The Interaction of Human Macrophages and Lymphocytes in the Phytohemagglutinin-Stimulated Production of Interferon

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ABSTRACT In studies of 13 normal adults to determine the blood cell types responsible for interferon production induced by phytohemagglutinin, the following observations were made. (*a*) In cultures containing 96–100% pure macrophages derived from blood monocytes, no interferon was detected in either the presence or the absence of phytohemagglutinin for up to 92 hr. (*b*) In cultures of 99.5–100% pure lymphocytes, low levels of interferon were detected in the presence, but not in the absence, of phytohemagglutinin. (*c*) An average fivefold increase in interferon titers occurred when pure lymphocytes were combined with the macrophages in culture with phytohemagglutinin. The peak response of interferon occurred at 68 hr after the initiation of the combined cultures. For maximum response, phytohemagglutinin was required for the duration of the culture, and both cell types in association were necessary. Medium from phytohemagglutinin-stimulated macrophages or lymphocytes could not substitute for the corresponding intact cell. However, frozen-thawed macrophages in combination with lymphocytes and phytohemagglutinin produced an intermediate interferon response. An increase in either cell type produced an increased response in the range studied: lymphocytes, $0.45–1.8 \times 10^6$ per ml; and macrophages, $0.5–2.1 \times 10^5$ per ml. Syngeneic fibroblasts, HeLa cells, or mouse macrophages could not substitute for the human macrophages in the combined cultures with phytohemagglutinin. (*d*) Although all cultures producing interferon showed some degree of transformation (thymidine-^{3}H incorporation into deoxyribonucleic acid), no direct correlation between the degree of phytohemagglutinin-induced lymphocyte transformation and the interferon titers was observed.

The demonstration of macrophage-lymphocyte interaction in the production of interferon is of interest in view of the known interrelationship of these same cell types in antibody synthesis and cellular immunity.

INTRODUCTION

Interferon is an antiviral protein produced by animal cells in response to viral infection. Its production can be stimulated by nonviral agents as well as by viruses. One such agent is phytohemagglutinin (PHA), a substance which transforms small lymphocytes into large, "blast-like" cells capable of enhanced RNA (1), new DNA (2) and protein synthesis (3), and subsequent division 4). In 1965, Wheelock observed the production of interferon in cultures of human leukocytes which contained PHA (5). This finding was confirmed and extended by Friedman and Cooper (6) and by Green, Cooperband, and Kibrick (7). The present studies were undertaken to determine the specific blood cell type or types responsible for PHA-induced interferon production.

METHODS

Preparation of AB serum. Clotted blood from six normal type AB blood donors was centrifuged at 12°C for 25 min at 2500 rpm. The supernatants were collected, pooled, and transferred to sterile bottles for freezing at −70°C. Several lots of serum were prepared in this manner and used for large groups of experiments.

Preparation of McCoy's medium. 5000 U of penicillin and 5 mg of streptomycin were added to each 100 ml of McCoy's medium (obtained from Grand Island Biological Co. (GIBCO), Berkeley, Calif.). In addition, whenever Mc-

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Coy's medium was employed to wash cells or to prevent their clumping before their inclusion in culture tubes, 0.5 ml of an aqueous sodium heparin solution (Lipo-Hepin, Riker Laboratories, Inc., Northridge, Calif., 1000 USP units/ml) was added to each 100 ml of medium. All final cultures (lymphocyte, macrophage, fibroblast, HeLa cell, mouse macrophage, or combined lymphocyte-macrophage or lymphocyte-fibroblast) were prepared with 30 ml of pooled human AB sera per 100 ml of McCoy's medium.

Preparation of PHA solution. The contents of each vial of PHA-P (Lot No. 533563 and No. 536280, Difco Laboratories, Detroit, Mich.) were dissolved in 5 ml of sterile phosphate-buffered saline, pH 7.4 (GIBCO). A 1/10 dilution of this solution was prepared and frozen in 3-ml aliquots for use as desired. For each 2-ml culture, 0.04 ml of the diluted PHA was employed, giving a final concentration of 34 μg of PHA-P per ml of culture.

Preparation of macrophage cultures. All blood donors were healthy adults with no recent history of viral illness and with WBC and differential counts within the normal range. Blood was withdrawn into two or three 50-ml plastic syringes, each of which contained 750 U of aqueous sodium heparin, and then dispersed into sterile, siliconized, 12-ml centrifuge tubes with silicone stoppers. The tubes were incubated at 37°C for 1½–2 hr or in a few instances, longer, until the erythrocytes settled well. The leukocyte-rich plasma was aspirated with a sterile siliconized pipette, mixed with an equal volume of McCoy's medium, and washed twice in an International PR-2 Centrifuge. A leukocyte count was performed and a Giemsa-stained cytocentrifuge preparation made (Shandon-Elliott Cytocentrifuge, London, England). The cells were adjusted to a concentration of 3×10^6 monocytes per ml in 30% AB serum McCoy's medium, and 1 ml aliquots were distributed to sterile Leighton tubes with silicone stoppers and glass coverslips. The tubes were kept in a horizontal position for 2 hr at 37°C, during which time many of the monocytes and some polymorphonuclear leukocytes adhered to the coverslips. The nonadherent cells were decanted and the Leighton tubes were washed three times with 1 ml of warm McCoy's medium. Freshly prepared 1-ml aliquots of 30% AB serum McCoy's medium were added to each tube and the cultures incubated at 37°C for 1 wk. Once during the week the cultures were washed again three times with warm McCoy's medium and fresh 30% AB serum McCoy's added. From each 100 ml of blood withdrawn from the donor, about 10–12 macrophage cultures could be obtained.

