Published November 1, 1973

FUNCTION OF MACROPHAGES IN ANTIGEN RECOGNITION BY GUINEA PIG T LYMPHOCYTES

I. Requirement for Histocompatible Macrophages and Lymphocytes

By ALAN S. ROSENTHAL and ETHAN M. SHEVACH

(From the Laboratory of Clinical Investigation and the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014)

(Received for publication 12 July 1973)

The activation of immunocompetent lymphocytes by antigen is dependent upon interaction between antigen and a specific lymphocyte recognition structure. For the bone marrow-derived or B lymphocyte, a number of studies have demonstrated easily detectable membrane immunoglobulin receptors that are capable of binding the antigen for which the cell is specific (1, 2). Upon interaction with antigen, B lymphocytes proliferate and/or differentiate into plasma cells that synthesize and secrete immunoglobulin molecules with binding properties identical with that of the receptors of their precursor B lymphocyte. The mechanism by which thymus-derived or T lymphocytes recognize antigen is a matter of considerable controversy. Although some workers have observed immunoglobulin on the membrane of T lymphocytes (3, 4), others have failed to detect it in significant quantities (5, 6). Specific antigen binding to T lymphocytes has also been quite difficult to demonstrate (7, 8). Antigen binding to T cells has been measured indirectly by exposing cells in vitro to [^{125}I]-antigen of high specific activity with subsequent killing or "suicide" of the specific antigen-binding T lymphocytes (9). More recently, theta-positive, surface immunoglobulin-negative antigen-binding cells have been visualized directly after incubation of primed mouse spleen cells with relatively high concentrations of ^{125}I-labeled antigen (10) and by the cytoadherence techniques (11).

Regardless of whether or not T cells efficiently bind soluble antigens, little or no data is available on the mechanism by which these cells are activated or triggered by antigen. Indeed, a number of studies suggest that T-cell activation, as measured by lymphocyte proliferation, involves the cooperation of macrophages and lymphocytes (12–15). In the guinea pig recent studies have shown that stimulation of T-lymphocyte proliferation in vitro involves an initial uptake of soluble protein antigens by macrophages (16). This uptake is maximally acquired after only brief exposure of the macrophages to antigen (30–60 min at 37°C) (15, 17) and requires the expenditure of metabolic energy. Thus, the uptake of antigen by macrophages appears to represent more than simple surface binding (13, 15, 17).

Other studies on the genetic control of specific immune responses have raised the possibility that molecules other than immunoglobulin may play a role in antigen recognition by the T lymphocyte (18). Thus, alloantisera can specifically block the

Published November 1, 1973

activation of T lymphocytes by antigens, the response to which is linked to the presence of histocompatibility specificities against which the alloantisera are directed (19). It was concluded from these observations that the immune response genes produce a cell surface-associated product and that this product plays a role in the mechanism of antigen recognition by the T lymphocyte.

It seemed reasonable, therefore, to evaluate the importance of macrophage and T-lymphocyte histocompatibility determinants in the expression of antigen recognition. We will demonstrate in this report that the recognition of soluble protein antigens by guinea pig T lymphocytes requires the presentation of antigen on histocompatible macrophages and that this interaction between macrophage and T lymphocyte can be blocked by alloantisera. These data are interpreted as supporting the existence of a specific associative event between macrophage-bound antigen and T lymphocyte that is mediated by histocompatibility determinants themselves or by membrane surface products of genes linked to the major histocompatibility region.

Materials and Methods

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

Guinea pigs were immunized with an emulsion of antigen or saline in complete Freund's adjuvant (containing 0.4 mg/ml *Mycobacterium tuberculosis* $H_{37}RA$; Difco Laboratories, Detroit, Mich.). Each animal received 0.1 ml emulsion in each footpad for a total dose of antigen of 100 μg per guinea pig.

Reagents.—Guinea pig albumin (GPA)[1] was purchased from Pentex, Biochemical, Kankakee, Ill. 2,4-Dinitrophenyl (DNP)-GPA was prepared as previously described (1). Purified protein derivative of tuberculin (PPD) was obtained from Connaught Medical Research Laboratories, Willowdale, Ontario, Canada. The strain 2 anti-strain 13 serum and the strain 13 anti-strain 2 serum were prepared and assayed as previously described (19). These sera were also used to histocompatibility type lymph node lymphocytes from outbred animals as described (20).

Cell Collection and Purification.—The techniques for collection of peritoneal exudate cells (PEC) have been described in detail previously (21). In brief, animals were injected intraperitoneally with 25 ml of sterile mineral oil (Marcol 52; Humble Oil and Refining Co., Houston, Tex.). 3–4 days later the exudate cells were harvested by lavaging the peritoneal cavity with 150 ml of Hanks' balanced salt solution. Erythrocytes were removed from the pooled exudate cells by treatment with buffered NH_4Cl (22) at 4°C and washed four times before further manipulation. The PEC population consisted of about 75% monocytes-macrophages, 10% neutrophils, and 15% lymphocytes. Peritoneal exudate lymphocytes (PELs), a population of highly enriched antigen-reactive T lymphocytes (21, 23), were obtained by purification of the PEC over adherence columns (7). After column purification the

[1] *Abbreviations used in this paper:* DNP, 2,4-dinitrophenyl; FCS, fetal calf serum; GPA, guinea pig albumin; H, histocompatibility; LNLs, lymph node lymphocytes; MLR, mixed leukocyte reaction; Mϕ, macrophage; NGPS, normal guinea pig serum; PECs, peritoneal exudate cells; PELs, peritoneal exudate lymphocytes; PHA, phytohemagglutinin; PPD, purified protein derivative of tuberculin; [^{3}H]TdR, tritiated thymidine.

Published November 1, 1973

PEL population contains about 90% lymphocytes, 2–5% neutrophils, and 5–8% monocytes or macrophages.

Lymph node cell suspensions were prepared from trimmed lymph nodes by teasing with a needle and forceps and depleted of macrophages and B lymphocytes by passage over adherence columns as previously described (7). Lymph node lymphocytes (LNLs) contained about 0.5% macrophages when tested by latex bead phagocytosis and were greater than 98% viable by trypan blue exclusion.

"Purified" macrophages were prepared from the PEC population by allowing the cells to adhere to the surface of glass Petri dishes in the presence of 10% fetal calf serum (Gray Industries, Inc., Fort Lauderdale, Fla.) at 37°C. After 3 h, the cells were washed and overlaid with fresh media containing 10% fetal calf serum (FCS) and then cultured for an additional 24 h. Nonadherent cells were again washed away; the medium was replaced with several washes of iced saline; and the dishes were cooled at 4°C. After 30 min the cells were gently scraped off the glass with a rubber policeman and washed. This cell preparation contained greater than 98% macrophages with a viability of greater than 90%.

