SYNERGISTIC INTERACTION OF MACROPHAGES AND LYMPHOCYTES IN ANTIGEN-INDUCED TRANSFORMATION OF LYMPHOCYTES*

BY ROBERT C. SEEGER, M.D., AND JOOST J. OPPENHEIM, M.D.

(From the Cell Biology Section, Laboratory of Biochemistry, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20014)

(Received for publication 9 February 1970)

In recent years, the role of the macrophage in immunological responses has received much attention. Fishman and Adler focused attention on macrophage "processing" of antigen when they reported that RNA extracted from peritoneal exudate (PE)[1] macrophages after incubation with bacteriophage was able to stimulate synthesis of phage-neutralizing antibody by normal lymph node cells in diffusion chambers (1). Askonas and Rhodes reported that such RNA extracts contained antigen and suggested that macrophages " . . . may process antigen in some way so as to make it highly immunogenic" (2). Subsequently, Adler, Fishman, and Dray described a second type of RNA extracted from peritoneal exudate cells (PE cells). It induced synthesis of specific phage-neutralizing antibody by lymph node cells which had the allotype marker of the PE cells rather than the lymph node cells. This implied that genetic information had been transferred from the PE cells to the antibody-synthesizing cells (3).

Another approach has been to study macrophage-lymphocyte interaction in vitro. Mosier demonstrated that primary antibody production in vitro by mouse spleen cells against sheep erythrocytes depended on the interaction of glass-adherent ("macrophage rich") and nonadherent ("lymphocyte rich") cells. Removal of either population interfered with the response (4).

Stimulation of transformation of lymphocytes in vitro by a specific antigen affords the opportunity to investigate the interaction of an antigen with various cells involved in immunological responses (5). Oppenheim, Leventhal, and Hersh demonstrated that in vitro transformation of human lymphocytes by specific antigen was markedly inhibited when phagocytic cells were removed from human leukocyte suspensions by passage down a glass bead column (6). Restoration of the response was achieved by adding macrophages to lymphocytes purified by glass bead column (Hersh and Harris, 7).

---

* A preliminary report of this work was presented at the Fourth Leukocyte Culture Conference on June 27, 1969, and is to be published in the proceedings of that conference (29).

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CFA, complete Freund's adjuvant; cpm, counts per minute; HBSS-GPS, Hank's balanced salt solution containing guinea pig serum; ^{3}H-TdR, ^{3}H-thymidine; PE, peritoneal exudate; PPD, purified protein derivative; LFU, Lf unit.

In the present study we have employed inbred histocompatible guinea pigs to investigate the role of macrophages and lymphocytes during antigen-induced proliferation of lymphocytes in vitro. Our findings indicate that the proliferative response of sensitized lymphocytes to specific antigen is greatly enhanced by macrophages but that the immunological memory requisite to elicit specific transformation responses is a property of the lymphocytes and not the macrophage.

Materials and Methods

Animals.—Male and female NIH strain 13 guinea pigs weighing 500–800 g were used for these studies. These animals are highly inbred and fail to reject skin grafts (8). Such histocompatible guinea pigs permit the use of cells from immunized and unimmunized animals without the complication of in vitro mixed leukocyte reactions. In vitro mixed leukocyte reactions between homologous guinea pig leukocytes would increase the background incorporation of ^{3}H-thymidine and thus interfere with the detection of lymphocyte transformation by soluble antigens.

Immunization.—Guinea pigs were immunized once with either complete Freund's adjuvant (CFA, *Mycobacterium tuberculosis*, 8 mg/ml, 0.1 ml into each footpad; provided by Dr. S. Stone) or tetanus toxoid (provided by Dr. L. Levine, Massachusetts Department of Public Health) emulsified in CFA (10 LFU/ml and 4 mg/ml respectively; 0.1 ml into each footpad). 2–4 wk later, such immunized guinea pigs ("immune") were used for experimental procedures. Control guinea pigs, except as indicated, received no immunization ("nonimmune").

Skin Tests and Antibody Determination.—Skin reactivity was assessed after intracutaneous injection of purified protein derivative (PPD; 10 μg in 0.1 ml; Lederle Laboratories, Pearl River, N. Y.) or tetanus toxoid (1 LFU in 0.1 ml) and evaluated at 4–6, 24, and 48 hr. Immunized animals manifested delayed hypersensitivity to PPD and tetanus toxoid which was maximal at 24 hr (greater than 10 mm diameter erythema and induration) and decreasing by 48 hr. The presence of antibody was determined by Ouchterlony immunodiffusion. Sera from animals that were immunized with tetanus toxoid contained detectable anti-tetanus antibody, whereas those immunized with CFA contained no detectable anti-PPD antibody.

Preparation of Lymphocytes.—Guinea pigs were exsanguinated via intracardiac puncture 2–4 wk after immunization. Within 30 min popliteal, flank, and axillary lymph nodes were removed and placed in Hank's balanced salt solution which contained 2.5% normal guinea pig serum (Hartley strain), penicillin (50 units/ml), and streptomycin (50 μg/ml) (HBSS-GPS). Submental and omental lymph nodes were also removed from nonimmune animals. The nodes were teased apart with forceps, and the cell suspension was filtered through a cup-shaped stainless steel sieve (60-mesh). Cells were washed in HBSS-GPS and concentrated by centrifugation at 450 *g* for 10 min at room temperature. Immunized animals yielded an average of 9×10^8 of these "lymph node cells" which consisted of 95% small and medium lymphocytes, 3% blasts, 1% monocytes, and 1% neutrophils and plasma cells; 80% of these cells excluded trypan blue. Control animals yielded approximately 4×10^8 lymph node cells, and 95% of these cells excluded trypan blue. Glass adherent cells were removed from lymph node cells by passage down a 12 inch high glass bead column (9, 6). The effluent cells which had not adhered to glass (lymphocytes) consisted of 98–99% small and medium lymphocytes, less than 1% blasts, and less than 0.5% monocytes; 95% or more of these cells excluded trypan blue. The average lymphocyte yield from such a procedure was 30–40% of the lymph node cells which were originally placed on the column.

Preparation of Macrophages.—Peritoneal exudate cells (PE cells) were obtained 4 days after

intraperitoneal injection of either a 2.5% solution of hydrolyzed starch (Connaught Medical Research Laboratories, Toronto, Canada) in buffered saline (10) or pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemical Co., Milwaukee, Wis.). The peritoneal cavity was rinsed with 200–300 ml of cold HBSS-GPS which contained heparin (1 unit/ml). The usual yield after intraperitoneal injection of starch was 40–80 × 10^6 PE cells, of which 80–85% were macrophages, 15–20% were lymphocytes, and 2–5% were neutrophils; 95% of these cells excluded trypan blue. Macrophages were identified in stained smears (Jenner-Giemsa) as medium to large mononuclear cells with indented nuclei and abundant cytoplasm. The cytoplasm frequently contained starch or oil-filled vacuoles depending on the irritant used to induce the exudate. Incubation of PE cells at 37°C for 1–2 hr with polystyrene latex particles (1.3 μ diameter; Dow Chemical Co., Midland, Mich.) resulted in phagocytosis of the particles by these mononuclear cells. Lymphocytes were identified by the criteria of small size, scanty cytoplasm, darkly staining nuclei, and an inability to phagocytose polystyrene particles. These PE cells were used as the source of macrophages in most experiments. After injection of pristane, approximately 200 × 10^6 cells were obtained, and they consisted of 50–60% macrophages, 30–40% lymphocytes, and 10% neutrophils. To obtain pure macrophages, the pristane-induced exudate was placed on top of a 17% bovine serum albumin (BSA) solution (Pathocyte 5, Pentex Inc., Kankakee, Ill.) and was centrifuged at 10,000 *g* for ½ hr. Cells which remained layered over the albumin after centrifugation consisted of 99% macrophages.