To quantitate the number of macrophages in representative culture tubes from each experiment, the following technique was used to remove the macrophages from the surface of the coverslips. 0.1 ml of phosphate-buffered saline containing 3.3 mg of disodium EDTA (Sigma) was added to each 1 ml macrophage culture to be counted and the tubes were shaken well. They were kept in an incubator at 40°C for ½ hr, during which time they were shaken briskly a few times. The cells were counted and Giemsa-stained cytocentrifuge preparations were examined for morphology and purity.

To check further the purity of the macrophage preparations, phagocytosis and response to PHA-P were studied. In some experiments heat-killed *Candida albicans* were added at a ratio of 10 organisms per macrophage. The cultures were incubated at 37°C for ½ hr and then the coverslips removed, washed, and studied for evidence of phagocytosis. Additional checks for purity of macrophage cultures were made by adding 0.04 ml of $\frac{1}{10}$ PHA-P solution and, after 3 days at 37°C, studying the uptake of thymidine-^{3}H into DNA by radioautography. This technique separates lymphocytes, which respond, from macrophages, which do not.

In some experiments the macrophage cultures were destroyed by freeze-thawing the contents of the Leighton tubes 12 times at -70°C in a dry ice–acetone bath. Cytocentrifuge preparations of such frozen-thawed macrophages indicated complete disruption of the cells. No phagocytosis occurred in the frozen-thawed macrophages, confirming their disrupted state.

Isolation of lymphocytes. Blood for isolation of lymphocytes was always obtained from the same donor who 1 wk previously had donated blood for the preparation of macrophage cultures. The procedure employed was a modification of that employed by Levis and Robbins (8). The contents of a Leukopac Fenwal Co., Morton Grove, Ill.) were washed with distilled water for 2 hr and the nylon fibers dried overnight in an oven. An 8 inch, water-jacketed column, I.D. 0.5. inch, was then packed with the nylon fibers for a length of 8 inches, connected to a 37°C water bath, and rinsed with 30 ml of 30% AB serum McCoy's medium.

Leukocyte-rich plasma was obtained by gravity sedimentation of erythrocytes in nonsiliconized glassware at 37°C. The leukocytes were washed, resuspended in 40 ml of 30% AB serum McCoy's medium with 0.2 ml aqueous sodium heparin, and delivered to the column via a separatory funnel, whose flow was adjusted to 10–15 drops per min. After the cell suspension had been applied to the column, 40 ml of 30% AB serum McCoy's medium was added via a second separatory funnel to elute additional lymphocytes. The volume and the number of cells in the effluent was noted and 200–500 cells examined on Giemsa-stained cytocentrifuge preparations for a differential count. The cells were washed and resuspended in fresh 30% AB serum McCoy's medium at a concentration of 1.8×10^6 lymphocytes per ml. For routine cultures containing only lymphocytes, 1 ml of this cell suspension was combined with 1 ml of 30% AB serum McCoy's medium (thus giving a final concentration of 0.9×10^6 lymphocytes per ml) in Leighton tubes with coverslips and kept at a 30° angle at 37°C as long as desired for each experiment. For some experiments the concentration of lymphocytes was varied between 0.45×10^6 and 1.8×10^6 per ml. From each 100 ml of blood obtained, $60–80 \times 10^6$ purified lymphocytes were recovered.

Purity of the lymphocyte suspensions was determined for each experiment by morphologic criteria. In numerous instances the lack of phagocytosis of heat-killed *C. albicans* (method described above) was used as an additional criterion for identification of lymphocytes.

The viability of many of the lymphocyte preparations was determined by the trypan blue exclusion test. 0.1 ml of each lymphocyte cell suspension was added to 0.1 ml phosphate-buffered saline and 0.1 ml 1.5% trypan blue solution. After 5 min the cells were examined microscopically for the presence of blue nonviable cells.

Preparation of fibroblasts. Sterile punch biopsies of skin were taken with the aid of Dr. Sharon Lantis, University of California in San Francisco. The skin was immersed in a few drops of Earle's balanced salt minimal essential medium with 20% fetal calf serum, and the minced fragments were sandwiched between two coverslips in Leighton tubes for 15 min. 1 ml of serum-containing medium was added to each tube and the cultures gassed with 5% CO_2 and sealed. Every 3 days, half the medium was decanted and replaced with fresh medium until 2 wk, when the cultures were divided and subcultured. 1 ml of 0.25% pronase solution Calbiochem, Los Angeles, Calif.) was added to each Leighton tube, followed shortly by 1 ml of medium with 20% fetal

calf serum. The cultures were washed and subdivided until the desired number of Leighton tubes was obtained, usually within 1 month. They were adapted to growth in 30% AB serum McCoy's medium just before use in these experiments.