Technique of Brief Antigen Exposure.—PECs or monolayer-purified macrophages at a concentration of 15×10^6/ml in the presence of 30 μg/ml mitomycin C (Nutritional Biochemicals Corp., Cleveland, Ohio) were allowed to equilibrate at 37°C. The appropriate concentration of antigen was then added and the cell mixtures were maintained at 37°C for 60 min (15). At the end of the exposure period, the cell suspensions were washed four times with media. Residual antigen after this washing procedure was determined using ^{125}I-labeled PPD and found to be consistently less than 0.1 μg of PPD per 10^6 macrophages after an initial 100 μg PPD/ml exposure.

In Vitro Assay of Antigen-Induced DNA Synthesis.—Antigen-pulsed macrophages at a concentration of 1×10^6/ml were mixed with immune PELs or LNLs at a concentration of 2×10^6/ml in Eagle's minimal essential media, Spinner modification (S-MEM; Microbiological Associates, Bethesda, Md.) supplemented with glutamine (300 μg/ml), penicillin 100 U/ml, streptomycin 100 μg/ml, and 10% FCS or 10% normal guinea pig serum (NGPS). Aliquots (0.2 ml) of these mixtures were added to each of four wells in a sterile round bottom microtiter plate (Cooke Engineering Co., Alexandria, Va.) covered and incubated at 37°C in a humidified atmosphere of 95% air, 5% CO_2. After 24 or 48 h, 1 μCi of tritiated thymidine ([^{3}H]thymidine, 6.7 Ci/mM; New England Nuclear, Boston, Mass.) was added to each well. 18 h later the amount of [^{3}H]thymidine incorporated into cellular DNA was measured with the aid of a semiautomated microharvesting device (24). Radioactivity was counted in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.) and expressed as either total counts per minute (cpm) per culture or difference between control and antigen-stimulated culture (Δcpm per culture). The overall scheme of the experiments is shown in Fig. 1.

RESULTS

Requirement for Syngeneic Macrophages for Activation of T Lymphocyte Proliferation.—We have shown previously that a log linear relationship exists between the numbers of syngeneic PPD-pulsed macrophages added to immune lymphocytes and the resultant DNA synthesis at ratios of macrophages to lymphocytes less than 1:1.[2] Moreover [^{3}H]TdR incorporation into new DNA was proportional to the log of the concentration of antigen used to

[2] Rosenstreich, D. L., and A. S. Rosenthal. Peritoneal exudate lymphocyte. III. Dissociation of antigen-reactive lymphocytes from antigen binding cells in T-lymphocyte-enriched populations in the guinea pig. Manuscript submitted for publication.

Published November 1, 1973

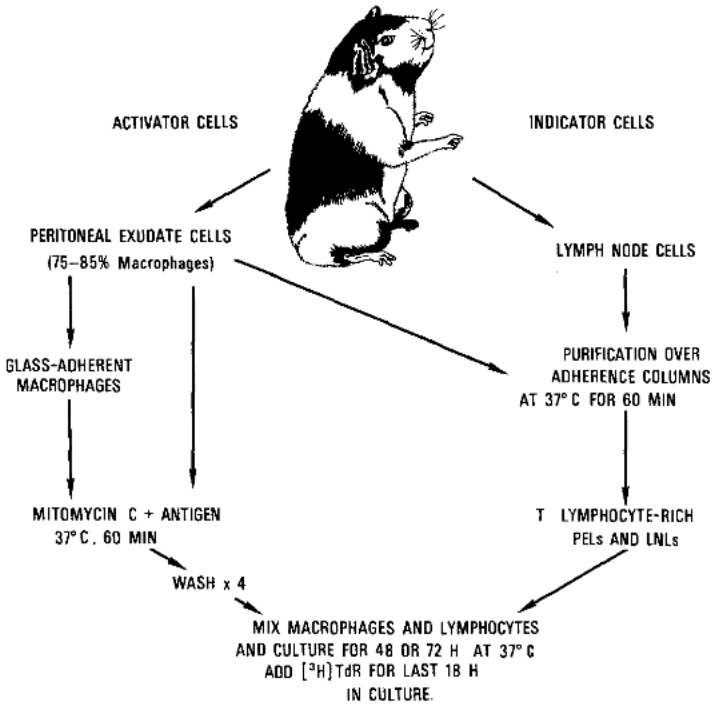

FIG. 1. Schematic diagram of the assay of macrophage-associated antigen activation of guinea pig T-DNA lymphocyte synthesis.

pulse the macrophages up to a concentration of 100 μg/ml. Subsequent experiments, therefore, routinely employed 0.1×10^6 macrophages pulsed with 100 μg/ml PPD for 60 min at 37°C and mixed with 0.2×10^6 lymphocytes.

The initial studies examined the ability of strain 2 and 13 macrophages to activate immune strain 2 and 13 PELs. The antigen-dependent incorporation of [^{3}H]TdR into new DNA was assessed at 72 h and expressed as cpm $\times 10^{-3}$ (Table I). Strain 2 macrophage-associated antigen ($M\phi_2$) initiated immune strain 2 PEL DNA synthesis quite effectively ($M\phi_2$-PPD 180,390 cpm vs. $M\phi_2$-control 1,250 cpm), while the same macrophages mixed with immune strain 13 PELs stimulated little DNA synthesis ($M\phi_2$-PPD 13,540 vs. $M\phi_2$-control 7,400). Similarly, antigen-pulsed strain 13 macrophages induced little DNA synthesis when mixed with immune strain 2 PELs ($M\phi_{13}$-PPD 16,790 cpm vs. $M\phi_{13}$-control 6,360 cpm) but considerable DNA synthesis in immune strain 13 lymphocytes ($M\phi_{13}$-PPD 58,700 vs. $M\phi_{13}$-control 550 cpm). Also of note was the small but significant mixed leukocyte reaction (MLR) seen in the absence of antigen in mixtures of allogeneic macrophage-rich PECs and PELs.

The experimental approach shown in Table I was repeated, but in this series

Published November 1, 1973

of experiments column-purified lymph node cells (LNLs) were used as the reactive population. The ability of (2 × 13)F_1 LNL to respond to parental macrophages and the ability of F_1 macrophages to activate parental LNL were also examined. The data presented in Table II using LNLs confirm the observa-

TABLE I

The Requirement for Histocompatible Macrophages in Antigen-Mediated DNA Synthesis in Immune Guinea Pig Peritoneal Exudate Lymphocytes

Macrophage*		Lymphocyte DNA synthesis	
Strain	Antigen pulse	Strain 2	Strain 13
		[^3H]*TdR incorporation* (*cpm* × 10^{-3})	
2	0	1.25 ± 0.88	7.40 ± 3.04
2	+	180.39 ± 21.79	13.54 ± 4.60
13	0	6.36 ± 2.01	0.55 ± 0.28
13	+	16.79 ± 1.64	58.70 ± 11.41

* Macrophage-rich PEC from nonimmunized strain 2 and 13 guinea pigs were incubated with mitomycin C and/or PPD for 60 min at 37°C, washed, and each mixed with both immune strain 2 and 13 PELs as described in Materials and Methods. The [^{3}H]TdR incorporation is expressed as mean cpm × 10^{-3} ± SE of three experiments.

