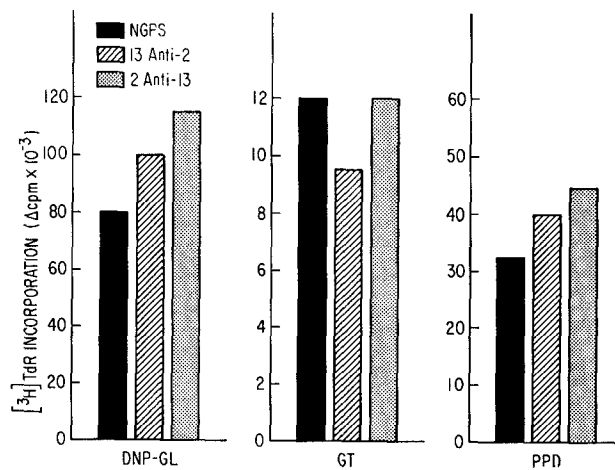


FIG. 3. Stimulation of $(2 \times 13)F_1$ PELs by antigen-pulsed $(2 \times 13)F_1$ macrophages—effect of alloantisera during antigen-pulse step. F₁ macrophages were pulsed with either DNP-GL, GT, or PPD in the presence of NGPS, 13 anti-2, or 2 anti-13 sera. After a 60 min incubation at 37°C, the macrophages were washed and added to $(2 \times 13)F_1$ PELs and cultured for 72 h in the presence of NGPS. Results are expressed as Δ cpm per culture.

alloantisera. To further rule out the possibility that alloantisera might inhibit the uptake of antigen by the macrophage, strain 2 macrophages were pulsed with DNP-GL and PPD in the presence of NGPS or anti-2 serum, and strain 13 macrophages were pulsed with GT or PPD in NGPS or the anti-13 serum. These macrophages were then washed and added to F₁ PELs. No significant difference is noted (Fig. 4) in the stimulation of F₁ PELs by parental macrophages pulsed in NGPS or alloantisera. We conclude from these observations that alloantisera fail to block the uptake of antigen by macrophages and hence it is unlikely that the distribution of antigen on the macrophage is related to histocompatibility antigen.

Alloantisera Inhibit Antigen-Induced Lymphocyte Proliferation Both by Blocking Macrophage-Lymphocyte Interaction and by Blocking the Products of the Ir Gene.—The results presented in the first paper (11) suggested that alloanti-

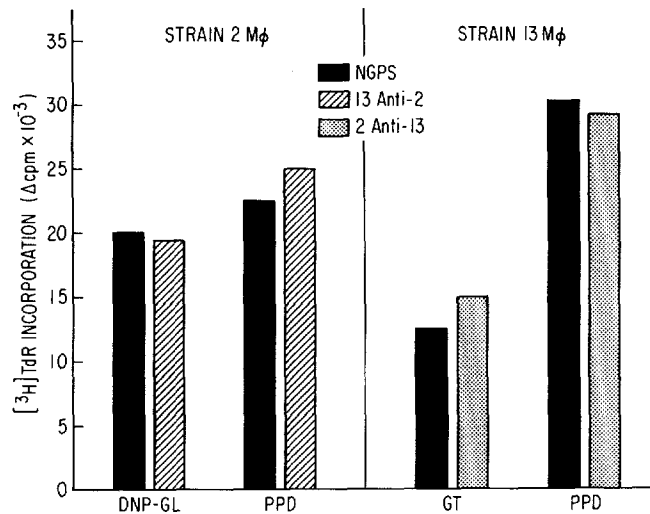


FIG. 4. Stimulation of $(2 \times 13)F_1$ PELs by antigen-pulsed parental macrophages—effect of alloantisera during antigen-pulse step. Strain 2 macrophages were pulsed with DNP-GL or PPD in the presence of NGPS or 13 anti-2 serum; strain 13 macrophages were pulsed with GT or PPD in the presence of NGPS or 2 anti-13 serum. After a 60 min incubation at 37°C, the macrophages were washed and added to $(2 \times 13)F_1$ PELs and cultured for 72 h in the presence of NGPS. Results are expressed as Δ cpm per culture.