Preparation of HeLa cells. HeLa cells growing in suspension were obtained from Dr. Leon Levintow, University of California in San Francisco, and adapted to growth in 30% AB serum McCoy's medium at a concentration of 2×10^5 cells per ml in Leighton tubes.

Preparation of mouse macrophage cultures. 5 ml of 30% AB serum McCoy's was injected intraperitoneally into six white Swiss mice. The peritoneal fluid containing 64×10^6 cells, 16% of which were monocytes, was collected and spun at 1200 rpm for 10 min at room temperature in a siliconized 40 ml centrifuge tube. The cell button was washed and resuspended in 10 ml of 30% AB serum McCoy's medium, and Leighton tube cultures were prepared with 1×10^6 monocytes per ml. The cultures were maintained at 37°C for 1 wk, during which time the medium was changed once and the cells washed three times with warm McCoy's medium.

Preparation of combined cultures. 1 ml of lymphocyte suspension in 30% AB McCoy's was added to each 1 ml culture of either macrophages, fibroblasts, HeLa cells, or mouse macrophages in Leighton tubes. The final concentration of lymphocytes in most combined cultures was 0.9×10^6 per ml, and macrophages $0.5–1.0 \times 10^5$ per ml. All combined human lymphocyte-macrophage and lymphocyte-fibroblast cultures were syngeneic.

Preparation of parabiotic chamber cultures. For each experiment sets of parabiotic chambers Bellco Glass, Inc., Vineland, N. J.) were set up, some of which had 0.45 μ Millipore filters interposed between the two sides. The chambers were set up in duplicates as follows:

Lymphocytes + PHA –Filter –Medium + PHA

Lymphocytes + PHA –Filter –Macrophages + PHA

Lymphocytes + macrophages + PHA –No filter–Lymphocytes + macrophages + PHA

Medium + PHA –Filter –Macrophages + PHA

Harvesting techniques. Cultures were harvested from 1 to 92 hr after addition of PHA-P. Thymidine-^{3}H (New England Nuclear Co., Boston, Mass.; specific activity 2.1 Ci/mmole, Lot No. 368-193 and No. 444-239) was diluted with sterile phosphate-buffered saline to 10 μCi/ml, and 0.1 ml of this solution was added to each 2 ml Leighton tube culture 1 hr before the termination of each culture. At the time of harvesting, the contents of each Leighton tube were transferred to sterile centrifuge tubes and spun at 1000 rpm for 10 min in an International PR-2 Centrifuge at 5°C. The supernatants were stored in sterile plastic tubes at 5°C for future interferon assay. The Leighton tubes were rinsed twice with 3 ml of cold, phosphate-buffered saline, and the rinses were added to the cell buttons in the centrifuge tubes. The contents of the centrifuge tubes were then spun at 1000 rpm for 10 min at 5°C and the cell buttons washed again with 6 ml of cold phosphate buffer. The cell buttons were frozen for future determinations of thymidine-^{3}H incorporation into DNA.

To rule out the possibility that significant numbers of thymidine-^{3}H-labeled cells remained in the Leighton tubes after the washing procedure, some tubes with their coverslips were frozen for future studies of thymidine-^{3}H incorporation into DNA. The coverslips from other Leighton tubes were air dried and fixed in absolute methyl alcohol (MeOH) for eventual radioautographic studies.

Method for determination of thymidine-^{3}H incorporation into DNA. The frozen cell buttons or Leighton tubes were thawed and washed twice with cold 5% trichloroacetic acid (TCA) and twice with cold absolute MeOH. The precipitate was dissolved in 0.5 ml Nuclear-Chicago solubilizer 1:3 in toluene (Nuclear-Chicago, Chicago, Ill.) and transferred in 10 ml of scintillation fluid to glass counting vials. The scintillation fluid was prepared with 3.79 liters of toluene, 15.16 g of 2,5-diphenyloxazole, and 0.189 g of 1,4-bis-[2-(5-phenyloxazolyl)-benzene. All samples were counted in a Packard Tri-Carb Liquid Scintillator for 10 min, and suitable corrections were made for background and quenching if necessary.

Radioautography. Coverslips attached with Permount and placed face up on microscope slides were dipped in NTB-2 liquid emulsion (Eastman Kodak Co., Rochester, N. Y.) Radioautograms were exposed for 1 wk, developed, and stained according to the procedure of Pasanen and Epstein (9).

Interferon assay. A plaque reduction assay employing human neonatal foreskin fibroblasts and bovine vesicular stomatitis virus (10) was used for interferon assay. Interferon titer was defined as that dilution of sample which, in a 4 ml volume, produced 50% reduction in the number of plaques. In most cases triplicate samples, but occasionally duplicates, were run for each type of culture. Control culture supernatants with medium or medium plus PHA were also tested. At least three separate dilutions of each sample were run along with the controls for the interferon assay, i.e., fibroblast monolayers with virus and no sample to be tested, and fibroblast monolayers with virus and standard interferon dilutions.