TABLE II

The Requirement for Histocompatible Macrophages in Antigen-Mediated DNA Synthesis in Immune Guinea Pig Lymph Node Lymphocytes

Macrophage*		Lymphocyte DNA synthesis		
Strain	Antigen pulse	Strain 2	Strain 13	(2 × 13)F_1
		[^3H]*TdR incorporation* (*cpm* × 10^{-3})		
2	0	0.92 ± 0.28	5.68 ± 1.08	1.60 ± 0.30
2	+	26.38 ± 8.27	8.61 ± 2.08	6.98 ± 0.80
13	0	4.63 ± 1.86	1.66 ± 0.37	1.78 ± 0.47
13	+	3.12 ± 0.67	19.89 ± 4.47	7.81 ± 1.75
(2 × 13)F_1	0	1.91 ± 0.99	4.27 ± 0.34	1.66 ± 0.53
(2 × 13)F_1	+	12.42 ± 3.19	11.81 ± 1.98	12.57 ± 2.33

* Experimental design identical to Table I except that (2 × 13)F_1 macrophages and lymphocytes have been added and column-purified lymph node cells have been used as indicators instead of PELs. [^{3}H]TdR incorporation is expressed as mean cpm × 10^{-3} ± SE of six experiments.

tion that macrophage-rich PEC pulsed with antigen efficiently activate only syngeneic lymphocytes. Thus, the requirement for syngeneic macrophages and lymphocytes for T-lymphocyte recognition is not a peculiarity of the source of the T lymphocytes. In addition, these data show that F_1 macrophage-associated antigen activates parental lymphocytes only about 50% as well as parental macrophages ($M\phi F_1 \rightarrow LNL_2$ 12,420 cpm vs. $M\phi_2 \rightarrow LNL_2$ 26,380

Published November 1, 1973

and $M\phi F_1 \rightarrow LNL_{13}$ 11,810 cpm vs. $M\phi_{13} \rightarrow LNL_{13}$ 19,890 cpm). Moreover, parental 2 and 13 macrophage-associated antigen activated immune F_1 lymphocyte DNA synthesis only about 50% as well as F_1 macrophage-associated antigen ($M\phi_2 \rightarrow LNL_{F_1}$ 6,980 cpm; $M\phi_{13} \rightarrow LNL_{F_1}$ 7,810 cpm vs. $M\phi_{F_1} \rightarrow LNL_{F_1}$ 12,570 cpm).

Purified Allogeneic Macrophages Fail to Activate T-Lymphocyte Proliferation.—The experiments presented thus far have used the PEC population as the source of macrophages. Inasmuch as this population of cells is composed of 75–85% macrophages, it seemed appropriate to evaluate the stimulation of immune T lymphocytes by highly purified allogeneic macrophages. Strain 13 macrophages were purified by adherence plating, washed, and pulsed with either 100 μg/ml DNP-GPA, or 10 μg/ml PPD; they were then mixed with either strain 2 or strain 13 lymphocytes (Table III). The results demonstrate again that antigen on macrophages stimulate only syngeneic lymphocyte proliferation. In addition, a similar requirement for syngeneic macrophages was shown for another antigen DNP-GPA; this demonstrates that the requirement for syngeneic macrophages is not a peculiarity of the antigen PPD. Of note is the absence of a mixed leukocyte reaction in combinations of allogeneic-purified macrophages and lymphocytes. The data show that macrophage-rich PEC and the more highly purified macrophages when pulsed with antigen consistently gave similar results when assessed for their ability to initiate allogeneic and syngeneic lymphocyte proliferation. Because of the difficulty in purifying macrophages, in sufficient numbers, the macrophages used in the remainder of this paper are the PEC population.

Absence of T-Lymphocyte Activation by Allogeneic Macrophage-Associated Antigen is Not the Result of Differences in the Kinetics of Activation.—Since DNA synthesis was measured at only a single time, the possibility remained that activation of lymphocytes by antigen on allogeneic macrophages might occur either before or after that time. The kinetics of activation of strain 2

TABLE III

Activation of Strain 2 and 13 PELs by Purified Strain 13 Macrophages

Strain 13 macrophages*	Lymphocyte DNA synthesis	
	Strain 2	Strain 13
	[^{3}H]TdR incorporation (cpm × 10^{-3})	
Control	0.37 ± 0.10	0.55 ± 0.07
DNP-GPA pulsed	0.30 ± 0.08	18.03 ± 4.91
PPD pulsed	0.63 ± 0.14	6.01 ± 0.53

* Monolayer purified macrophages from nonimmunized strain 13 guinea pigs were incubated at 37°C for 60 min in the presence of media alone, 100 μg/ml DNP-GPA, or 100 μg/ml PPD. The cells were then washed and mixed 1:2 with immune strain 2 and 13 PELs. The data presented are the mean cpm × 10^{-3} ± SE of three experiments.

Published November 1, 1973

lymphocytes by strain 2 and 13 macrophage-associated antigen was examined. As shown in Fig. 2, at neither 24, 48, nor 72 h was significant activation of strain 2 PELs by strain 13 macrophages observed.

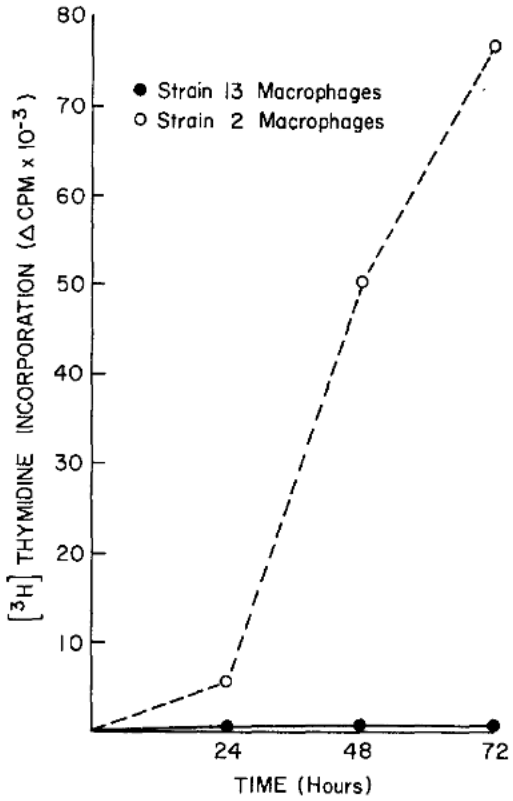

FIG. 2. Kinetics of macrophage-associated antigen activation of immune lymphocyte DNA synthesis. Strain 2 and 13 macrophages from a nonimmunized guinea pig were pulsed for 60 min at 37°C with mitomycin C alone or mitomycin C plus 100 μg/ml PPD, washed, and mixed with immune strain 2 PELs. Mixtures of syngeneic and allogeneic macrophages and lymphocytes were cultured 24, 48, and 72 h and [^{3}H]TdR incorporation expressed as Δcpm × 10^{-3}. Note that nonsyngeneic macrophages do not activate significant PEL DNA synthesis at any time in culture.