Pulmonary macrophages were obtained by cannulating the trachea with a No. 13 trochar and rinsing the lungs 5–6 times with 5 ml of HBSS-GPS. This yielded approximately 10–20 × 10^6 cells, and they consisted of 85–90% macrophages, 10% lymphocytes, and 5% neutrophils. More than 95% of these cells excluded trypan blue.

Culture Conditions.—Cells were cultured in 1 ml of either Eagle's basal medium No. 2 (EM) or RPMI-1640 (Roswell Park Memorial Institute) (NIH Media Unit, Bethesda, Md.) with 10% normal guinea pig serum (Hartley strain), penicillin (50 units/ml), and streptomycin (50 μg/ml) (EM-GPS or RPMI-GPS). No stimulant, 20 μg PPD, or 2 LFU tetanus toxoid was added to the cultures. The cultures were incubated in stationary flat-bottomed 1 dram vials (Demuth Glassworks, Inc., Parkersburg, W. Va.) with loose fitting covers at 36.5–37.5°C in a humidified atmosphere of 5% CO_2 in air. Duplicate or triplicate cultures were prepared in each experiment.

The response of concentrations of immune lymph node cells and of immune lymphocytes ranging from 125 × 10^3/ml to 4 × 10^6/ml to PPD was determined. Significant responses were obtained only when 5 × 10^5/ml or more lymph node cells or lymphocytes were present. The optimal response to PPD by lymph node cells and lymphocytes was obtained with 2 × 10^6 cells per ml ($P < 0.001$). Subsequently this concentration was routinely used. The response of purified lymphocytes to PPD and tetanus toxoid persisted even after successive passages through two glass bead columns.

^{3}H-Thymidine Incorporation.—The kinetics of ^{3}H-thymidine incorporation by immune lymph node cells and by immune lymphocytes which were stimulated with PPD was determined. Two μCi of ^{3}H-thymidine (^{3}H-TdR), specific activity 6.0 Ci/mM, Schwartz Bio Research, Inc., Orangeburg, N. Y.) in 0.07 ml nutritional mixture (11) and 0.03 ml normal guinea pig serum were added to each culture for a 14 hr period beginning at 24, 48, 72, and 120 hr of culture. Then the cultures were harvested, and the amount of radioactivity present in trichloroacetic acid-precipitable material was determined using a Packard scintillation counter by methods previously described (12). The greatest incorporation of ^{3}H-thymidine by stimulated cultures occurred in the 72–86 hr period. In all subsequent experiments ^{3}H-thymidine was added at 72 hr, and cultures were harvested at 86 hr.

Irradiation of Macrophages.—Macrophages (2 × 10^5) were irradiated in the culture vials in 0.5 ml of culture media. Various doses of irradiation (673 R/min) were delivered with a West-

inghouse Quadrocondex X-ray machine (200 kv, 15 ma, 0.25 mm Cu and 0.55 mm Al filters) at a target distance of 25 cm.

Preincubation of Macrophages.—Macrophages (2×10^5 in 0.5 ml of culture media) were incubated in the culture vials with either no stimulant, PPD (20 μg), or tetanus toxoid (2 LFU) for various periods of time at 36.5–37.5°C and then were washed three times with 4 ml of HBSS-GPS. These macrophages were then either irradiated with 3000 R or were not irradiated. Lymphocytes in 0.5 ml of culture media were then added to the macrophages, and cultures were incubated as previously described.

Morphology of Macrophage-Lymphocyte Interaction.—Lymphocytes were cultured with macrophages under the usual conditions in vials containing 9×9 mm coverslips. After 24, 48, and 86 hr of culture, the cover slips were gently removed from the vials and stained (Jenner-Giemsa) (12). In some experiments polystyrene latex particles were added to the cultures 2 hr before the cover slips were fixed and stained to facilitate identification of macrophages.

Statistical Analysis.—Logarithmic transformation of the counts per minute (cpm) of ^{3}H-TdR incorporation was done to obtain normally distributed data, and Student's t tests of paired comparisons were then performed. Geometric means were calculated. The "stimulation index" was calculated as follows: stimulation index = geometric mean cpm of stimulated cultures/geometric mean cpm of unstimulated cultures. (The computer program was prepared by Drs. C. MacLean and H. L. Cooper.)

RESULTS

Effect of Macrophages on Immune Lymph Node Cells.—Cultures of lymph node cells responded significantly to PPD and tetanus toxoid (Table I). However, addition of 2×10^5 immune macrophages to 2×10^6 unpurified immune lymph node cells (Table I) increased the ^{3}H-thymidine incorporation 7.6-fold ($P < 0.001$) with PPD and 3.6-fold ($P < 0.001$) with tetanus toxoid as compared to antigen stimulated lymph node cells alone. The stimulation index for lymph node cells with macrophages was 2.99 with PPD and 5.73 with tetanus toxoid, whereas it was 1.24 and 4.84, respectively, for lymph node cells alone. This indicates that the macrophages present in immune lymph node cell suspensions were quantitatively or qualitatively inadequate for optimal transformation.

Effect of Macrophages on Immune Lymphocytes.—Although macrophages enhanced transformation of immune lymph node cells, it seemed reasonable that the role of macrophages could be evaluated more clearly by testing their effect on lymphocytes which were purified by passage through a glass bead column. Such purified lymphocytes consisted of a more homogeneous population of nonadherent cells whose viability was 95% as compared to 80% for lymph node cells.

Purified immune lymphocytes responded significantly to PPD (Table II). However, addition of immune macrophages in doses ranging from 12.5×10^3 to 400×10^3 per 2×10^6 immune lymphocytes enhanced the ^{3}H-thymidine incorporation in cultures which were stimulated with PPD (Fig. 1). Incorporation of ^{3}H-thymidine increased progressively with addition of increasing percentages of macrophages ranging from 0.65 to 20% of the lymphocytes present.

When 2×10^5 immune macrophages were added to 2×10^6 immune lymphocytes the response to PPD was 19-fold ($P \ll 0.001$) greater than the response to PPD by lymphocytes alone. The stimulation index increased from 3.96 for lymphocytes alone to 10.34 for lymphocytes which were cultured with macrophages (Table II). Immune macrophages (PE cells) by themselves did not respond significantly to PPD which indicated that the purified lymph node lymphocytes rather than the macrophages or PE lymphocytes which contaminated them were responsible for this increased incorporation of ^{3}H-thymidine.

TABLE I

*Incorporation of ^{3}H-Thymidine by Unseparated Immune Lymph Node Cells: Effect of Adding Immune Macrophages**

Cell Population	cpm ^{3}H-TdR‡			Comparison of							
				Stimulated vs. unstimulated				Cell populations, P value			
				PPD		Tetanus toxoid					
	Unstimulated	PPD	Tetanus toxoid	S.I.§	P value‖	S.I.	P value	Population	Unstimulated	PPD	Tetanus toxoid
1. Lymph node cells¶	1,045	1,285	5,062	1.23	< 0.05	4.84	< 0.001	1 vs.			
								2	<0.001	<0.001	<0.001
								3	n.s.	n.s.	<0.025
2. Lymph node cells + immune macrophages**	3,260	9,754	18,680	2.99	< 0.001	5.73	≪ 0.001	2 vs.			
								3	< 0.001	< 0.001	< 0.001
3. Immune macrophages	721	849	1,012	1.18	n.s.‡‡	1.40	n.s.				