RESULTS

General observations on the cultures. The macrophage cultures used in these experiments resulted from the growth and differentiation of glass-adherent monocytes. Elimination of lymphocytes from these cultures was achieved by repeated washing of the monolayers at both the time of initiation and the time of refeeding of the cultures. Spontaneous degeneration of polymorphonuclear leukocytes occurred within the first few days, long before the cultures were used in these experiments. At the end of 7 days most cultures contained 99–100% macrophages, with eosinophils comprising the other 1%. The macrophages were identified by their characteristic eccentric nuclei, abundant, foamy, pale-staining cytoplasm, and large size (see Fig. 1 A). Further indication of the purity of these macrophage preparations was the fact that there was no increase in incorporation of thymidine-^{3}H into DNA of macrophage cultures with the addition of PHA. The macrophages phagocytized heat-killed *C. albicans* particles avidly, as evidence of their viability. In the samples tested, greater than 98% of the cells had phagocytized one or more particles.

The lymphocytes isolated by their passage through a sterile nylon fiber column were uniformly 99.5–100% pure (see Fig. 1 B). The remaining 0.5% were either degenerating polymorphonuclear leukocytes, monocytes, or metamyelocytes. Of the identifiable lymphocytes, 98% were small or medium sized with characteristic dense, heterochromatic nuclei and scant cytoplasm; 2% were large lymphocytes. Studies with trypan blue indicated that 99.8–100% of the lymphocytes were viable after passage through the nylon fiber column. None of the cells identified as lymphocytes phagocytized heat-killed *C. albicans*.

When freshly isolated lymphocytes were combined with 7-day-old macrophage cultures from the same donor, no morphologic changes were noted in either cell type for periods of up to 4 days.

Interferon titers in cultures prepared without PHA. In 21 macrophage, 36 lymphocyte, and 20 combined syngeneic macrophage-lymphocyte cultures prepared without PHA, no interferon (titer < 3) was produced in up to 92 hr of culture. Microscopic examination of the cultures indicated no evidence of transformation of small lymphocytes to large, blast-like cells; this was confirmed by radioautography and scintillation counting in which there was virtually no incorporation of thymidine-^{3}H into DNA.

Interferon titers in macrophage cultures prepared with PHA. No interferon was found in 23 cultures of macrophages grown in the presence of PHA for 20–92 hr.

Temporal sequence of interferon production. Two pilot experiments were performed to determine whether lymphocytes cultured in the presence of PHA could produce interferon and whether the addition of macrophages to lymphocytes in the presence of PHA would affect interferon production. Furthermore, by harvesting the cultures at various intervals from 20 to 92 hr, the temporal sequence of interferon production could be observed. The results are shown in Fig. 2, in which each point represents the mean for duplicate or triplicate cultures. In the presence of PHA a small amount of interferon was produced by lymphocytes alone. The addition of macrophages to the PHA-containing lymphocyte cultures resulted in a marked augmentation of interferon production, the peak of which occurred at 68 hr.

When the macrophages were combined with lymphocytes in the presence of PHA, typical rosettes such as the one found in a 68 hr culture and illustrated in Fig. 1 C resulted. The lymphocytes, in all stages of transformation to large, blast-like cells, were in intimate contact with the central macrophages and with each other as well.

Comparison of amount of interferon produced in lymphocyte and combined syngeneic lymphocyte-macrophage cultures with PHA. To determine the extent of the macrophage effect, studies with the blood of 13 additional normal donors were performed, and all cultures were harvested at 68 hr. Each of the points in Fig. 3 represents the mean value of triplicate and occasional duplicate cultures for each donor. In 12 of the 13 donors studied, combined syngenic lymphocyte-macrophage cultures with PHA produced more interferon than just lymphocytes with PHA. The mean titer observed in the PHA-stimulated lymphocyte cultures was 35 and that of the combined cultures 173, a fivefold increase. Using