The Mixed Leukocyte Reaction Does Not Inhibit Macrophage-Associated Antigen Induction of Lymphocyte Proliferation.—To evaluate whether or not an inhibitor of blastogenesis might be released in mixtures of histoincompatible cells and thus account for the lack of stimulation in allogeneic mixtures of macrophages and lymphocytes, a number of experiments was carried out. First, strain 2 and strain 13 macrophages were combined, pulsed with antigen, and then added to either strain 2 or strain 13 lymphocytes; the resulting DNA

Published November 1, 1973

synthesis was compared with that of individual syngeneic and allogeneic combinations (Table IV). The results demonstrate that the presence of allogeneic macrophages does not inhibit the induction of proliferation of strain 2 or strain 13 lymphocytes by syngeneic macrophages. Second, strain 2 or strain 13 macrophages were pulsed with antigen and then added to either strain 2, strain 13, or an equal mixture of 2 and 13 lymphocytes, and the resultant DNA synthesis was assessed. As can be seen from Table IV, the presence of allogeneic lymphocytes does not inhibit the activation of strain 2 or strain 13 lymphocytes by syngeneic macrophages.

TABLE IV

The Effect of the Mixed Lymphocyte Reaction on Macrophage-Associated Antigen Activation of DNA Synthesis by Immune Guinea Pig Lymphocytes

Macrophages		Lymph node lymphocyte strain		
Strain	Antigen pulse	2	13	2 + 13*
		[^3H]*TdR incorporation* ($cpm \times 10^{-3}$)		
2	0	1.25 ± 0.25	5.47 ± 0.79	5.99 ± 1.01
2	+	39.90 ± 4.87	6.43 ± 0.47	59.92 ± 1.97
13	0	3.68 ± 0.55	2.71 ± 0.27	4.05 ± 0.31
13	+	3.83 ± 0.60	23.55 ± 1.22	15.48 ± 0.58
2 + 13‡	0	3.67 ± 0.18	6.03 ± 0.06	N.T.§
2 + 13	+	26.21 ± 0.38	21.96 ± 1.57	N.T.

Experimental conditions were identical with those in Table I. [^{3}H]TdR incorporation is expressed as mean cpm $\times 10^{-3} \pm$ SE of three experiments.

* A mixture containing 1.0×10^6/ml each of 2 and 13 lymphocytes.

‡ Mixtures containing 0.5×10^6/ml each of 2 and 13 macrophages.

§ Not tested.

An alternative approach to rule out the presence of an inhibitor of blastogenesis in mixtures of allogeneic macrophages and lymphocytes is to examine whether allogeneic macrophages will inhibit the response of lymphocytes to PPD (100 μg/ml) present for the duration of the culture or to phytohemagglutinin (PHA) (1 μg/ml). Although LNLs are obtained by column purification, they still contain sufficient (~0.5%) syngeneic macrophages to respond partially to continuous antigen or PHA. Strain 2 or strain 13 macrophages were pulsed with PPD, washed, and then added to strain 13 LNL. Again activation of significant DNA synthesis was seen only in the syngeneic combination (Table V, column 1). When PPD or PHA were then added, the strain 13 LNLs respond irrespective of the presence of strain 2 or strain 13 macrophages. The response to added PPD is greater in the syngeneic mixture and this is probably due to the fact that LNLs are relatively deficient in endogenous macrophages, and the addition of syngeneic macrophages either with or without previous antigen exposure enhances lymphocyte proliferation. These and

Published November 1, 1973

TABLE V

Allogeneic Macrophages Do Not Inhibit Lymphocyte DNA Synthesis on Continuous Antigen or Mitogen Exposure

Macrophage		Additions to cultures of macrophages and strain 13 lymphocytes*		
Strain	Antigen pulse	None	PPD	PHA
		[^3H]TdR incorporation (cpm × 10^{-3})		
2	0	5.66 ± 0.66	13.36 ± 1.67	217.01 ± 41.19
2	+	7.63 ± 1.58	13.43 ± 2.60	331.64 ± 32.17
13	0	1.78 ± 0.45	21.79 ± 0.95	357.29 ± 25.32
13	+	18.47 ± 3.77	27.29 ± 2.75	407.94 ± 47.31

* Mixture of macrophages and lymphocytes were cultured for 48 h without further addition or with addition of 100 μg/ml PPD or 1 μg/ml PHA continuously. Experimental conditions identical to Table I except that strain 2 and 13 macrophages were assayed only on strain 13 LNLs. [^{3}H]TdR incorporation is expressed as mean cpm × 10^{-3} ± SE of six experiments.

the previous experimental series are interpreted as indicating that no inhibitor of blastogenesis was released sufficient to account for the almost total absence of stimulation of lymphocyte DNA synthesis by allogeneic macrophage-associated antigen.

Studies Using Outbred Animals as the Source of Macrophages.—Random-bred guinea pigs have previously been shown to possess strain 2 and 13 histocompatibility determinants (25, 26). In order to evaluate whether the failure of strain 2 or strain 13 macrophages to activate allogeneic lymphocytes is related to the major serologically defined histocompatibility antigens, we studied the capacity of macrophages from outbred animals of serologically defined histocompatibility type to stimulate strain 2 and strain 13 lymphocytes. Outbred Hartley guinea pigs were individually tested with 13 anti-2 and 2 anti-13 sera for the presence of strain 2 and strain 13 determinants and grouped into three groups, [(2+, 13+), (2+, 13−), (2−, 13+)]. No (2−, 13−) guinea pigs have been identified in our colonies. Macrophages from these guinea pigs were pulsed with PPD and mixed with immune strain 2 and 13 lymphocytes. The data are given as the arithmetic mean and with individual points reflecting the data from single animals (Fig. 3). A general correlation exists between the sharing of strain 2 or 13 histocompatibility determinants and the ability of macrophages from outbred guinea pigs to stimulate the immune lymphocytes. Thus, 2+ macrophages, whether 13− or 13+, are superior to 2− macrophages in activation of strain 2 lymphocytes. Similarly, 13+ macrophages, whether 2+ or 2−, are superior to 13− macrophages in stimulation of 13 lymphocytes.