* The term "immune" is used to indicate the immunological history of the animal from which the cells were obtained.
‡ Values are geometric mean of data from six experiments. Cells were cultured 72 hr, and then pulsed with ^{3}H-thymidine (^{3}H-TdR) for 14 hr.
§ S.I., stimulation index $= \frac{\text{geometic mean cpm of stimulated cultures}}{\text{geometric mean cpm of unstimulated cultures}}$.
‖ After logarithmic transformation of cpm, Student's t test was performed on paired cultures.
¶ "Lymph node cells", unseparated lymph node cells.
** "Macrophages", unseparated peritoneal exudate cells (80% macrophages, 20% lymphocytes).
‡‡ n.s., not significant.

In all subsequent macrophage-lymphocyte interaction experiments 2×10^5 macrophages were cultured with 2×10^6 lymphocytes.

Morphological examination of PPD or tetanus toxoid-stimulated macrophage-lymphocyte cultures incubated for 24, 48, and 86 hr revealed that 5–15 lymphocytes were clustered around many of the macrophages to form rosettes. Medium and large lymphoblasts as well as small lymphocytes were present in such rosettes. Unstimulated cultures only occasionally contained rosettes.

In one study it was determined whether a freely diffusable macrophage product caused the enhancement of lymphocyte transformation. The effect on purified immune lymphocytes of supernatant fluids from macrophages (PE cells) which were incubated without any antigen, PPD, or tetanus toxoid for

1 ½ or 5 ½ hr was tested. The supernatant fluids did not stimulate greater transformation of purified lymphocytes than did culture media, PPD, or tetanus toxoid which had not been incubated with macrophages. After removal of antigen these same macrophages were incubated in fresh medium. Supernatant fluids which were then collected from the 2nd to 6th, 6th to 48th, and 48th to 120th hr of culture had no antigen-specific stimulatory capacity. The 48 to

TABLE II

*Incorporation of ^{3}H-Thymidine by Immune Lymphocytes: Effect of Adding Immune Macrophages**

Cell population	Exp. No.	CPM ^{3}H-TdR‡		Comparison of				
				Unstimulated vs. PPD		Cell populations, *P* value		
		Unstimulated	PPD	S.I.	*P* value	Population	Unstimulated	PPD
1. Lymphocytes§	1	258	697			1 vs.		
	2	2,813	8,490			2	≪0.001	≪0.001
	3	916	1,882			3	≪0.001	n.s.
	4	74	6,452					
	5	533	4,801					
	6	327	1,552					
	7	132	488					
	Mean	611	2,422	3.96	<0.001			
2. Lymphocytes + immune macrophages	1	3,609	94,832					
	2	10,027	53,860			2 vs.		
	3	2,871	17,808			3	<0.01	≪0.001
	4	4,076	64,435					
	5	3,426	64,595					
	6	—	—					
	7	2,105	37,199					
	Mean	4,517	46,689	10.34	≪0.001			
3. Immune macrophages	1	5,667	23,480					
	2	11,389	7,643					
	3	2,266	955					
	4	8,734	6,115					
	5	10,087	6,603					
	6	—	—					
	7	3,205	4,721					
	Mean	6,628	4,891	0.74	n.s.			

* See footnotes to Table I.

‡ Values are geometric mean cpm for experiments 1 through 7 and the geometric mean for combined data from these seven experiments.

§ "Lymphocytes", nonadherent lymphocytes which were separated from lymph node cells by passage through a glass bead column.

120 hr supernatants, whether from macrophage cultures which had initially been pulsed with antigen or from cultures which had not been pulsed with antigen, increased lymphocyte incorporation of ^{3}H-thymidine about 75%. In contrast, addition of macrophages to purified lymphocytes increased their response 44-fold to PPD and to 18-fold tetanus toxoid. Therefore, the marked enhancing effect of macrophages in this assay system is dependent upon the

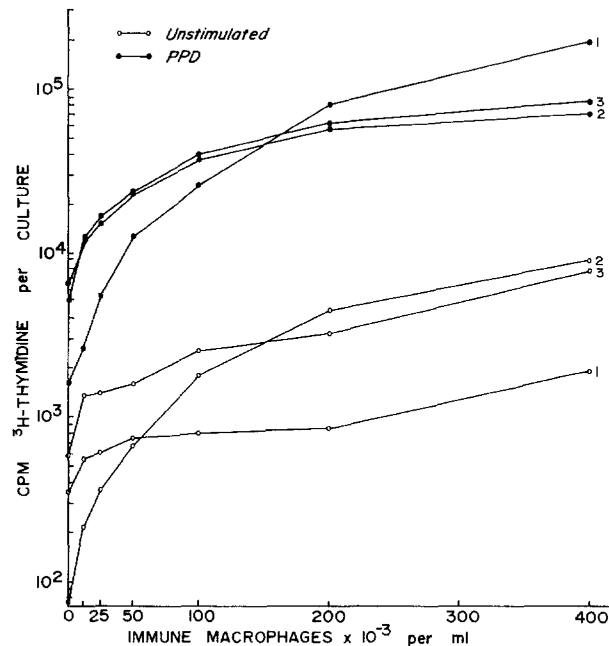

FIG. 1. Effect of dose of macrophages on the response of immune lymphocytes to PPD. Immune lymphocytes (2×10^6/ml) were cultured alone or with immune macrophages at doses ranging from 12.5×10^3 to 400×10^3 per ml. Cultures either were unstimulated or were stimulated with PPD (20 μg/ml). The results of three different experiments in which each point represents the geometric mean of duplicate or triplicate cultures are shown. The numbers 1, 2, and 3 refer to experiments 1–3.

presence of macrophage cells and is not due to either a freely diffusable immunologically specific or nonspecific macrophage cell product.

Effect of Irradiated Macrophages on Immune Lymphocytes.—To further insure that the enhanced incorporation of ^{3}H-thymidine in cultures containing both macrophages (PE cells) and lymphocytes was not due to proliferation of macrophages or contaminating lymphocytes from the peritoneal exudate, PE cells were irradiated to block significant ^{3}H-thymidine incorporation by them. Macrophages (PE cells) were incubated with antigen for 2–4 hr and then

irradiated with 3000 R. The addition of such irradiated macrophages to purified immune lymphocytes still increased their response to PPD 31-fold ($P \ll 0.001$) and to tetanus toxoid 11-fold ($P \ll 0.001$). The stimulation index for macrophage-lymphocyte cultures was 37.47 with PPD and 69.81 with tetanus toxoid, whereas for lymphocyte cultures it was 2.74 with PPD and 14.44 with tetanus toxoid (Table III; Fig. 2). In other experiments PPD or tetanus toxoid was added to macrophages 2–4 hr after they were irradiated; these macrophages increased the lymphocyte response to PPD 20-fold ($P < 0.001$) and to tetanus toxoid 9-fold ($P < 0.005$). The stimulation index for these macrophage-lympho-

TABLE III

*Incorporation of ³H-Thymidine by Immune Lymphocytes: Effect of Adding Immune Macrophages Which Were Irradiated 2–4 Hr after Being Exposed to Antigen**

Cell population	cpm ³H-TdR‡			Comparison of							
				Stimulated vs. unstimulated cells				Cell populations, P value			
				PPD		Tetanus toxoid					
	Unstimulated	PPD	Tetanus toxoid	S.I.	P value	S.I.	P value	Population	Unstimulated	PPD	Tetanus toxoid
1. Lymphocytes	699	1,915	10,087	2.74	< 0.001	14.44	≪ 0.001	1 vs.			
								2	< 0.005	≪0.001	< 0.001
								3	< 0.010	≪0.001	≪0.001
2. Lymphocytes + irradiated immune macrophages§	1,606	60,183	112,124	37.47	≪ 0.001	69.81	≪ 0.001	2 vs.			
								3	< 0.001	≪0.001	≪0.001
3. Irradiated immune macrophages§	174	132	210	0.76	n.s.	1.21	n.s.				

* See footnotes to Table I.

‡ Values are the geometric mean of data from seven experiments.