sera inhibit antigen-induced proliferation by blocking macrophage-lymphocyte interaction. In view of the results presented thus far, we can now examine the effects of alloantisera on the inhibition of T-cell proliferation when the sera are directed at determinants present solely on the T lymphocyte. F_1 , strain 2, or strain 13 macrophages were pulsed with DNP-GL, GT, PPD, or PHA in the presence of 10% NGPS. After a 60 min incubation at 37°C, they were washed and added to immune F_1 PELs and cultured for 3 days in the presence of NGPS, anti-2, or anti-13 sera. The results of a typical experiment are presented in Table II. Stimulation of F_1 T-cell proliferation to all three antigens and the mitogen PHA is observed when the F_1 cells are cultured with pulsed F_1 macrophages in the presence of NGPS. When the same cells are cultured in the presence of anti-2 serum the response to DNP-GL is completely abolished; when these cells are cultured in the presence of anti-13 serum, the response to GT is specifically inhibited. This confirms our previous report (10) of specific inhibition of Ir gene-controlled responses by alloantisera. When F_1 cells are mixed with strain 2 macrophages that have been pulsed with GT, much less stimulation (3,572 \rightarrow 8,388 cpm) is seen than when strain 13 (2,346 \rightarrow 24,532 cpm) or F_1 (3,377 \rightarrow 14,824 cpm) GT-pulsed macrophages are used. When strain 2 macrophages and F_1 T cells are cultured in the presence of anti-2 serum, the response of the F_1 cells to all of the antigens but not the mitogen

TABLE II
Activation of F₁ Lymphocyte Proliferation: Effect of Alloantisera

Macrophage-associated antigen*	Serum		
	NGPS	13 anti-2	2 anti-13
F ₁ - Mφ			
0	3,377*	4,479	2,349
DNP-GL	17,916	2,889	18,866
GT	14,824	18,357	3,420
PPD	31,494	23,812	20,887
PHA	38,087	54,687	62,447
Strain 2 - Mφ			
0	3,572	3,435	2,940
DNP-GL	26,974	5,275	32,308
GT	8,388	3,205	4,308
PPD	25,491	5,602	29,053
PHA	58,593	64,684	81,903
Strain 13 - Mφ			
0	2,346	2,485	1,385
DNP-GL	5,338	2,554	1,465
GT	24,532	24,820	2,324
PPD	30,394	34,133	1,504
PHA	69,884	77,537	78,834

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

PHA is abolished; when this combination of cells is cultured in the presence of anti-13 serum, only the response to GT is inhibited. When strain 13 macrophages are cultured with F₁ cells in NGPS, the response to DNP-GL-pulsed macrophages is much less (2,346 → 5,338 cpm) than that produced by F₁ (3,377 → 17,916 cpm) or strain 2 macrophages (3,572 → 26,974 cpm) pulsed with DNP-GL. When this combination of cells is cultured in anti-13 serum the response to all the antigens, but not PHA is abolished; in anti-2 serum only the response to DNP-GL is inhibited.

The results of three separate experiments are summarized in Fig. 5 and the data arranged in groups according to the antigen used. When F₁ T cells are mixed with DNP-GL-pulsed macrophages (Fig. 5 A) from the different strains and cultured in the different sera, less stimulation is seen when the DNP-GL is presented on strain 13 macrophages than when it is on strain 2 or F₁ macrophages; however, the response to the strain 13 macrophage-associated DNP-GL is completely inhibited by culturing the cells in either the anti-2 serum or the anti-13 serum. In the former situation, the anti-2 serum blocks determinants present only on the responder T lymphocytes; in the latter situation, the anti-13 serum blocks determinants present on both macrophage and T lymphocyte (but not linked to the Ir gene product controlling the response to DNP-GL) and presumably inhibits in this case by blocking macrophage-lymphocyte