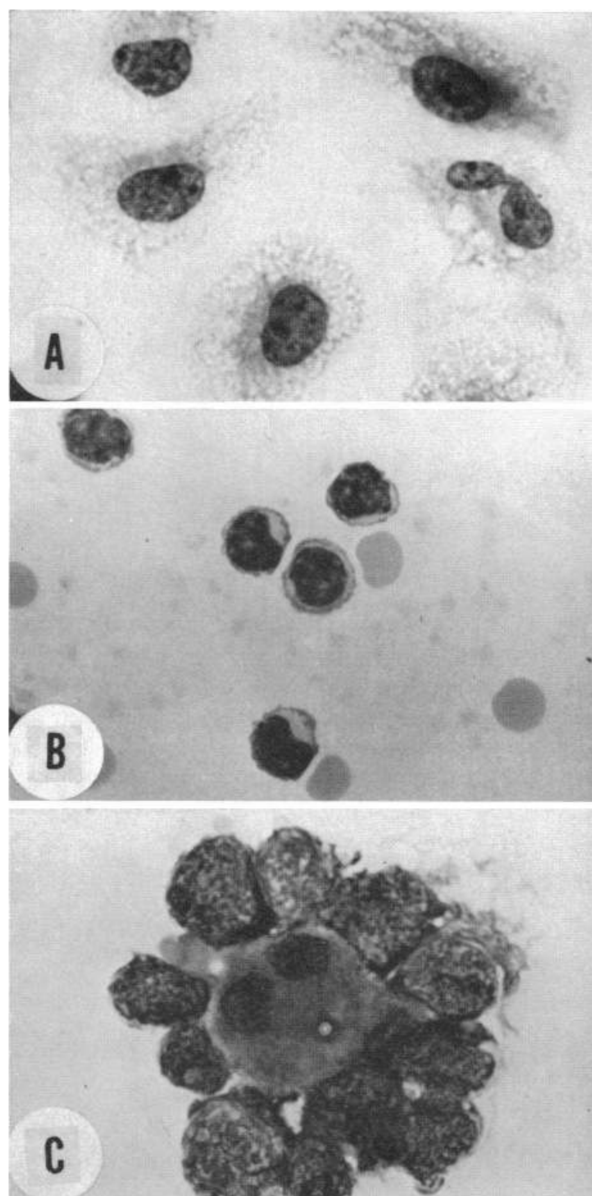

FIGURE 1 (A) Pure macrophage culture without PHA. (B) Pure lymphocyte suspension without PHA. (C) Rosette from combined syngeneic lymphocyte-macrophage culture with PHA after 68 hr in culture. × 1250.

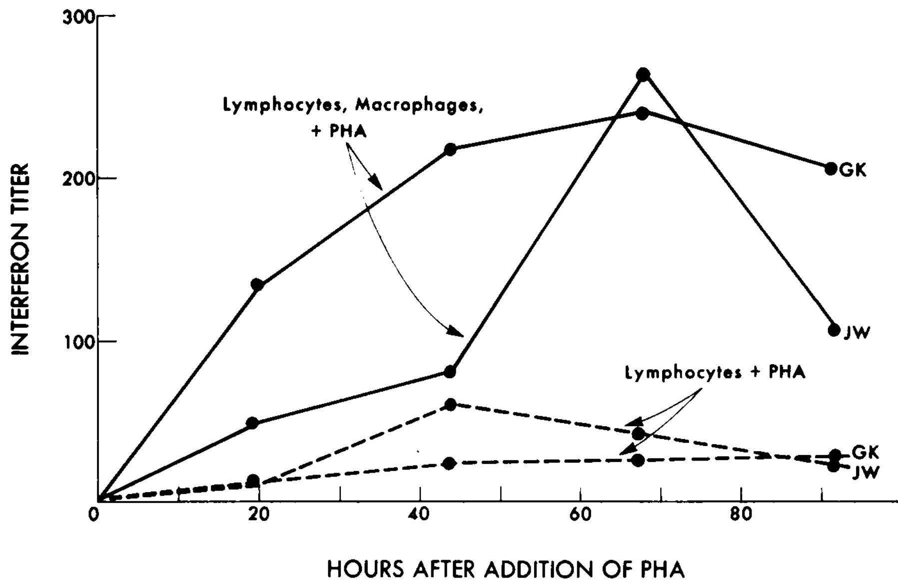

FIGURE 2 Time course study of interferon production in cultures of lymphocytes, and syngeneic lymphocytes and macrophages with PHA.

the Wilcoxon signed rank test (11), this difference between the two groups was found to be highly significant ($P < 0.01$). Although the same number of lymphocytes and roughly the same number of macrophages were employed in all of the combined cultures, the amount of interferon produced varied greatly from donor to donor.

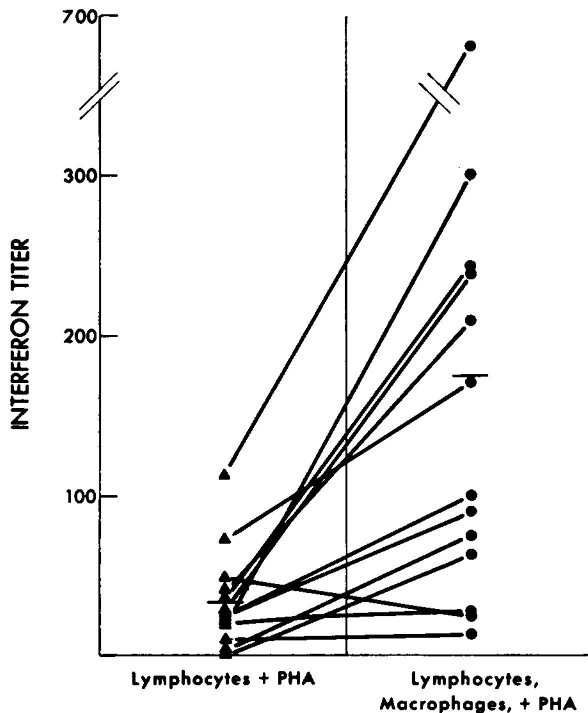

FIGURE 3 Comparison of interferon titers in cultures of lymphocytes with PHA, and syngeneic lymphocytes and macrophages with PHA.