§ "Macrophages", immune unseparated peritoneal exudate cells which were irradiated with 3000 R 2–4 hr after exposure to antigen.

cyte cultures was 45.83 with PPD and 113.24 with tetanus toxoid, as compared to 3.13 with PPD and 17.98 with tetanus toxoid for lymphocyte cultures (Table IV). Irradiation of macrophages with doses of 500 R, 1000 R, or 6000 R also failed to block their ability to enhance transformation of lymphocytes. These experiments demonstrated that the increased incorporation of ³H-thymidine in cultures containing macrophages and lymphocytes was due to lymphocyte proliferation.

Effect of Previous Immunological Experience on Macrophages.—Macrophages from either sensitized or unsensitized guinea pigs were cultured with immune lymphocytes (Table V). In these experiments, immune lymphocytes by themselves responded significantly to PPD ($P < 0.001$), but their response was

increased 19-fold ($P \ll 0.001$) by adding 10% immune macrophages and 21-fold ($P \ll 0.001$) by adding 10% nonimmune macrophages. The stimulation index for macrophage-lymphocyte cultures was 10.34 with immune macrophages and 11.63 with nonimmune macrophages, as compared to 3.96 for

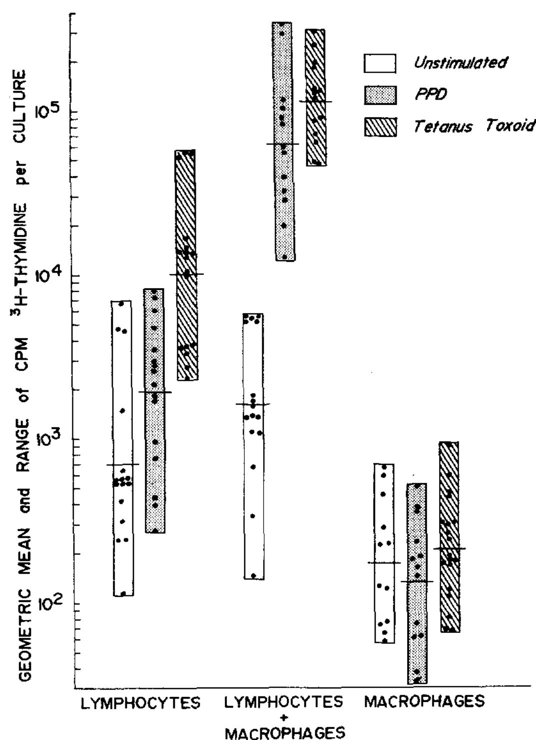

FIG. 2. Effect of macrophages which were irradiated 2–4 hr after being exposed to antigen on the response of immune lymphocytes to PPD or tetanus toxoid. Cultures contained lymphocytes (2×10^6/ml), immune irradiated macrophages (2×10^5/ml) and lymphocytes, or only macrophages with PPD (20 μg/ml), tetanus toxoid (2 LFU/ml), or no antigen. Data is from the seven experiments, the means of which are provided in Table III. Each point represents a single culture and the geometric mean is given by the horizontal bar.

lymphocytes alone. There was no significant difference between the effect of immune and nonimmune macrophages on the response of immune lymphocytes. Thus previous immunological experience did not influence the ability of macrophages to enhance transformation of immune lymphocytes.

Effect of Previous Immunological Experience on Lymphocytes.—Lymphocytes from unsensitized donors generally fail to transform in vitro in response to specific antigen (5). However, the report that messenger RNA was transferred

from macrophages to lymphocytes (3) raised the possibility that macrophages, particularly immune macrophages, could induce transformation of nonimmune lymphocytes. Therefore, nonimmune lymphocytes were cultured with nonimmune or immune macrophages (Table VI). In these experiments the same macrophages which had enhanced the transformation of immune lymphocytes (Table V) were used. Neither nonimmune lymph node cells nor nonimmune lymphocytes by themselves responded significantly to PPD (stimulation index of 1.06 and 0.64, respectively). Addition of 2×10^5 macrophages, whether immune or nonimmune, to nonimmune lymphocytes resulted in a significant

TABLE IV

*Incorporation of ^{3}H-Thymidine by Immune Lymphocytes: Effect of Adding Immune Macrophages Which were Irradiated 2–4 Hr Before Being Exposed to Antigen**

Cell population	cpm ^{3}H-TdR‡			Comparison of							
				Stimulated vs. unstimulated cells				Cell populations, *P* value			
	Unstimulated	PPD	Tetanus toxoid	PPD		Tetanus toxoid		Population	Unstimulated	PPD	Tetanus toxoid
				S.I.	*P* value	S.I.	*P* value				
1. Lymphocytes	390	1,220	7,014	3.13	< 0.001	17.98	$\ll 0.001$	1 vs. 2	n.s.	< 0.001	< 0.005
2. Lymphocytes + irradiated immune macrophages§	528	24,221	59,839	45.83	< 0.001	113.24	$\ll 0.001$	3	< 0.005	< 0.005	< 0.001
								2 vs. 3	< 0.005	$\ll 0.001$	$\ll 0.001$
3. Irradiated immune macrophages§	76	169	120	2.21	n.s.	1.58	n.s.				

* See footnotes to Table I.

‡ Values are the geometric mean of data from four experiments.

§ "Macrophages", immune unseparated peritoneal exudate cells which were irradiated with 3000 R 2–4 hr before exposure to antigen.

increase in the incorporation of ^{3}H-thymidine. However, the stimulation index for macrophage–nonimmune lymphocyte cultures was only 0.82 with immune macrophages and 0.99 with nonimmune macrophages. Control cultures which contained only macrophages incorporated ^{3}H-thymidine to the same extent as cultures which contained immune or nonimmune macrophages and nonimmune lymphocytes. Thus the increased incorporation of ^{3}H-thymidine by cultures containing nonimmune lymphocytes and macrophages was due to nonspecific incorporation of ^{3}H-thymidine by peritoneal exudate cells. Similarly, nonimmune lymphocytes from animals which were immunized with CFA did not respond to tetanus toxoid either in the presence or in the absence of immune or nonimmune macrophages. Neither immune nor nonimmune macrophages

were able to produce a significant response by nonimmune lymphocytes. Thus, the previous immunological experience of the lymphocyte rather than the macrophage provides the necessary immunological memory for the in vitro transformation response to antigen.

Effect of Pure Macrophages on Immune Lymphocytes.—To demonstrate that

TABLE V

*Incorporation of ^{3}H-Thymidine by Immune Lymphocytes: Effect of Adding Immune or Nonimmune Macrophages**

Cell population	cpm ^{3}H-TdR‡		Comparison of				
			Unstimulated vs. PPD		cell populations, P value		
	Unstimulated	PPD	S.I.	P value	Population	Unstimulated	PPD
1. Lymphocytes	611	2,422	3.96	<0.001	1 vs.		
					2	≪0.001	≪0.001
					3	≪0.001	≪0.001
					4	≪0.001	n.s.
					5	≪0.001	n.s.
2. Lymphocytes + immune macrophages§	4,517	46,689	10.34	≪0.001	2 vs.		
					3	n.s.	n.s.
					4	<0.01	≪0.001
					5	<0.025	≪0.001
3. Lymphocytes + nonimmune macrophages§	4,351	50,614	11.63	≪0.001	3 vs.		
					4	n.s.	≪0.001
					5	<0.025	≪0.001
4. Immune macrophages§	6,628	4,891	0.74	n.s.	4 vs.		
					5	n.s.	n.s.
5. Nonimmune macrophages	7,163	4,819	0.67	n.s.			

* See footnotes to Table I.

‡ Values are the geometric mean of data from seven experiments.