These findings establish that macrophage-lymphocyte interaction is regulated by histocompatibility (H) antigen itself or by the product of a gene that is closely linked to the H-antigen gene. Of additional interest is that 2− outbred

Published November 1, 1973

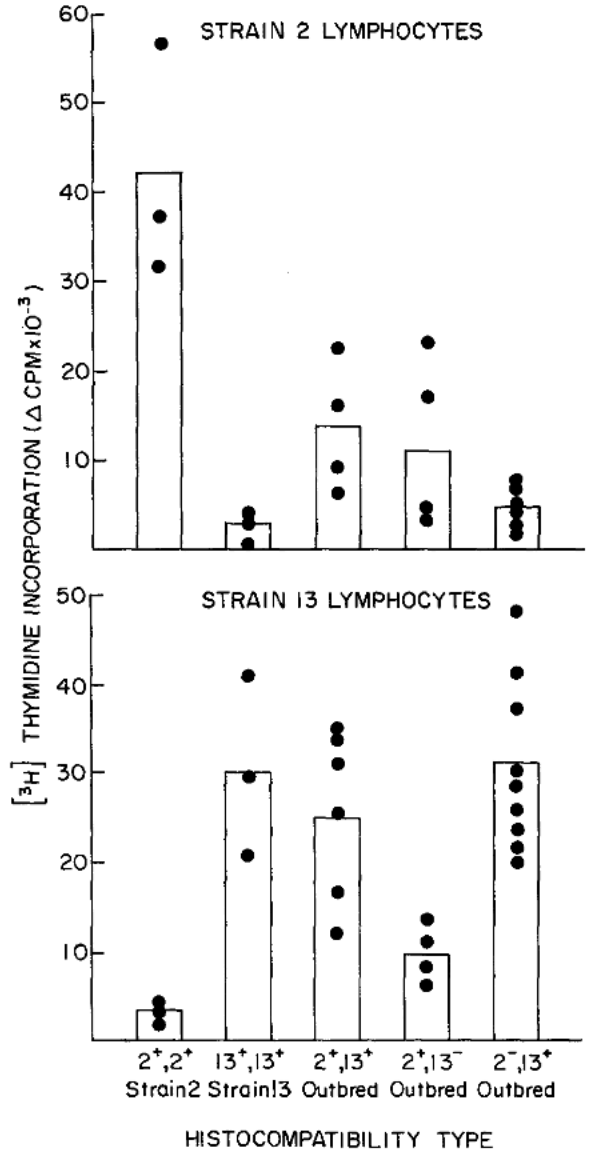

FIG. 3. The correlation of the histocompatibility type of outbred guinea pigs and the ability of their macrophages to stimulate DNA synthesis in immune strain 2 and 13 guinea pig lymphocytes. Experimental conditions are identical to Table I. LNLs are used as indicator lymphocytes. Outbred guinea pigs are categorized as (2+, 13+), (2+, 13−), and (2−, 13+). The bar graphs give the arithmetic mean cpm ± SE of the difference between stimulated and control culture. The individual value for each animal is represented by points about the mean.

Published November 1, 1973

macrophages activate strain 2 lymphocytes to a greater extent than do 2— inbreds (strain 13). Conversely, 13— outbreds activate strain 13 lymphocytes greater than do 13— inbreds (strain 2). The latter observations suggests that factors other than those serologically identifiable by the 13 anti-2 and 2 anti-13 sera may play some role in macrophage-lymphocyte cooperation.

Inhibition of Macrophage-Lymphocyte Cooperation by Alloantisera.—If indeed the interaction between macrophages and lymphocytes is mediated via histocompatibility antigen determinants or by membrane structures closely linked to these determinants, alloantisera might block this interaction. Strain 2, strain 13, or F_1 macrophages were pulsed with PPD or PHA, washed, and then mixed with F_1 PELs; these combinations were then cultured in normal guinea pig serum, 13 anti-2 serum, or 2 anti-13 serum (Fig. 4). Both the anti-2 and the anti-13 sera inhibited by about 30–40% the activation of F_1 PELs by F_1 macrophages pulsed with PPD. However, when strain 2 macrophages that had been pulsed with PPD were added to F_1 cells in the presence of the 13 anti-2 serum, the stimulation of the F_1 lymphocytes was markedly diminished; the strain 2 anti-strain 13 serum had essentially no effect on the activation of F_1 cells by strain 2 macrophages, but this serum did completely abolish the stimulation of F_1 cells by strain 13 macrophages. In no case did the alloantisera effect the activation of F_1 lymphocytes by macrophage-associated PHA. These data are interpreted as indicating that alloantisera block macrophage-lymphocyte interaction by acting on histocompatibility antigen determinants or sites closely linked to them; these studies do not localize on which cell the alloantisera exert their inhibitory effect.

Alloantisera Do Not Inhibit Macrophage-Lymphocyte Interaction by Acting Solely on the Macrophage.—In these experiments, F_1 macrophages were pulsed with PPD and then added to strain 2 or strain 13 PELs. When F_1 macrophages were added to strain 2 PELs, marked inhibition of the PPD response (Fig. 5) was seen in the presence of the 13 anti-2 serum and only minimal depression was seen with the 2 anti-13 serum. Similar results were observed when F_1 macrophages were added to strain 13 PELs; only in the presence of the 2 anti-13 serum was depression of the PPD response noted. These data demonstrate that the alloantisera only exert their inhibitory effect if they are directed against H-antigen determinants of both the macrophage and the lymphocyte.

DISCUSSION

The mechanism(s) by which T lymphocytes recognize antigenic signals is not known. The approach to the understanding of T-lymphocyte function used in this study is the analysis of the physiologic conditions under which T lymphocytes respond to soluble protein antigens. The assay of T-lymphocyte function employed in this study, antigen-induced proliferation, is a correlate of in vivo cellular immunity (27). Moreover, in the guinea pig, this in vitro response can be analyzed in terms of the cell types involved. T-lymphocyte antigen recognition in the guinea pig, as assessed by lymphocyte proliferation, has been shown to require macrophages (12–16). Recent

Published November 1, 1973

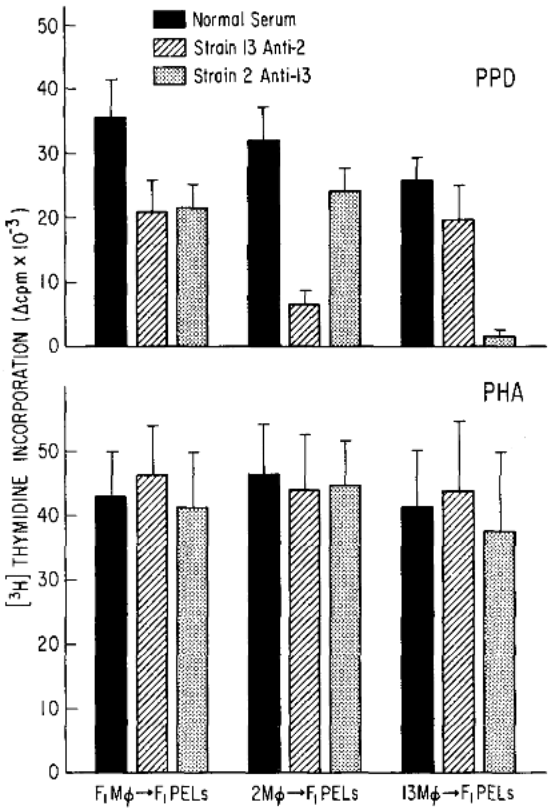

FIG. 4. The effect of alloantisera on macrophage-associated antigen activation DNA synthesis in (2 × 13)F_1 PELs. Strain 2, 13, and (2 × 13)F_1 macrophages obtained from a nonimmunized guinea pig were pulsed for 60 min at 37°C with mitomycin C and 100 μg/ml PPD or 10 μg/ml PHA. All cells were washed and mixed with immune (2 × 13)F_1 lymphocytes and each was cultured in normal guinea pig serum, strain 13 anti-2 serum, and strain 2 anti-13 serum. After 72 h [^{3}H]TdR incorporation was expressed as mean Δcpm × 10^{-3} ± SE. Note that both 13 anti-2 and 2 anti-13 sera suppress F_1 macrophage-associated antigen activation of F_1 lymphocytes about 40%. Strain 2 macrophage activation of F_1 lymphocyte DNA synthesis is suppressed by strain 13 anti-2 sera 80% and only 25% by strain 2 anti-13. In a reciprocal manner strain 13 macrophage activation of F_1 lymphocytes was suppressed 94% by strain 2 anti-13 and only 22% by strain 13 anti-2 antisera. The response of lymphocytes to macrophage pulsed with the nonspecific mitogen PHA is not appreciably altered by the source or specificity of the guinea pig sera used.