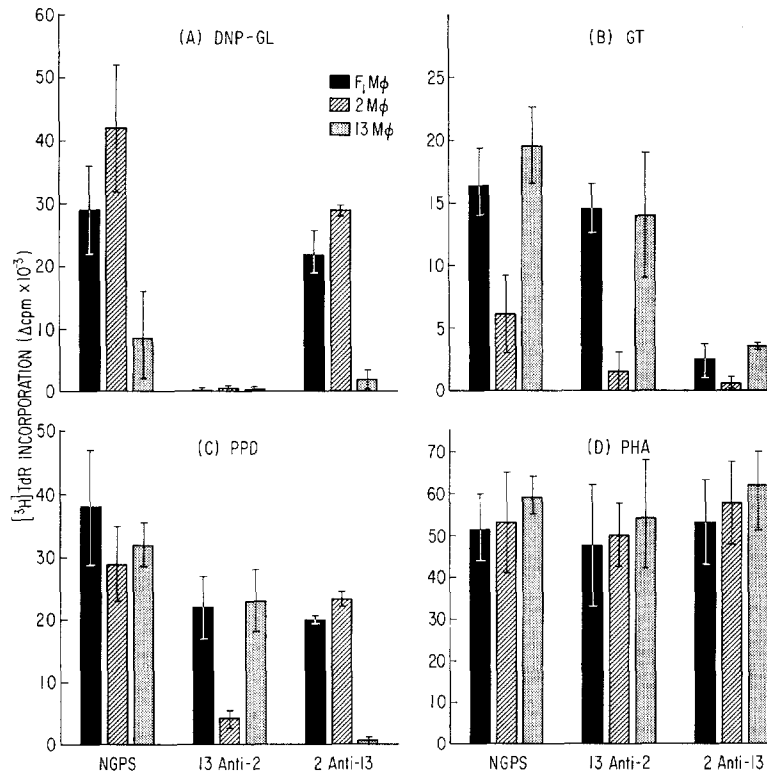


FIG. 5. Stimulation of $(2 \times 13)F_1$ PELs by antigen-pulsed parental or $(2 \times 13)F_1$ macrophages—effect of alloantisera during culture step. Strain 2, strain 13, or F_1 macrophages were pulsed with DNP-GL, GT, PPD, or PHA in the presence of NGPS. After a 60 min incubation at $37^\circ C$, the macrophages were washed and added to $(2 \times 13)F_1$ PELs and cultured in NGPS, 13 anti-2, or 2 anti-13 sera for 72 h. The results are expressed as Δcpm per culture. Each bar is the arithmetic mean of three experiments ± 1 SEM.

interaction. The anti-2 serum also blocks the stimulation of F_1 cells by DNP-GL-pulsed strain 2 or F_1 macrophages. The anti-13 serum has no effect on response of F_1 cells to pulsed strain 2 or F_1 macrophages.

When F_1 T cells are cultured with GT-pulsed macrophages (Fig. 5 B), macrophages of strain 2 origin produce less stimulation than F_1 or strain 13-pulsed macrophages. This stimulation is blocked both by the anti-2 and by the anti-13 serum. The anti-13 serum blocks determinants present only on the T lymphocyte, while the anti-2 serum blocks determinants present both on the lymphocyte and on the macrophage. The response of F_1 lymphocytes to GT presented on F_1 or strain 13 macrophages is also inhibited by the anti-13 serum but not by the anti-2 serum. The response of the F_1 lymphocytes to macrophages pulsed with PPD (Fig. 5 C) is only inhibited when parental macrophages are used and when the alloantisera are directed against determinants

present on both the lymphocyte and the macrophage. There is no inhibition of response of F_1 T lymphocytes to macrophages that have been pulsed with PHA (Fig. 5 *D*) and cultured in the different sera. This indicates that the mechanism of recognition of macrophage-associated PHA is different from that of macrophage-associated antigen and also demonstrates that all the sera used in this study support the growth of stimulated cells equally well.

DISCUSSION

The approach presented in this and the accompanying paper (11) to the understanding of antigen recognition by the T lymphocyte has been to analyze the conditions that regulate the *in vitro* proliferative response to antigen. We have already reviewed the experimental evidence that demonstrates that the *in vitro* proliferative response by T lymphocytes in the guinea pig requires that the antigen first be bound or "processed" by macrophages. The observation that the interaction of macrophage-associated antigen with immune T lymphocytes requires that both cells share histocompatibility antigens raised the question as to whether the macrophage played a role in the genetic control of the immune response or even if the macrophage was the primary cell in which the product of the Ir gene is expressed.