Comparison of interferon titers with degree of transformation. The cells remaining at the time of harvesting the cultures from the preceding experiments on 13 donors were studied for their incorporation of thymidine-^{3}H into DNA. The extent of incorporation reflects the degree of transformation of small lymphocytes into large, blast-like cells. The results for the 68-hr samples are shown in Fig. 4. Each point represents the mean value of triplicate and occasional duplicate cultures, each initiated with 1.8×10^6 lymphocytes. It was apparent that the presence of macrophages did not always increase the amount of transformation. The mean for cultures with lymphocytes and PHA was 52,157 cpm; and for combined lymphocyte-macrophage cultures with PHA it was 60,169 cpm. The difference between the two groups is not statistically significant and confirms similar observations made by Hersh and Harris (12). The wide variation in the ability of each donor's lymphocytes to respond to PHA was again apparent.

Although all cultures producing interferon showed some degree of transformation, no direct statistical correlation between the degree of PHA-induced lymphocyte transformation and the amount of interferon produced was observed. For some donors, the presence of macrophages did not necessarily enhance transformation; the presence of macrophages did, however, consistently enhance interferon production.

Physical and chemical characterization of the interferon produced in combined lymphocyte-macrophage cultures with PHA. Numerous studies were performed to determine whether the interferon produced in the combined lymphocyte-macrophage cultures with PHA had similar physical-chemical properties as human interferon produced in other situations. The PHA-stimulated interferon was not sedimented by centrifugation at 100,000 *g* for 1 hr, but was susceptible to the action of trypsin and was partially inactivated by heating for 1 hr at 56°C. Considerable activity was lost after exposure of the supernatants to pH 2 for 18 hr, as has also been previously observed by Wheelock (5). The virus-protective effect of the supernatants from combined lymphocyte-macrophage cultures with PHA was prevented by shortening to 2 hr the period in which the supernatants were allowed to act on the fibroblast monolayers. The usual period that the supernatants were left in contact with cells was 18–24 hr, during which time the interferon in the supernatants had sufficient time to exert its full antiviral effect. Incubation of the culture supernatants with bovine vesicular stomatitis virus had no effect on the plaque-forming ability of the virus, thus supporting the concept that interferon acts not by direct action on the virus but by conferring resistance to virus infection on cells. The interferon produced in the combined lymphocyte-macrophage cultures with PHA was species specific; it had no protective effect on mouse cell monolayers against the same challenge virus.

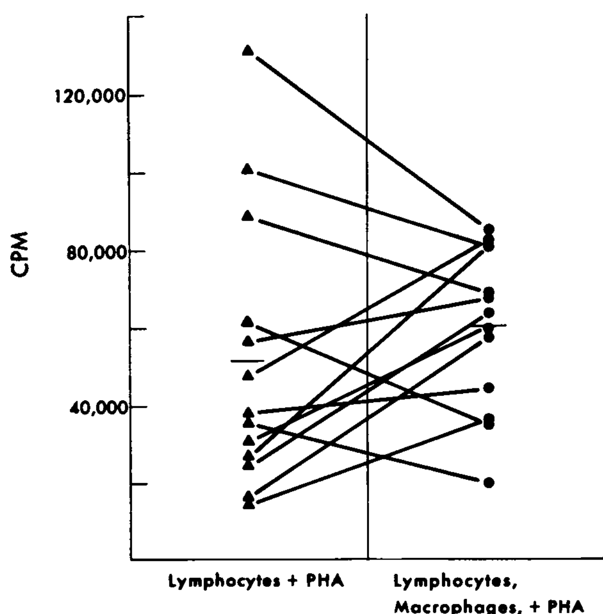

FIGURE 4 Comparison of incorporation of thymidine-^{3}H in cultures of lymphocytes with PHA, and syngeneic lymphocytes and macrophages with PHA.

TABLE I

*Effect of Lymphocyte and Macrophage Concentration on Interferon Titer in Combined Syngeneic Lymphocyte-Macrophage Cultures with PHA**

	Macrophages	Lymphocytes	Interferon titer
	cells/ml	*cells/ml*	
Experiment 1‡	0	0.45×10^6	11
	0	0.90×10^6	17
	0	1.80×10^6	69
	0.5×10^5	0.45×10^6	24
	0.5×10^5	0.90×10^6	48
	0.5×10^5	1.80×10^6	112
Experiment 2§	0.5×10^5	0.9×10^6	108
	1.0×10^5	0.9×10^6	171
	1.5×10^5	0.9×10^6	223
Experiment 3‖	1.4×10^5	0.9×10^6	680
	2.1×10^5	0.9×10^6	800
	2.8×10^5	0.9×10^6	680

* All cultures were harvested 68 hr after the addition of PHA.
‡ Each value represents the mean of six samples.
§ Each value represents the mean of two samples.
‖ Each value represents the mean of three samples.

Conditions in combined syngeneic lymphocyte-macrophage cultures which affect interferon production. Combined lymphocyte-macrophage cultures with PHA were prepared with a fixed concentration of macrophages and an increasing concentration of lymphocytes, as is shown in the data from experiment 1 in Table I. The counts on the macrophage cultures were obtained from parallel cultures of macrophages set up specifically for the purpose of counting. They were removed from the glass with a concentrated EDTA solution at elevated temperatures, as outlined in the section on methods. As can be seen from Table I, an increase in the concentration of lymphocytes from 0.45 to 1.8×10^6 per ml when the number of macrophages was kept constant or omitted resulted in an increase in the amount of interferon produced.