§ The terms "immune" and "nonimmune" are used to indicate the immunological history of the animal from which the cells were obtained.

the macrophages in the PE cells were responsible for enhancement of lymphocyte transformation, macrophage cell suspensions which were 99% pure were tested (Table VII). These purified macrophages, whether immune, nonimmune, irradiated, or unirradiated, were able to significantly enhance lymphocyte transformation. They increased the lymphocyte response 6-fold with PPD ($P < 0.001$) and 2.4-fold with tetanus toxoid ($P < 0.025$). The mean stimulation index of three experiments for lymphocytes alone was 7.92 with PPD and 36.4 with tetanus toxoid. For macrophages with lymphocytes it was 4.63 with

PPD and 8.64 with tetanus toxoid. Although the stimulation index was not increased by the pure macrophages, they considerably increased the ^{3}H-thymidine incorporation by purified lymphocytes. The stimulation increment (geometric mean cpm of stimulated cultures–geometric mean cpm of unstimulated

TABLE VI

*Incorporation of ^{3}H-Thymidine by Nonimmune Lymphocytes: Effect of Adding Immune or Nonimmune Macrophages**

Cell population	cpm ^{3}H-TdR‡		Comparison of stimulated vs. unstimulated cells		Comparison of cell populations, *P* value		
	Unstimulated	PPD	S.I.	*P* value	Population	Unstimulated	PPD
1. Lymph node cells	551	585	1.06	n.s.	1 vs.		
					2	<0.01	≪0.001
					3	<0.01	<0.01
2. Lymphocytes	287	183	0.64	n.s.	4	<0.005	≪0.001
					5	<0.005	<0.005
3. Lymphocytes + immune macrophages	4084	3363	0.82	n.s.	6	<0.001	≪0.001
					2 vs.		
					3	≪0.001	≪0.001
4. Lymphocytes + nonimmune macrophages	4699	4662	0.99	n.s.	4	≪0.001	≪0.001
					5	≪0.001	≪0.001
					6	≪0.001	≪0.001
5. Immune macrophages	7745	4925	0.64	n.s.	3 vs.		
					4	n.s.	n.s.
					5	<0.001	n.s.
6. Nonimmune macrophages	7426	4701	0.63	n.s.	6	<0.001	n.s.
					4 vs.		
					5	<0.01	n.s.
					6	<0.001	n.s.
					5 vs.		
					6	n.s.	n.s.

* See footnotes to Tables I and V.

‡ Values are the geometric mean for three experiments which were done concomitantly with three of the seven experiments shown in Table V.

cultures) for lymphocytes cultured alone was 2,101 cpm with PPD and 10,737 cpm with tetanus toxoid, whereas for lymphocytes cultured with pure macrophages it was 11,314 cpm with PPD and 23,779 cpm with tetanus toxoid. From these data we conclude that enhancement of lymphocyte transformation does occur with addition of pure macrophages. In contrast, addition of 10% syngeneic nonimmune lymph node cells or purified immune lymphocytes, whether irradiated or unirradiated, did not enhance transformation of immune lympho-

cytes. Therefore, macrophages are responsible for enhancing transformation of immune lymphocytes.

Effect of Alveolar Macrophages on Immune Lymphocytes.—Because macro-

TABLE VII

Incorporation of ^{3}*H-Thymidine by Immune Lymphocytes: Effect of Adding 99% Pure Macrophages**

Cell population	Exp. No.	cpm ^{3}H-TdR			Comparison of							
					Stimulated vs. unstimulated				Cell populations, P value			
					PPD		Tetanus toxoid					
		Un-stim-ulated	PPD	Tet-anus tox-oid	S.I.	P value	S.I.	P value	Pop-ula-tion	Un-stim-ulated	PPD	Tet-anus toxoid
1. Lymphocytes	1	250	1,271	9,648	5.08		38.6		1 vs.			
	2	295	1,908	—	6.47		—		2	< 0.001	< 0.001	< 0.025
	3	418	5,730	12,633	13.71		30.2		3	< 0.001	n.s.	< 0.001
	Mean	303	2,404	11,040	7.92	< 0.001	36.4	≪ 0.001				
2. Lymphocytes + immune macrophages‡	1	3,233	9,434	24,111	2.92		7.46					
	2	4,210	18,717	—	4.45		—		2 vs.			
	3	2,532	21,046	31,678	8.31		12.51		3	n.s.	≪ 0.001	< 0.001
	Mean	3,114	14,428	26,893	4.63	< 0.001	8.64	< 0.001				
3. Immune macrophages	1	895	1,045	860								
	2	1,978	2,453	—								
	3	3,603	2,886	1,467								
	Mean	1,671	1,783	775	1.07	n.s.	0.46	n.s.				
4. Lymphocytes + irradiated macrophages§	3	2,295	16,823	26,684	7.33		11.63					
5. Irradiated immune macrophages§	3	333	260	178	0.78		0.53					
6. Lymphocytes + nonimmune macrophages	3	4,431	44,357	50,564	10.01		11.41					
7. Nonimmune macrophages	3	4,637	3,210	3,264	0.69		0.70					
8. Lymphocytes + irradiated nonimmune macrophages	3	772	46,562	40,818	60.31		52.87					
9. Irradiated nonimmune macrophages	3	299	247	202	0.83		0.68					

* See footnotes to Table I.

‡ In these experiments macrophages were obtained 99% pure by centrifugation of peritoneal exudate cells through a 17% BSA solution.

§ Macrophages were irradiated with 3000 R 2–4 hr after being exposed to antigen.

phages which are obtained after intraperitoneal injection of an irritant may be metabolically activated, the ability of normally occurring alveolar macrophages to enhance lymphocyte transformation was tested. Addition of immune alveolar macrophages (Table VIII) to immune lymphocytes increased their response 144 fold to PPD ($P \ll 0.001$) and 28-fold to tetanus toxoid ($P < 0.025$). The stimulation indices for PPD and tetanus toxoid for macrophage-lymphocyte cultures were 7.01 and 4.49, whereas they were 1.49 and 4.87 for lymphocyte

TABLE VIII

*Incorporation of ³H-Thymidine by Immune Lymphocytes: Effect of Adding Immune Alveolar Macrophages**

Cell population	Exp. No.	cpm ³H-TdR			Comparison of							
					Stimulated vs. unstimualted				cell populations, P value			
		Un-stim-ulated	PPD	Tet-anus toxoid	PPD		Tetanus toxoid		Pop-ula-tion	Un-stim-ulated	PPD	Tet-anus toxiod
					S.I.	P value	S.I.	P value				
1. Lymphocytes	1	618	849	2,271	1.37		3.67		1 vs.			
	2	367	572	2,294	1.56		6.25		2	< 0.005	$\ll 0.001$	< 0.025
	Mean	469	697	2,282	1.49	< 0.025	4.87	< 0.01	3	< 0.001	< 0.001	< 0.001
									4	< 0.025	$\ll 0.001$	< 0.005
2. Lymphocytes +	1	9,382	89,174	44,955	9.50		4.79		5	< 0.025	< 0.001	< 0.025
immune	2	21,691	112,199	129,682	5.17		5.98					
macrophages‡	Mean	14,266	100,026	63,995	7.01	< 0.005	4.49	< 0.005	2 vs.			
3. Immune macro-	1	9,395	18,874	16,446	2.01		1.75		3	n.s.	< 0.001	< 0.05
phages‡	2	12,188	22,014	20,323	1.80		1.67		4	< 0.025	n.s.	n.s.
	Mean	10,701	20,383	18,263	1.90	< 0.01	1.71	< 0.005	5	$\ll 0.001$	$\ll 0.001$	< 0.025
4. Lymphocytes +	1	4,777	84,141	38,957	17.61		8.16		3 vs.			
irradiated	2	15,517	104,819	103,792	6.76		6.69		4	n.s.	< 0.001	< 0.025
immune	Mean	8,610	93,913	63,588	10.91	< 0.005	7.39	< 0.001	5	$\ll 0.001$	$\ll 0.001$	< 0.005
macrophages§												
5. Irradiated	1	115	163	195	1.42		1.70		4 vs.			
immune	2	174	122	95	0.70		0.55		5	< 0.001	$\ll 0.001$	< 0.005
macrophages§	Mean	142	141	136	0.99	n.s.	0.96	n.s.				