Although antigen-pulsed macrophages were incapable of initiating significant proliferation of allogeneic T cells, parental macrophages when pulsed with an antigen not known to be under genetic control (PPD) were capable of inducing proliferation of F_1 T cells. We therefore pulsed parental macrophages with an antigen, the response to which is controlled by an Ir gene not present in that parent; these macrophages were then mixed with T cells derived from a (nonresponder \times responder) F_1 and the resultant stimulation was measured. The initial series of experiments was performed using column-purified lymph node cells as the indicator population. Although parental macrophages pulsed with PPD were able to activate proliferation of F_1 T cells, no stimulation was seen when F_1 T cells were mixed with parental macrophages that had been pulsed with an antigen, the response to which is controlled by an Ir gene which the macrophages lacked. The result of this study suggested that the Ir gene might indeed be expressed in the macrophage and that a major defect in the nonresponder animal might be at the level of the antigen-processing cell rather than (or in addition to) the T lymphocyte.

However, when these same experiments were repeated using the more reactive PEL population as the indicator cells, parental macrophages pulsed with an antigen whose Ir gene they lacked were capable of initiating F_1 T-cell proliferation. The magnitude of stimulation was approximately $\frac{1}{10}$ that seen when macrophages from either the responder parent or the F_1 were used. It is unlikely that the stimulation seen when nonresponder macrophages were added to F_1 T cells was secondary to passive carry-over of antigen by these macrophages, because nonresponder macrophages pulsed with the same concentration of antigen did not activate allogeneic T cells. This result suggests that the macrophage, although involved in genetically controlled responses, is not the principal determinant of such responses.

The observations in this paper and the accompanying paper (11) suggest that macrophage-lymphocyte interaction is mediated via histocompatibility

antigen itself or via products of the major histocompatibility complex that are linked to serologically detectable histocompatibility antigen on the cell surface. Fig. 6 is a schematic diagram of the postulated surface structures on macrophages and lymphocytes that may be involved in the initiation of T-

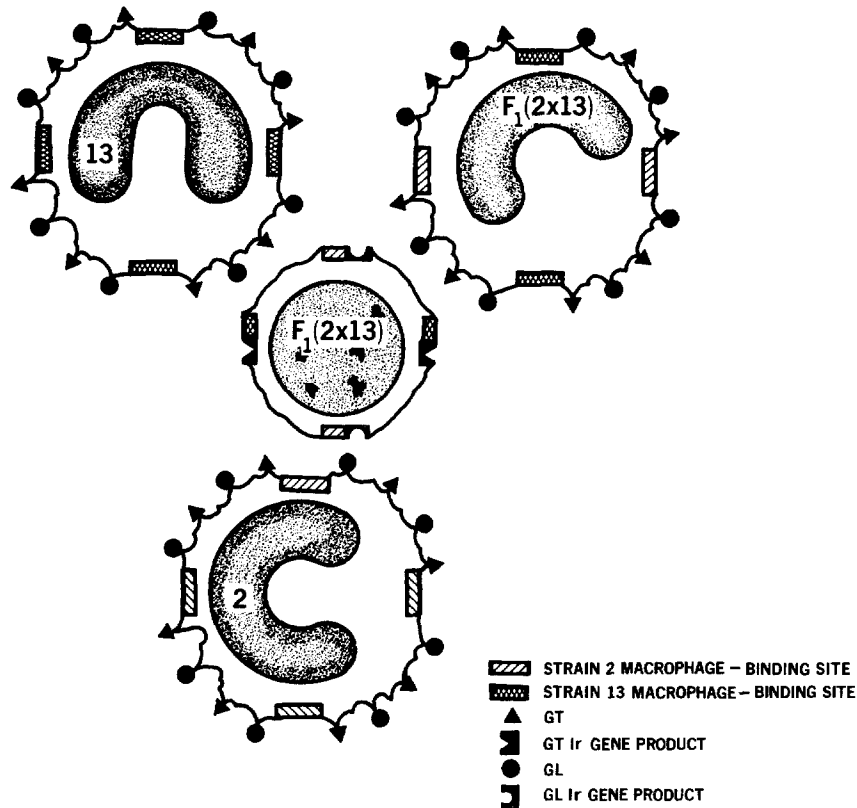


FIG. 6. Schematic diagram of some of the cell surface structures on macrophages and lymphocytes that are involved in the initiation of T-cell proliferation. There is no difference in the quantity or distribution of antigen on the surface of strain 2, strain 13, or $(2 \times 13)F_1$ macrophages. Macrophage-lymphocyte interaction is mediated via a macrophage-binding site that is either identical or closely linked to histocompatibility antigen. On the surface of the F_1 lymphocyte, the Ir gene product for GL is physically related to the strain 2 macrophage-binding site, while the Ir gene product for GT is physically related to the strain 13-binding site.