The effect of increasing the concentration of macrophages on interferon production was also examined. Cultures with increasing numbers of macrophages, verified by counting additional parallel cultures, were prepared by varying the initial inoculum of monocytes. The results, depicted in the lower portion of Table I, indicate that an increase in the concentration of macrophages from 0.5 to 2.1×10^5 per ml when the number of lymphocytes was kept constant resulted in an increase in the amount of interferon.

Three experiments were performed to determine whether PHA was necessary throughout the duration of the combined cultures for maximum interferon response and also to determine whether medium from cul-

tures of macrophages could substitute for the intact cells. The cultures, all run in duplicate or triplicate, were harvested at 92 hr in the first experiment and at 68 hr in the second and third. The design of the experiments was to pretreat the macrophage monolayers with PHA for 4 or 24 hr, collect the supernatants, and wash the macrophages six times with warm medium. Then the supernatants containing any residual PHA were added through a 0.45 μ Millipore filter to freshly isolated lymphocytes from the same donor, incubated for an additional 3 or 4 days, and harvested. Also, the macrophages washed six times were added to additional freshly prepared lymphocyte cultures. Appropriate controls with medium incubated with PHA for 4 or 24 hr, but with no exposure to macrophages, were also included. The results indicated that once PHA was removed from the cultures by thorough washing of the macrophages, no augmentation of interferon occurred, despite a prior exposure of the macrophages to PHA for 4 or 24 hr. Furthermore, the data indicated that medium from macrophages could not substitute for the intact cell in augmenting the production of interferon by cultures of lymphocytes with PHA.

To rule out the possibility that something produced by the lymphocytes in the presence of PHA was stimulating the macrophages in combined culture to produce interferon, two experiments were performed. Lymphocytes were pretreated with PHA for 4 or 24 hr; the supernatants were collected and then added through a 0.45 μ Millipore filter to cultures of macrophages. The lymphocytes were washed six times with warm medium and transferred to additional macrophage cultures. The results of these experiments confirmed the fact that if PHA is removed from the cultures, no augmentation of interferon production results. Supernatant fluids from PHA-stimulated lymphocytes could not substitute for the intact cell in the combined cultures.

Fig. 5 depicts the results from three experiments prepared to determine whether physically intact macrophages were necessary to augment interferon production in cultures of lymphocytes plus PHA. Cultures were prepared with lymphocytes alone, lymphocytes plus macrophages which were frozen-thawed 12 times to disrupt them totally, and lymphocytes plus intact macrophages—all in the presence of PHA. The PHA was added after the macrophages were frozen-thawed. The results demonstrated that frozen-thawed macrophages could augment the response of lymphocytes alone but to a lesser degree than intact macrophages. Frozen-thawed macrophages alone or with PHA did not produce interferon in culture. Microscopically the cultures with lymphocytes, frozen-thawed macrophages, and PHA showed numerous clumps of cells, often with fragments of macrophages and numerous lymphocytes in intimate contact.

In two experiments in which Millipore filters were interposed between lymphocytes and macrophages in parabiotic chambers, both in the presence of PHA, no interferon was found. Interferon could be detected only in two of four parabiotic chambers in which the Millipore filters were removed, thereby allowing the lymphocytes and macrophages intimate cell-to-cell contact in the presence of PHA.

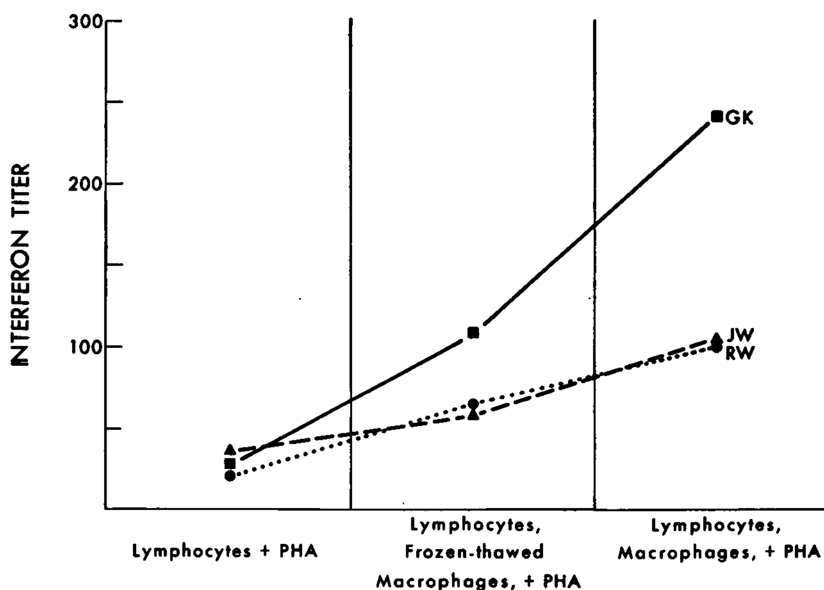

FIGURE 5 Comparison of effect of frozen-thawed and intact macrophages on interferon production by cultures of lymphocytes with PHA. G.K., J.W., and R.W. are the initials of the donors.