* See footnotes to Table I.

‡ Macrophages, unpurified alveolar cells (80–85% macrophages, 10–15% lymphocytes, 5% neutrophils).

§ Macrophages were irradiated with 3000 R 2–4 hr after being exposed to antigen.

cultures. Although the stimulation index for tetanus toxoid was similar for lymphocytes alone and lymphocytes with macrophages, the stimulation increment for tetanus toxoid was 1,813 cpm for lymphocytes alone and 49,729 cpm for lymphocytes with macrophages. Irradiation of alveolar macrophages did not block their ability to enhance transformation, but this lowered their incorporation of ³H-thymidine and thus increased the stimulation index to 10.91 for PPD and 7.40 for tetanus toxoid. Nonimmune alveolar macrophages, whether unirradiated or irradiated, also increased the response of immune lymphocytes to PPD and tetanus toxoid (Table IX). Thus, the ability of

macrophages to enhance transformation of immune lymphocytes is not restricted to macrophages derived from peritoneal exudates.

Preincubation of Macrophages with Antigen.—To determine if macrophages interact with antigen prior to lymphocyte-antigen interaction, macrophages were incubated with antigen, then washed, and finally incubated with immune

TABLE IX

*Incorporation of ^{3}H-Thymidine by Immune Lymphocytes: Effect of Adding Nonimmune Alveolar Macrophages**

Cell population	Exp. No.	cpm ^{3}H-TdR			Comparison of							
					Stimulated vs. unstimulated				Cell populations, P value			
		Unstimulated	PPD	Tetanus toxoid	PPD		Tetanus toxoid		Population	Unstimulated	PPD	Tetanus toxoid
					S.I.	P value	S.I.	P value				
1. Lymphocytes	1	1,430	1,705	11,403	1.19							
	2	618	849	2,271	1.37				1 vs.			
	3	367	572	2,294	1.56				2	≪ 0.001	≪0.001	< 0.005
	Mean (1, 2, 3)	628	939	3,902	1.50	< 0.01	6.21	< 0.001	3	< 0.05	<0.01	n.s.
	Mean (2, 3)	452	697	2,282	1.54	< 0.025	5.05	< 0.01	4	< 0.001	≪0.001	< 0.005
									5	< 0.025	< 0.005	< 0.005
2. Lymphocytes + nonimmune macrophages	1	17,743	52,654	44,386	2.97		2.50					
	2	20,048	124,339	59,886	6.20		2.99		2 vs.			
	3	23,648	197,968	138,428	8.37		5.85		3	< 0.01	≪0.001	≪0.001
	Mean	20,780	109,030	84,480	5.25	< 0.001	4.07	< 0.001	4	n.s.	< 0.025	< 0.01
									5	< 0.001	≪0.001	< 0.001
3. Nonimmune macrophages	1	1,407	3,710	1,332	2.64		0.95					
	2	5,312	5,933	4,690	1.12		0.88					
	3	11,197	11,243	8,389	1.00		0.75		3 vs.			
	Mean	4,374	6,278	3,742	1.44	n.s.	0.86	n.s.	4	< 0.005	< 0.001	< 0.001
									5	< 0.005	< 0.001	< 0.001
4. Lymphocytes + irradiated nonimmune macrophages	2	13,084	91,161	43,745	6.97		3.34		4 vs.			
	3	19,019	128,141	109,834	6.74		5.77		5	< 0.005	≪0.001	< 0.001
	Mean	16,789	111,824	80,810	6.67	< 0.001	4.81	< 0.001				
5. Irradiated nonimmune macrophages	2	338	114	129	0.34		0.38					
	3	182	128	105	0.70		0.58					
	Mean	248	121	116	0.49	n.s.	0.47	n.s.				

* See footnotes to Tables I and VIII.

lymphocytes (Fig. 3). Macrophages were irradiated (3000 R) after their interaction with antigen to decrease the background incorporation of ^{3}H-thymidine contributed by their proliferation and thus to maximize detection of lymphocyte transformation. Macrophages, which were incubated with PPD or tetanus toxoid for periods ranging from ½ to 6 hr and then washed, induced proliferation of added immune lymphocytes. The optimal time period was 4 hr. Such macrophage-induced transformation of lymphocytes was always less than that

of lymphocytes with macrophages which were continuously exposed to antigen but was usually (4 of 5 experiments) greater than that of lymphocytes alone with antigen. Both immune and nonimmune macrophages which were pre-

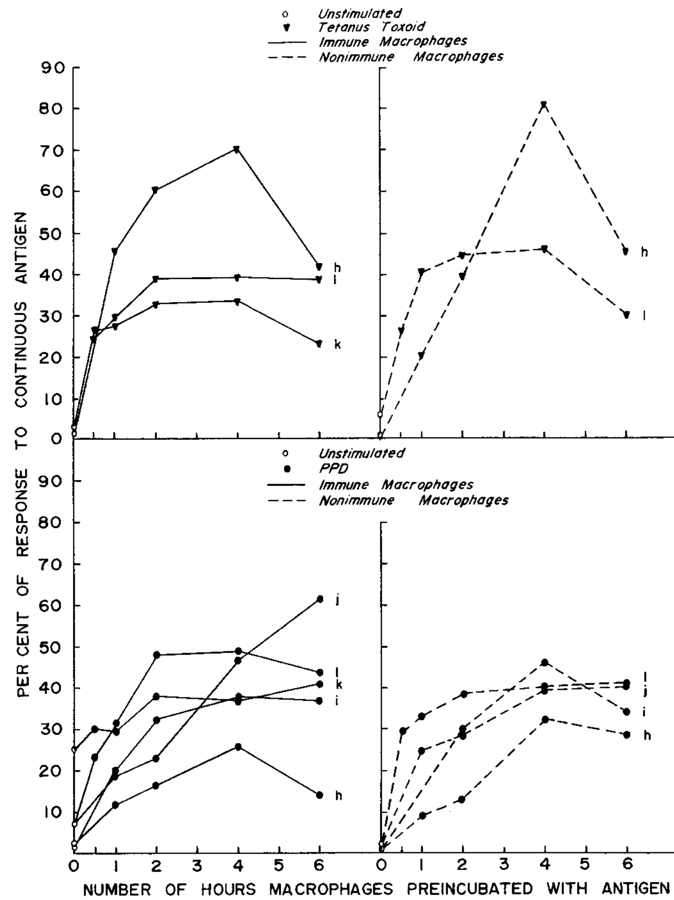

FIG. 3. Preincubation of macrophages with PPD or tetanus toxoid. Immune or nonimmune macrophages were incubated for ½–6 hr with PPD (20 μg/ml), tetanus toxoid (2 LFU/ml), or no antigen, washed, and then immune lymphocytes (2×10^6/ml) were added to them. The transformation response is expressed as follows: per cent of response to continuous antigen = (geometric mean cpm of preincubation cultures/geometric mean cpm of continuous antigen cultures) × 100. The letters *h*, *i*, *j*, *k*, and *l* indicate data from five different experiments.

incubated with antigen functioned equally well. Supernatant fluids which were obtained 2–4 hr after macrophages had been washed free of antigen did not stimulate transformation of immune purified lymphocytes. This indicated that the macrophages were thoroughly washed and were not releasing free im-

munogen into the medium. These experiments indicate that macrophage-antigen interaction can antecede lymphocyte-antigen interaction, and, as a result of such interaction, macrophages can stimulate transformation of immune lymphocytes.