cell proliferation. The distribution of antigen on the surface of the macrophage is probably random and unrelated to histocompatibility antigen. The evidence for this conclusion is derived from the studies of B. E. Cohen and W. E. Paul (unpublished observation) who demonstrated that the absolute amount of radiolabeled DNP-GL or GT bound does not differ between strain 2, strain 13,

or F₁ macrophages and this binding is not inhibited by alloantisera. Furthermore, in the current study we were unable to functionally impair the uptake of DNP-GL or GT by treating either parental macrophages or F₁ macrophages with alloantisera. Why then does the macrophage, which lacks a given Ir gene, when pulsed with an antigen the response to which is controlled by that gene, activate F₁ T-cell proliferation so inefficiently? As suggested in Fig. 6, the conclusion we have reached is that during immunologically relevant macrophage-lymphocyte interaction the two cells come into close functional contact in areas of shared histocompatibility. Furthermore, the Ir gene-controlled antigen recognition sites on the surface of the T lymphocyte are physically related to the macrophage-binding site and *both* are linked to the serologically determined histocompatibility antigen. Thus, DNP-GL-pulsed strain 13 macrophages activate F₁ cells poorly because the Ir gene-controlled antigen recognition sites for DNP-GL are physically related to the strain 2 macrophage binding sites, while the main contacts between the cells are at the strain 13 macrophage-binding sites. Thus, DNP-GL although brought to the general proximity of the T lymphocyte is, relatively speaking, unavailable to the Ir gene-controlled DNP-GL recognition sites. In similar fashion when strain 2 macrophages pulsed with GT interact with F₁ T cells, the interaction is mediated via a binding site for strain 2 macrophages and the Ir gene-controlled antigen recognition sites for GT are physically closely related to the strain 13 macrophage-binding sites, but not to the strain 2 macrophage-binding sites. Again, antigen presentation will be inefficient and a poor response ensues.

This analysis of macrophage-lymphocyte interaction offers an explanation for the interesting observations made several years ago by Green, Paul, and Benacerraf (14) that delayed cutaneous hypersensitivity was rarely transferred from outbred responder guinea pigs immunized with DNP-PLL or DNP-GL to outbred animals that lacked the PLL gene. These same cell populations were able to transfer skin test reactivity to DNP-GL or PLL responder guinea pigs. Furthermore, delayed cutaneous sensitivity to ovalbumin, an antigen not known to be under genetic control, could be transferred from PLL responder outbred to PLL nonresponder outbred. More recent studies of the histocompatibility types of outbred animals comparable to those used in these studies have shown that PLL responder outbreds are in the main phenotypically 2⁺, 13⁺ while PLL negative outbreds are uniformly 2⁻, 13⁺. If the delayed cutaneous reaction is an *in vivo* analogue to the *in vitro* proliferative response, then the production of lymphokines by the donor lymphocytes in the passive transfer model probably requires initial binding of antigen by macrophages in the skin; these macrophages would most likely be of host origin. In the case of transfer from PLL responder to PLL responder in the outbred situation, the host's macrophages could cooperate with donor's lymphocytes (both sharing the strain 2 histocompatibility complex) and lead to a positive skin test. However, when responder lymphocytes were transferred to a nonresponder recipient, the host's macrophages, lacking the strain 2 site, would interact very inefficiently with the donor lymphocyte antigen recognition site for

PLL that would be physically related to a 2-histocompatibility site. On the other hand, the transfer of the response to ovalbumin that is not under unigenic control could be achieved because the responder and nonresponder animals shared strain 13 histocompatibility specificities and ovalbumin could be presented to relevant recognition sites presumably linked to the strain 13 histocompatibility complex. These data suggest that in the passive transfer of delayed hypersensitivity with any antigen, processing of the injected antigen by host macrophages is required before the interaction with donor lymphocytes can take place.