studies have indicated that the macrophage is the initial cell to interact with antigen (15, 16). Thus, when T-lymphocyte populations are fractionated by passage over adherence columns to deplete macrophages, exposed to high concentrations of antigens for 1–24 h, washed extensively to remove residual antigen, and then cultured for 2–3 days, no proliferation is seen (16). Indeed, no proliferation occurs even if the antigen-pulsed lymphocytes are reconstituted with macrophages before culture. However,

Published November 1, 1973

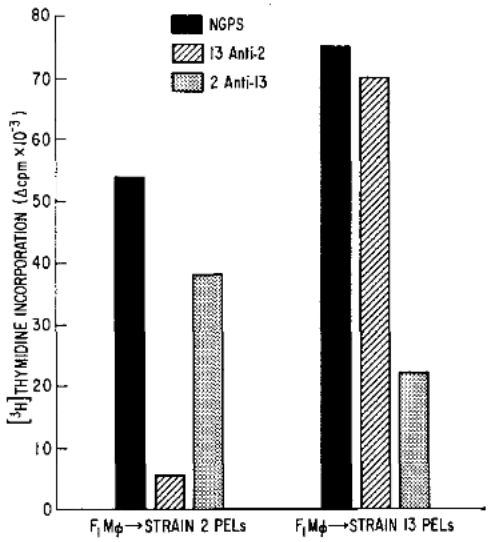

FIG. 5. The effect of alloantisera on macrophage-associated antigen activation of DNA synthesis in strain 2 or strain 13 animals. F_1 macrophages were pulsed for 60 min at 37°C with 100 μg/ml PPD in the presence of 30 μg/ml mitomycin C. The macrophages were washed and then added to either strain 2 or strain 13 PELs in the presence of NGPS, 13 anti-2, or 2 anti-13 sera. After 72 h, [^{3}H]TdR incorporation was measured and the results are expressed as Δcpm × 10^{-3}. The inhibition of F_1 macrophage activation of parental T cell DNA synthesis is only seen when the alloantisera are directed against determinants present both on the macrophage and the T lymphocyte.

macrophages from nonimmunized animals exposed to antigen for as little as 60 min at 37°C, washed, and mixed with immune lymphocytes induce a proliferative response comparable to that of unfractionated lymphoid-rich populations (15). Lymphocytes, fibroblasts, thymocytes, guinea pig L_2C leukemia cells, or killed macrophages similarly exposed to antigen, washed, and mixed with immune lymphocytes do not induce proliferation. Furthermore, culture supernatants derived from antigen-pulsed macrophages are unable to induce immune lymphocyte proliferation (13). These data indicate that an initial and obligatory uptake of antigen by macrophages precedes immunospecific T-lymphocyte recognition and suggest that a direct physical interaction between macrophage and T lymphocyte is required for initiation of proliferation (16).

The data presented clearly establish that efficient interaction of macrophage-associated antigen with the immunospecific T lymphocyte as measured by antigen-induced lymphocyte proliferation occurs only when the macrophage and T lymphocyte are syngeneic. It is likely that this interaction is mediated by histocompatibility antigens themselves or by the products of genes closely linked to the major H-antigen complex. Combinations of semiallogeneic macrophages and T cells did lead to T-lymphocyte proliferation although this activation was somewhat less efficient than the syngeneic combinations. This

Published November 1, 1973

indicates that the presence of a foreign H-antigen determinant does not completely block macrophage-lymphocyte cooperation. It is also unlikely that the requirement for syngeneic macrophages to initiate DNA synthesis is a peculiarity of the principal antigen used in these studies (PPD) as it was also seen with another soluble protein antigen DNP-GPA and as will be shown in the companion paper with the random copolymers of L-glutamic acid, L-lysine and L-glutamic acid, and L-tyrosine.

Although a small but significant MLR was seen in most experiments, the failure of allogeneic macrophages to activate T lymphocytes was not due to the presence of an inhibitor of blastogenesis liberated in mixtures of histoincompatible cells. Thus, when strain 2 and strain 13 macrophages were intentionally mixed and added to lymphocytes from one of the strains, the activation of the syngeneic lymphocytes by the syngeneic macrophages was not inhibited; conversely, when the lymphocytes of the two strains were intentionally mixed and macrophages of one strain added, again no inhibition of the syngeneic macrophage-T cell interaction was observed. Furthermore, the presence of allogeneic macrophages did not inhibit the response of column-purified LNLs to high dose continuous antigen or PHA; the activation of the T lymphocytes in this situation is presumably mediated via the residual syngeneic macrophages in the LNL population. Although the activation of lymphocytes by allogeneic macrophages might exhibit a different kinetic pattern than their activation by syngeneic macrophages, no evidence of T-cell proliferation was seen when allogeneic mixtures of antigen-pulsed macrophages and lymphocytes were harvested over a 2–4 day period.

The precise role of the macrophage in a number of in vitro cell culture systems has not been defined. Indeed, it has recently been suggested that the requirement for macrophages is an in vitro "artifact" and their presence is merely required to prevent lymphocyte death (28); in the Mishell-Dutton antibody-forming system in the mouse, the plaque-forming cell capacity of nonadherent cells can be restored completely by 2-mercaptoethanol. We have already stated the evidence derived from in vitro studies of lymphocyte proliferation in the guinea pig that the macrophage is the cell that initially binds and subsequently presents antigen to the T lymphocyte. In addition, we have been unable to replace macrophage function in this system by 2-mercaptoethanol.

It has been demonstrated in a number of experimental systems that aggregation of lymphocytes occurs around macrophages and that this aggregation may be a requirement for the in vitro proliferative response (29, 30). More recent studies in this laboratory have shown that guinea pig macrophages possess an antigen-independent receptor for thymocytes as well as mature lymphocytes (36). Binding of lymphoid cells to macrophages requires active macrophage metabolism and is not mediated by lymphocyte membrane-associated immunoglobulin. The characteristics and specificities of this receptor indicate that it is distinct from other known macrophage receptors. Whether this receptor plays a functional role in macrophage-associated antigen activation or lymphocyte proliferation remains to be established.