DISCUSSION

Lymph node cells from sensitized guinea pigs that had delayed cutaneous hypersensitivity reactions to PPD and both delayed hypersensitivity and circulating precipitating antibodies to tetanus toxoid were stimulated by these antigens in vitro to incorporate ^{3}H-thymidine. The response of immune lymph node cells to PPD and tetanus toxoid was significantly enhanced by addition of macrophages. This suggests either that inadequate numbers of macrophages are present in lymph node cell suspensions for the culture conditions or that the macrophages in lymph node cell suspensions are not functionally competent. Such incompetence might result from cell damage sustained in the preparation of the cell suspensions or destruction of optimal in vivo intercellular relationships inherent in the lymph node architecture.

Passage of lymph node cells through glass bead columns removed all but a few of the lymph node macrophages. This separation of glass-adherent from nonadherent cells significantly reduced the ^{3}H-thymidine incorporation of both unstimulated and antigen-stimulated lymphocyte (nonadherent cells) cultures, but the stimulation index was the same for purified lymphocytes as for unpurified lymph node cells. This persistance of the response of purified guinea pig lymph node lymphocytes differs from the observation that the response of purified human peripheral blood lymphocytes to antigens is usually significantly reduced (6, 7). This difference may result from any of the following: (*a*) sufficient macrophages (less than 0.5%) or fragments of macrophages contaminate the guinea pig lymphocytes after glass bead column purification to allow transformation; (*b*) purified immune lymph node lymphocytes are able to respond to antigen in vitro without the aid of macrophages because macrophage-dependent events have already occurred in vivo; or (*c*) lymph node lymphocytes are able to respond to antigen without the aid of macrophages. The first explanation is favored by the observation that addition of as few as 0.65% macrophages enhanced transformation of immune lymphocytes. In preliminary experiments, the response to PPD or tetanus toxoid by purified immune lymphocytes was enhanced by culturing them at 3–8 times the usual cell density for the first 8–24 hr of the 86 hr culture period.[2] The response of purified human lymphocytes to antigens is also restored if they are cultured at high cell densities (5). Increasing the cell density possibly allows more effective interaction between the few contaminating macrophages and lymphocytes. However, it is

[2] Seeger, R. C. Unpublished observations.

possible that lymphocyte-lymphocyte interactions are enhanced by increased cell density and that such interactions, without a requirement for macrophages, are capable of inducing transformation. In macrophage-enriched cultures we have observed that lymphocytes form rosettes around macrophages[2] as have Salvin and Nishio (13), Mosier (14), and Lamvik (15). Such rosette formation suggests that macrophages may facilitate lymphocyte proliferation by providing foci of increased cell density and thus promote macrophage-lymphocyte as well as lymphocyte-lymphocyte interaction.

The most effective enhancement of transformation was obtained when 10% macrophages were added to immune purified lymphocytes. This effect of macrophages on lymphocyte transformation was considerably more when macrophages were added to purified lymphocytes than when they were added to unpurified lymph node cells. Approximately this same percentage of macrophages has been found to be most effective in restoring the response of pure human lymphocytes to antigen (7, 16). Mosier (4) and Pierce (17) also have shown that 10% macrophages are most effective in producing the primary antibody response to sheep erythrocytes in vitro.

Macrophages (PE cells) were irradiated to insure that the only cell capable of proliferation in the cultures was the lymph node lymphocyte and to determine the effect of irradiation on macrophage-antigen interaction. Irradiation of macrophages either before or after their first exposure to antigen did not impair their ability to enhance transformation of immune lymphocytes. Hersh and Harris recently reported that human macrophages which were irradiated 7 hr prior to being exposed to antigen were able to restore transformation of purified lymphocytes (16). Similarly, Roseman has shown that irradiation of the glass-adherent (macrophage-rich) fraction of spleen cells either before or after their exposure to sheep erythrocytes did not affect their ability to promote a primary in vitro antibody response by nonadherent (lymphocyte-rich) cells (18).

In contrast, in vivo studies by Gallily and Feldman (19) and Mitchison (20) have indicated that irradiation of macrophage donors 24 hr before exposure to antigen resulted in an inability of these macrophages to stimulate antibody formation when transferred to normal syngeneic recipients. However, Unanue and Askonas found that irradiation of macrophage donors had no effect on the ability of macrophages to stimulate antibody production when transferred to normal recipients (21). The reason for these observed differences in the effect of irradiation on macrophage function is yet to be resolved.

That macrophage-antigen interaction does occur and can antecede lymphocyte-antigen interaction was indicated by studies in which macrophages which were preincubated with antigen for ½–6 hr and then washed were able to stimulate transformation of lymphocytes. Similarly, Hersh and Harris (16) recently demonstrated that human lymphocyte transformation can be induced

by macrophages which are preincubated with antigen for 10 or more min. Cline and Sweet (2) reported that human macrophages which were preincubated with PPD for 2 hr were able to stimulate transformation of lymphocytes. In the in vitro anti-sheep erythrocyte antibody system employed by Mosier (4) and Pierce (17) specific antibody was produced when glass-adherent (macrophage-rich) mouse spleen cells were preincubated with antigen for ½ hr, washed, and then cultured with nonadherent (lymphocyte-rich) spleen cells.

Macrophage-antigen interaction has been studied in vivo by transferring macrophages to syngeneic recipients. Mitchison showed that BSA bound to peritoneal exudate cells is more potent than free BSA in inducing primary but not secondary immunization (20). Unanue and Askonas reported that macrophage-bound hemocyanin was highly effective both in priming for a secondary response and in inducing a secondary response (21). Argyris showed that macrophages which contained sheep erythrocytes elicited antibody synthesis after being transferred to syngeneic recipients (23).

To investigate whether macrophages or lymphocytes (or both) are responsible for immunological memory, immune and nonimmune macrophages were cultured with immune or nonimmune lymphocytes. Transformation of immune lymphocytes was increased to an equal extent by either immune or nonimmune macrophages which were pulsed with antigen for short periods or which were continuously exposed to antigen. These results suggest that cytophilic antibody does not influence the ability of macrophages to enhance lymphocyte transformation. Conversely, nonimmune lymphocytes did not respond to either PPD or tetanus toxoid in the presence or absence of immune or nonimmune macrophages. Similarly, Levis and Robbins recently studied a pair of identical twins in which the macrophages from either the immune or nonimmune twin restored the antigen-induced transformation response by purified lymphocytes from the immune twin (24). Hersh and Harris made similar observations in another pair of identical twins and in addition reported that neither immune nor nonimmune macrophages were able to induce transformation of nonimmune lymphocytes (16). Harris demonstrated that macrophages from both tolerant and nonimmune rabbits took up BSA and stimulated DNA synthesis in immune spleen cell suspensions (25). Mitchison demonstrated that macrophages from mice tolerant to BSA took up BSA and induced anti-BSA antibody synthesis in normal but not in tolerant syngeneic recipients (20). Therefore, because the macrophage is not influenced by past immunological experiences, it interacts with antigen nonspecifically and does not provide immunological memory for the immune response. In contrast, the lymphocyte is influenced by past immunological experiences, does interact with antigen specifically, and does provide immunological memory for the immune response.