The model of macrophage-lymphocyte interaction depicted in Fig. 6 allows us to interpret the mode of action of alloantisera by dissecting out the different components involved. Thus, when parental macrophages are pulsed with *any* antigen and then added to F₁ T cells, an alloantiserum directed against parental histocompatibility antigen reacts with both the lymphocyte and the macrophage and thereby inhibits macrophage-lymphocyte interaction, abolishing antigen-induced lymphocyte transformation. The stimulation of F₁ cells by strain 13 macrophages that have been pulsed with either GT, PPD, or DNP-GL is blocked for this reason by the 2 anti-13 serum. However, the stimulation of F₁ cells by strain 13 macrophages pulsed with DNP-GL is also inhibited by the 13 anti-2 serum. In this situation the anti-2 serum can only act on the lymphocyte and must in some manner interfere with the DNP-GL recognition site. The most likely mechanism for this inhibition is steric interference with the DNP-GL Ir gene product on the T-cell surface by blockade of physically related strain 2 histocompatibility antigen. The previously observed (10) specific inhibition of the response to DNP-GL by anti-2 serum and of the response to GT by anti-13 serum seen when F₁ macrophages are added to F₁ lymphocytes probably involves both of these mechanisms because the Ir gene product is closely related both to histocompatibility antigen on the cell surface and to the site of macrophage-lymphocyte interaction. The latter two determinants, indeed, may be identical.

An alternative explanation for all of the results described in this report is that both histocompatibility antigen and the Ir gene product antigen recognition sites exhibit allelic exclusion in the F₁ animal. Thus, the inefficiency of activation of F₁ T cells by strain 13 macrophages would result because the antigen-sensitive cell for DNP-GL in the F₁ animal expresses only strain 2 histocompatibility determinants on its surface, and macrophage-lymphocyte interaction occurs very poorly across the allogeneic barrier. We believe this explanation to be unlikely although we are not able to exclude the possibility that allelic exclusion of this type exists on a small percentage of T lymphocytes. Preliminary studies² have failed to demonstrate evidence for expression of only strain 2 or only strain 13 histocompatibility antigens on F₁ T cells either by indirect immunofluorescence or cytotoxicity testing.

This report does not establish whether or not the Ir gene product is the prime antigen-binding receptor of the T lymphocyte. Indeed, the Ir gene product might still represent a nonclonally distributed substance found on

² Ben-Sasson, Z., E. Shevach, W. E. Paul, and I. Green. Manuscript in preparation.

the surface of T cells and capable of interacting with antigen as an auxiliary to the prime antigen-binding receptor of the T lymphocyte. We have for this reason used the term Ir gene-controlled antigen recognition site rather than receptor. The data presented in this and the companion study strongly support the conclusion that antigen recognition by T lymphocytes is a complex multicellular event involving more than simple antigen binding to a specific lymphocyte receptor. Rather, antigen recognition by the T lymphocyte appears to involve a complex membrane unit consisting of an antigen-binding site and a specific site for macrophage-lymphocyte interaction. The simplest model one can deduce from these studies is that the Ir gene product is the prime antigen-binding site and histocompatibility antigen itself or a structure closely linked to it is the site of macrophage-lymphocyte interaction and both of these structures are linked on the surface of the cell. A more complex model is that the antigen recognition unit consists of a prime antigen-binding receptor, such as immunoglobulin, an Ir gene product as an auxiliary antigen recognition structure, and a separate macrophage-lymphocyte interaction site.

SUMMARY

A number of recent studies have suggested that the main functional role of the product of the immune response (Ir) genes is in the process of antigen recognition by the T lymphocyte. The observation in the accompanying report that the interaction of macrophage-associated antigen with immune T lymphocytes requires that both cells share histocompatibility antigens raised the question as to whether the macrophage played a role in the genetic control of the immune response or even if the macrophage were the primary cell in which the product of the Ir gene is expressed. In the current study, parental macrophages were pulsed with an antigen, the response to which is controlled by an Ir gene lacking in that parent; these macrophages were then mixed with T cells derived from the (nonresponder \times responder) F_1 and the resultant stimulation was measured. No stimulation was seen when column-purified F_1 lymph node lymphocytes were mixed with antigen-pulsed macrophages from the non-responder parent. However, when the highly reactive peritoneal exudate lymphocyte population was used as the indicator cells, parental macrophages pulsed with an antigen whose Ir gene they lacked were capable of initiating F_1 T-cell proliferation. The magnitude of stimulation was approximately $\frac{1}{10}$ that seen when macrophages from either the responder parent or the F_1 were used. In order to explain this observation, we hypothesize that antigen recognition sites on the T lymphocyte are physically related to a macrophage-binding site and both are linked to the serologically determined histocompatibility antigens. Thus, parental macrophages pulsed with an antigen, whose Ir gene they lack, activate F_1 cells poorly because the recognition sites for the antigen are physically related to the macrophage-binding site of the responder