Published November 1, 1973

Studies of macrophages from outbred animals strongly suggest that at least one component of the cell surface structures mediating macrophage lymphocyte interaction is the product of the major histocompatibility gene complex. Thus, a good correlation was seen between the ability of macrophages obtained from an outbred animal to activate strain 2 or 13 T cells and the presence of serologically detectable strain 2 or strain 13 determinants. However, the specificity of the interaction was not as great as that observed with inbred cells. Thus, macrophages from outbred animals that lacked strain 2 determinants did induce some lymphocyte proliferation in strain 2 animals. It should also be noted that according to the classification of guinea pig leukocyte antigens proposed by Sato and de Weck (31), strain 2 and strain 13 animals share a major histocompatibility antigen. Perhaps this could account for the small amount of stimulation seen when strain 2 macrophages are mixed with strain 13 T cells or vice versa; alternatively, if the major histocompatibility complex in the guinea pig is bipartite in structure and similar to that of mice and man, it is possible that strain 2 and strain 13 animals could share one major sublocus but the gene controlling macrophage-lymphocyte interaction is at or near the second sublocus. Resolution of these problems must wait further study of the guinea pig H antigens.

Although the requirement in the in vitro antigen-induced proliferative response for histocompatible macrophages and lymphocytes has not been examined in detail in another species, a number of observations suggest the possibility that a similar situation may exist in man. Cline and Swett reported that human monocytes that had been pulsed with tuberculin were capable of inducing lymphocyte proliferation only when they were derived from the same individual (12). On the other hand, Blaese et al. (32) observed that allogeneic monocytes could activate T-lymphocyte proliferation although in most cases this activation was significantly less than in the syngeneic combination. However, in this study the HL-A types of the cell donors were not determined and hence partial cooperation between allogeneic lymphocyte and macrophage may have occurred because the cells shared some HL-A specificities. Although the responding lymphocyte populations used in the study of Blaese et al. were obtained by adherence column purification and did not respond to stimulation by continuous antigen unless reconstituted with macrophages, it is possible that allogeneic macrophages could have provided the "feeder" or nutritive function of syngeneic macrophages and enough syngeneic macrophages remained after column purification to function as the antigen-presenting cells.

Kindred and Shreffler (33) have shown that the cooperation between T and B cells in vivo in congenitally athymic (*nu/nu*) mice requires that the participating cells be syngeneic. These observations have been confirmed and extended by Katz, Hamaoka, and Benacerraf (34) who have shown that both in vivo and in vitro T and B cell cooperation only occurs between T and B cells derived from syngeneic or semisyngeneic donors. In contrast to the rigid requirement for syngeneic T and B cells necessary for antibody production, Katz and Unanue (35) have demonstrated that syngeneic and allogeneic antigen-pulsed macrophages activate in vitro secondary anti-DNP responses of mouse spleen cells equally well. These observations differ

Published November 1, 1973

markedly from our results and a number of comments should be made about this discrepancy. In the studies reported by Katz and Unanue, the role of the macrophage has not been firmly established. Indeed when they attempted to assess this role by depleting macrophages on Petri dishes, the adherent cell-depleted populations were capable of mounting secondary in vitro responses in the absence of added macrophages, but the addition of macrophages that had been pulsed with antigen resulted in substantial improvement in the response particularly in the case of IgG antibody production. Furthermore, Katz and Unanue demonstrated that the antigen-presenting function of macrophages could be replaced although considerably less efficiently by antigen-pulsed fibroblasts. The differences in macrophage function in the mouse in contrast to those described in the present study in the guinea pig may represent either species differences or alternatively differences in the assay itself. With respect to the latter point, it should be noted that in the in vitro lymphocyte proliferation assay as used in our studies, a log linear relationship exists between the amount of macrophage-associated antigen and the amount of [^{3}H]TdR incorporated into new DNA. By contrast, no evidence has been presented in the mouse assays as to what the quantitative relationship was between the T helper activity and the resultant number of plaques. If T helper activity is present in excess, then significant differences in the efficiency of syngeneic vs. allogeneic macrophages might go unobserved. It is apparent that resolution of the apparent discrepancies in the function of macrophages in the mouse and guinea pig must await further studies.

Alloantisera directed against the histocompatibility antigens of the two inbred strains of guinea pigs are able to markedly inhibit macrophage-lymphocyte interaction as measured by the inhibition of antigen-mediated cell proliferation. This conclusion is derived from the experiments where parental macrophages are added to F_1 lymphocytes. In this situation inhibition of the proliferative response to PPD is only seen when the alloantisera are directed against histocompatibility antigens present on both the macrophage and T lymphocyte; when the alloantisera are directed against the determinants present only on the lymphocyte, no inhibition is seen. Conversely, when F_1 macrophages are added to parental lymphocytes, inhibition of the PPD response was again seen only when the alloantisera were directed against determinants present on both the macrophage and the lymphocyte; when the alloantiserum is directed only against specificities present on the macrophage, no inhibition is seen. This latter experiment makes it very unlikely that the inhibition of the DNA synthetic response produced by the alloantisera is secondary to their ability to kill or agglutinate macrophages, because the F_1 macrophages have both the strain 2 and the strain 13 determinants on their surface.

The conclusion to be drawn from this study is that the activation of immune lymphocytes by antigen-pulsed macrophages is dependent on the interaction of cell surface structures that are the products of the major histocompatibility complex, and this interaction can be blocked by sera that are directed against these structures or against membrane components close to these structures.

Published November 1, 1973

SUMMARY

Antigen activation of DNA synthesis in immune thymus-derived lymphocytes of guinea pigs requires the cooperation of macrophages and lymphocytes. We have investigated the role of histocompatibility determinants in this macrophage-lymphocyte interaction using cells from inbred strain 2 and 13 guinea pigs. The data demonstrate that efficient presentation of macrophage-associated antigen to the lymphocyte requires identity between macrophage and lymphocyte at some portion of the major histocompatibility complex. The failure of allogeneic macrophages to effectively initiate immune lymphocyte proliferation was not the result of the presence of an inhibitor of blastogenesis released in mixtures of allogeneic cells, peculiarities of the antigen or lymphoid cells employed, nor differing kinetics of activation by allogeneic macrophages. In addition, data were presented that demonstrated that alloantisera inhibit lymphocyte DNA synthesis by functional interference with macrophage-lymphocyte interaction.