It is apparent from both in vitro and in vivo studies that a product of macrophage-antigen interaction can be immunogenic. The nature of such interaction

and of the immunogenic substance(s) remains to be clearly defined. Thus instructional RNA (messenger RNA) may be transferred from macrophage to lymphocyte (3). However, it is difficult to explain the facilitative effect of macrophages on lymphocyte transformation by such transfer of messenger RNA from macrophage to lymphocyte because immune macrophages did not induce nonimmune lymphocytes to respond to antigen and nonimmune macrophages enhanced the transformation of immune lymphocytes. Another possibility is that antigen may be "processed" and bound to RNA to form a "super antigen" (2, 3, 26). Alternatively, the antigen may remain partially or completely intact bound to macrophage cell membranes (27, 28). Our study shows that the interaction of antigen with nonimmune macrophages results in a product which specifically stimulates only previously sensitized lymphocytes. The simplest basis for recognition by immune lymphocytes, but not by nonimmune lymphocytes, of the product of nonimmune macrophage-antigen interaction is that such a product contains antigen. Thus, we favor the view that the immunogenic product of macrophage-antigen interaction contains antigen. We are not able to distinguish whether such a product is super antigen or antigen bound to cell membranes.

After interacting with antigen, macrophages may provide a focus for both lymphocyte-antigen and lymphocyte-lymphocyte interaction. The interaction of lymphocytes with antigen and with one another may be more efficient in such foci of lymphocytes aggregated around macrophages.

SUMMARY

The role of macrophages and lymphocytes in antigen-induced transformation of lymphocytes has been investigated. Lymphocytes and macrophages were obtained from inbred strain 13 guinea pigs which were either unimmunized or immunized with complete Freund's adjuvant (CFA) or tetanus toxoid in CFA. The transformation response to PPD or tetanus toxoid was assayed by tritiated thymidine incorporation.

Addition of macrophages to immune lymphocytes significantly increased their response to purified protein derivative (PPD) or tetanus toxoid. This was observed if the macrophages were (*a*) "immune" or "nonimmune", (*b*) unirradiated or irradiated (3000 R), (*c*) 99% pure, and (*d*) peritoneal or alveolar in origin. Neither immune nor nonimmune macrophages were able to induce nonimmune lymphocytes to respond to PPD or tetanus toxoid. When macrophages were incubated with PPD or tetanus toxoid and then washed, they stimulated immune lymphocytes to transform. An incubation time of ½ hr was adequate, however, 2–4 hr was optimal.

These studies indicate (*a*) that antigen-induced transformation of lymphocytes is greatly enhanced by macrophages; (*b*) that macrophage-antigen interaction can antecede lymphocyte-antigen interaction and results in macrophages

which are able to stimulate lymphocyte transformation; and (*c*) that the immunological memory requisite to elicit specific transformation responses is a property of the lymphocyte and not the macrophage.

We wish to thank Dr. Herbert L. Cooper for helpful discussions, Dr. Richard Asofsky for helpful advice regarding cell separation with bovine serum albumin density gradients, and Mrs. Diane Mizel, Mrs. Melinda Seeger, and Mrs. Thelma Prather for excellent technical assistance.

BIBLIOGRAPHY

1. Fishman, M., and F. L. Adler. 1963. Antibody formation initiated in vitro. II. Antibody synthesis in X-irradiated recipients of diffusion chambers containing nucleic acid derived from macrophages incubated with antigen. *J. Exp. Med.* **117**:595.
7. Askonas, B. A., and J. M. Rhodes. 1965. Immunogenicity of antigen-containing ribonucleic acid preparations from macrophages. *Nature (London).* **205**:470.
3. Adler, F. L., M. Fishman, and S. Dray. 1966. Antibody formation initiated *in vitro*. III. Antibody formation and allotypic specificity directed by ribonucleic acid from peritoneal exudate cells. *J. Immunol.* **97**:554.
4. Mosier, D. E. 1967. A requirement for two cell types for antibody formation *in vitro*. *Science (Washington).* **158**:1573.
5. Oppenheim, J. J. 1968. Relationship of *in vitro* lymphocyte transformation to delayed hypersensitivity in guinea pigs and man. *Fed. Proc.* **27**:21.
6. Oppenheim, J. J., B. G. Leventhal, and E. M. Hersh. 1968. The transformation of column-purified lymphocytes with non-specific and specific antigenic stimuli. *J. Immunol.* **101**:262.
7. Hersh, E. M., and J. E. Harris. 1968. Macrophage-lymphocyte interaction in the the antigen induced blastogenic response of human peripheral blood leukocytes. *J. Immunol.* **100**:1184.
8. Bauer, J. A., Jr. 1958. Histocompatibility in inbred strains of guinea pigs. *Ann. N. Y. Acad. Sci.* **73**:663.
9. Rabinowitz, Y. 1964. Separation of lymphocytes, polymorphonuclear leukocytes and monocytes on glass columns, including tissue culture observations. *Blood J. Hematol.* **23**:811.
10. Turk, J. L., and L. Polak. 1967. Studies on the origin and reactive ability *in vivo* of peritoneal exudate cells in delayed hypersensitivity. *Int. Arch. Allergy Appl. Immunol.* **31**:403.
11. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* **126**:423.
12. Oppenheim, J. J., R. A. Wolstencroft, and P. G. H. Gell. 1967. Delayed hypersensitivity in the guinea pig to a protein hapten conjugate and its relationship to *in vitro* transformation of lymph node, spleen, thymus, and peripheral blood lymphocytes. *Immunology.* **12**:89.
13. Salvin, S. B., and J. Nishio. 1969. "In vitro" cell reactions in delayed hypersensitivity. *J. Immunol.* **103**:138.
14. Mosier, D. E. 1969. Cell interactions in the primary immune response in vitro: a requirement for specific cell clusters. *J. Exp. Med.* **129**:1969.

15. Lamvik, J. O. 1969. The role of phagocytic cells in the immune reaction of rabbit blood lymphocytes in antigen stimulated cultures. *Scand. J. Haematol.* **6:**149.
16. Hersh, E. M. and J. E. Harris. 1970. Further studies of macrophage-lymphocyte interaction. *In* Proceedings of the Fourth Leukocyte Culture Conference. O. R. McIntyre, editor. Appleton-Century-Crofts, New York. In press.
17. Pierce, C. W. 1969. Immune responses in vitro. I. Cellular requirements for the immune response by nonprimed and primed spleen cells in vitro. *J. Exp. Med.* **130:**345.
18. Roseman, J. 1969. X-ray resistant cell required for the induction of in vitro antibody formation. *Science (Washington).* **165:**1125.
19. Gallily, R., and M. Feldman. 1967. The role of macrophages in the induction of antibody in X-irradiated animals. *Immunology.* **12:**197.
20. Mitchison, N. A. 1969. The immunogenic capacity of antigen taken up by peritoneal exudate cells. *Immunology.* **16:**1.
21. Unanue, E. R., and B. A. Askonas. 1968. The immune response of mice to antigen in macrophages. *Immunology.* **15:**287.
22. Cline, M. J., and V. C. Sweet. 1968. The interaction of human monocytes and lymphocytes. *J. Exp. Med.* **128:**1309.
23. Argyris, B. F. 1967. Role of macrophages in antibody production: immune response to sheep red blood cells. *J. Immunol.* **99:**744.
24. Levis, W. R., and J. H. Robbins. 1969. Function of glass adherent cells in lymphocyte cultures. *Fed. Proc.* **28:**566.
25. Harris, G. 1967. Macrophages from tolerant rabbits as mediators of specific immunological response *in vitro. Immunology.* **12:**159.
26. Gottleib, A. A. 1969. Macrophage ribonucleoprotein: nature of the antigenic fragment. *Science (Washington).* **165:**592.
27. Pierce, C. W. 1969. Immune responses in vitro. II. Suppression of the immune response in vitro by specific antibody. *J. Exp. Med.* **130:**365.
28. Unanue, E. R., and J. C. Cerotini. 1969. Persistence of antigen on the surface of macrophages. *Nature (London).* **222:**1193.
29. Seeger, R. C., and J. J. Oppenheim. 1970. Macrophage-lymphocyte synergy in lymphocyte transformation. *In* Proceedings of the Fourth Leukocyte Culture Conference. O. R. McIntyre, editor. Appleton-Century-Crofts, New York. In press.