parent while the main contacts between the cells are at the nonresponder binding sites. Experiments performed with alloantisera lend support to this hypothesis. Thus, when parental macrophages are pulsed with any antigen and added to F₁ T cells, an alloantiserum directed against parental histocompatibility antigens reacts with both the lymphocyte and the macrophage and thereby inhibits macrophage-lymphocyte interaction and abolishes antigen-induced lymphocyte transformation. When the alloantisera are directed at determinants present solely on the T lymphocyte, they only inhibit the recognition of antigens controlled by the Ir gene linked to the histocompatibility antigen against which they are directed. We conclude from these studies that antigen recognition by the T lymphocyte is a complex multicellular event involving more than simple antigen binding to a specific lymphocyte receptor.

We wish to thank Doctors I Green and W. E. Paul for helpful discussions and critical review of the manuscript. We also wish to thank Mr. J. Thomas Blake and Mrs. Linda Lee for expert technical assistance.

REFERENCES

1. Mitchell, G. F., F. C. Grumet, and H. O. McDevitt. 1972. Genetic control of the immune response. The effect of thymectomy on the primary and secondary antibody response of mice to poly-L-(Tyr, Glu)-poly-D,L,-Ala--poly-L-Lys. *J. Exp. Med.* **135**:126.
2. Benacerraf, B., I. Green, and W. E. Paul. 1967. The immune response of guinea pigs to hapten-poly-L-lysine conjugates as an example of the genetic control of the recognition of antigenicity. *Cold Spring Harbor Symp. Quant. Biol.* **32**:569.
3. Green, I., W. E. Paul, and B. Benacerraf. 1966. The behavior of hapten-poly-L-lysine conjugates as complete antigens in genetic responder and as haptens in nonresponder guinea pigs. *J. Exp. Med.* **123**:859.
4. Davie, J. M., W. E. Paul, and I. Green. 1972. Genetic control of the immune response of guinea pigs to 2,4-dinitrophenyl guinea pig albumin. Frequency of antigen binding cells and avidity of antibodies secreted by plaque-forming cells. *J. Immunol.* **109**:193.
5. Dunham, E. K., E. R. Unanue, and B. Benacerraf. 1972. Antigen binding and capping by lymphocytes of genetic nonresponder mice. *J. Exp. Med.* **136**:403.
6. Hammerling, G. J., T. Masuda, and H. O. McDevitt. 1973. Genetic control of the immune response. Frequency and characteristics of antigen-binding cells in high and low responder mice. *J. Exp. Med.* **137**:1180.
7. Schlossman, S. 1972. *In* Genetic Control of Immune Responsiveness. H. O. McDevitt and M. Landy, editors. Academic Press, New York. 60.
8. 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.
9. Ellman, L., I. Green, and B. Benacerraf. 1970. Identification of the cell population responding to DNP-GL in lethally irradiated strain 13 chimeric guinea pigs

- reconstituted with strain 13 bone marrow and (2×13) F₁ lymph node and spleen cells. *Cell. Immunol.* **1**:445.
10. 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.
 11. 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.
 12. Kantor, F. S., A. Ojeda, and B. Benacerraf. 1963. Studies on artificial antigens. I. Antigenicity of DNP-poly-lysine and DNP-copolymer of lysine and glutamic acid in guinea pigs. *J. Exp. Med.* **117**:55.
 13. Rosenthal, A. S., J. M. Davie, D. L. Rosenstreich, and J. T. Blake. 1972. Depletion of antibody-forming cells and their precursors from complex lymphoid cell populations. *J. Immunol.* **108**:279.
 14. Green, I., W. E. Paul, and B. Benacerraf. 1967. A study of the passive transfer of delayed hypersensitivity to DNP-poly-L-lysine and DNP-GL in responder and nonresponder guinea pigs. *J. Exp. Med.* **126**:959.