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. Davie, J. M., and W. E. Paul. 1971. Receptors on immunocompetent cells. II. Specificity and nature of receptors on dinitrophenylated guinea pig albumin-^{125}I-binding lymphocytes of normal guinea pigs. *J. Exp. Med.* **134:**495.
2. Davie, J. M., A. S. Rosenthal, and W. E. Paul. 1971. Receptors on immunocompetent cells. III. Specificity and nature of receptors on dinitrophenylated guinea pig albumin-^{125}I-binding cells of immunized guinea pigs. *J. Exp. Med.* **134:**517.
3. Marchalonis, J. J., R. E. Cone, and J. L. Atwell. 1972. Isolation and partial characterization of lymphocyte surface immunoglobulin. *J. Exp. Med.* **135:**956.
4. Hammerling, U., and K. Rajewsky. 1971. Evidence for surface associated immunoglobulin on T and B lymphocytes. *Eur. J. Immunol.* **1:**447.
5. Vitetta, E. S., C. Bianco, V. Nussenzweig, and J. W. Uhr. 1972. Cell surface immunoglobulin. IV. Distribution among thymocytes, bone marrow cells, and their derived populations. *J. Exp. Med.* **136:**81.
6. Perkins, W. D., M. J. Karnovsky, and E. R. Unanue. 1972. An ultrastructural study of lymphocytes with surface-bound immunoglobulin. *J. Exp. Med.* **135:** 267.
7. 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.
8. Lamelin, J. P., B. Lisowska-Bernstein, A. Matter, J. E. Ryser, and P. Vassalli. 1972. Mouse thymus-independent and thymus-derived lymphoid cells. I. Immunofluorescent and functional studies. *J. Exp. Med.* **136:**984.
9. Basten, A., J. F. A. P. Miller, N. L. Warner, and J. Pye. 1971. Specific inactiva-

Published November 1, 1973

tion of thymus-derived (T) and non-thymus derived (B) lymphocytes by ^{125}I-labeled antigen. *Nat. New Biol.* **231:**104.

10. Roelants, G., L. Forni, and B. Pernis. 1973. Blocking and redistribution ("capping") of antigen receptors on T and B lymphocytes by anti-immunoglobulin antibody. *J. Exp. Med.* **137:**1060.
11. Ashman, R. F., and M. C. Raff. 1973. Direct demonstration of theta-positive antigen-binding cells, with antigen-induced movement of thymus dependent cell receptors. *J. Exp. Med.* **137:**69.
12. Cline, M. J., and V. C. Swett. 1968. The interaction of human monocytes and lymphocytes. *J. Exp. Med.* **128:**1309.
13. Hersh, E. M., and J. E. Harris. 1968. Macrophage-lymphocyte interaction in antigen-induced blastogenic response of human peripheral blood leukocytes. *J. Immunol.* **100:**1184.
14. Seeger, R. C., and J. J. Oppenheim. 1970. Synergistic interaction of macrophages and lymphocytes in antigen-induced transformation of lymphocytes. *J. Exp. Med.* **132:**44.
15. Rosenstreich, D. L., and A. S. Rosenthal. 1973. Peritoneal exudate lymphocyte. II. *In vitro* lymphocyte proliferation induced by brief exposure to antigen. *J. Immunol.* **110:**934.
16. Waldron, J. A., R. G. Horn, and A. S. Rosenthal. 1973. Antigen induced proliferation of guinea pig lymphocytes *in vitro*: obligatory role of macrophages in the recognition of antigen by immune T-lymphocytes. *J. Immunol.* **111:**58.
17. Rosenthal, A. S., D. L. Rosenstreich, J. T. Blake, P. E. Lipsky, and J. W. Waldron. 1973. Mechanism of antigen recognition by T lymphocytes. *In* Proceedings Seventh Leukocyte Culture Conference. F. Daguillard, editor. Academic Press, New York. 201.
18. Benacerraf, B., and H. O. McDevitt. 1972. Histocompatibility-linked immune response genes. *Science* (*Wash. D.C.*). **175:**273.
19. 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.
20. Shevach, E. M., D. L. Rosenstreich, and I. Green. 1973. The distribution of histocompatibility antigen on T and B cells in the guinea pig. *Transplantation.* **6:**126.
21. 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.
22. Roos, D., and J. A. Loos. 1970. Changes in the carbohydrate metabolism of mitogenically stimulated human peripheral lymphocytes. *Biochim. Biophys. Acta.* **222:**565.
23. Rosenstreich, D. L., E. M. Shevach, I. Green, and A. S. Rosenthal. 1972. The uropod-bearing lymphocyte of the guinea pig. Evidence for thymic origin. *J. Exp. Med.* **135:**1037.
24. Harzman, R. J., M. L. Bach, F. H. Bach, G. B. Thurman, and K. W. Sell. 1972. Precipitation of radioactively labeled samples: a semi-automatic multiple sample processor. *Cell. Immunol.* **4:**182.

Published November 1, 1973

25. Martin, W. J., L. Ellman, I. Green, and B. Benacerraf. 1970. Histocompatibility type and immune responsiveness in random bred Hartley strain guinea pig. *J. Exp. Med.* **132:**1259.

26. Bluestein, H. G., I. Green, and B. Benacerraf. 1971. Specific immune response genes of the guinea pig. IV. Demonstration in random-bred guinea pigs that responsiveness to a copolymer of L-glutamic acid and L-tyrosine is predicated upon the possession of a distinct strain 13 histocompatibility specificity. *J. Exp. Med.* **134:**1538.

27. Oppenheim, J. J. 1968. Relationship of in vitro lymphocyte transformation to delayed hypersensitivity in guinea pigs and man. *Fed. Proc.* **27:**21.

28. Chen, C., and J. G. Hirsch. 1972. The effects of mercaptoethanol and of peritoneal macrophages on the antibody-forming capacity of nonadherent mouse spleen cells in vitro. *J. Exp. Med.* **136:**604.

29. Mosier, D. E. 1969. Cell interactions in the primary immune response in vitro. A requirement for specific cell clusters. *J. Exp. Med.* **129:**351.

30. Hanifin, J. M., and M. J. Cline. 1970. Human monocytes and macrophages: interaction with antigen and lymphocytes. *J. Cell Biol.* **46:**97.

31. Sato, W., and A. L. de Weck. 1972. Leucocyte typing in guinea pigs. *Z. Immunitaetsforsch.* **144:**S.49.

32. Blaese, R. M., J. J. Oppenheim, R. C. Seeger, and T. A. Waldmann. 1972. Lymphocyte-macrophage interaction in antigen induced in vitro lymphocyte transformation in patients with the Wiskott-Aldrich syndrome and other diseases with anergy. *Cell. Immunol.* **4:**228.

33. Kindred, B., and D. C. Shreffler. 1972. H-2 dependence of cooperation between T and B cells *in vivo*. *J. Immunol.* **109:**940.

34. Katz, D. H., T. Hamaoka, and B. Benacerraf. 1973. Cell interactions between histoincompatible T and B lymphocytes. II. Failure of physiologic cooperative interactions between T and B lymphocytes from allogeneic donor strains in humoral response to hapten protein conjugates. *J. Exp. Med.* **137:**1405.

35. Katz, D. H., and E. R. Unanue. 1973. Critical role of determinant presentation in the induction of specific responses in immunocompetent lymphocytes. *J. Exp. Med.* **137:**967.

36. Lipsky, P. E., and A. S. Rosenthal. 1973. Macrophage-lymphocyte interaction. I. Characteristics of the antigen-independent binding of guinea pig thymocytes and lymphocytes to syngeneic macrophages. *J. Exp. Med.* In press.