TABLE II

Effect of Syngeneic Fibroblasts and Macrophages on Thymidine-^3H Incorporation and Interferon Titer of Lymphocyte Cultures with PHA

Components of cultures	Experiment 1		Experiment 2	
	cpm $\times 10^{-4}$	IF titer	cpm $\times 10^{-4}$	IF titer
Lymphocytes + macrophages + PHA	11.07	181	3.40	293
Lymphocytes + macrophages	0.12	10	0.07	10
Macrophages + PHA	0.07	10	0.01	10
Macrophages	0.01	10	0.02	10
Lymphocytes + PHA	6.65	57	5.38	10
Lymphocytes	0.02	10	0.01	10
Lymphocytes + fibroblasts + PHA	1.26	31	0.39	10
Lymphocytes + fibroblasts	1.29	10	0.23	10
Fibroblasts + PHA	0.66	10	0.33	10
Fibroblasts	0.56	17	0.14	10

Specificity of macrophage in augmenting interferon production by cultures of lymphocytes with PHA. Cultures were prepared in the following combinations: either lymphocytes, macrophages, or fibroblasts alone or with PHA, and lymphocyte-macrophage or lymphocyte-fibroblast cultures with or without PHA. Cell cultures were prepared in triplicate, occasionally in duplicate, and were harvested at 68 hr. The results of two such experiments with syngenic fibroblasts are depicted in Table II. No augmentation of interferon production was noted in combined lymphocyte-fibroblast cultures with PHA as compared with cultures of just lymphocytes with PHA. The degree of thymidine-^3H incorporated by lymphocytes with PHA was markedly depressed by the presence of fibroblasts. Radioautograms indicated that the incorporation of thymidine-^3H into DNA by fibroblasts and lymphocytes in combined cultures with PHA was considerably depressed as compared with each cell type by itself with PHA. In the presence of PHA the lymphocytes were in intimate contact with the fibroblasts and aggregrated all over their surfaces. Without PHA no aggregation or intimate contact was noted.

Similar experiments performed with HeLa cells and mouse macrophages also demonstrated that these two cell types could not substitute for human macrophages in augmenting interferon production by cultures of human lymphocytes. Assay of the culture supernatants from the combined human lymphocyte-mouse macrophage plus PHA cultures on mouse monolayers indicated that no mouse interferon was produced. This was further evidence that the macrophage itself does not produce the interferon in combined PHA-stimulated cultures.

DISCUSSION

These experiments demonstrated that the addition of macrophages to cultures of lymphocytes with PHA greatly augments interferon production. A fivefold increased occurred over that observed in cultures with just lymphocytes and PHA. The interferon produced in the combined syngeneic lymphocyte-macrophage cultures with PHA had similar physical and chemical properties to that induced by Newcastle disease virus in cultures of human leukocytes (5).

The identification of the cell which produces interferon in the combined cultures is tentative. However, because of the fact that pure lymphocytes alone in the presence of PHA can produce small amounts of interferon whereas pure preparations of intact or frozen-thawed macrophages cannot produce or release interferon, it is likely that it is the lymphocyte which is producing interferon in the combined cultures as well. Whether all lymphocytes or only a few produce interferon is yet to be determined.

The specificity of the macrophage in its ability to augment interferon production in this system was indicated by the results of the experiments in which either syngeneic fibroblasts, HeLa cells, or mouse macrophages failed to substitute for the human macrophages in combined cultures with PHA.

Several observations were made which help define the role of the macrophage in augmenting interferon production in cultures of lymphocytes with PHA. Intimate contact between the macrophages and lymphocytes was necessary for a maximum interferon response, as was demonstrated by the parabiotic chamber culture experiments. Moreover, examination of the PHA-stimulated, combined lymphocyte-macrophage cultures revealed the presence of numerous rosettes in which lymphocytes were in intimate contact with macrophages and with each other. The macrophages required the continuous presence of PHA throughout the duration of culture for maximum interferon response. Apparently no soluble substance was elaborated by the macrophages in the presence of PHA that could substitute for the macro-

phages in the combined lymphocyte-macrophage cultures with PHA. Frozen-thawed macrophages in combination with lymphocytes and PHA gave a somewhat better interferon response than just lymphocytes plus PHA, but not as marked an interferon increase as intact macrophages.

Exactly how the macrophage produces the increase in interferon titers in the combined cultures with PHA is not known. One possibility is that the membrane of the macrophage, whether intact or not, binds PHA and that the resulting PHA-membrane complex is capable of stimulating interferon production by the lymphocytes to a greater extent than is PHA alone.

Certain observations are pertinent to the relationship between interferon production and blastogenic transformation. Interferon production was always associated with transformation and was never observed in those cultures prepared without PHA; however, the amount of interferon produced in the combined lymphocyte-macrophage cultures with PHA was not related to the extent of transformation as estimated by the incorporation of thymidine-^{3}H into DNA. As long as some transformation occurred, interferon could be produced. The work of Green, Cooperband, and Kibrick (7) parallels these observations. In their studies of antigen-induced interferon production, they found less transformation in antigen-stimulated leukocyte cultures than in those stimulated with PHA; yet oftentimes the amount of interferon produced in antigen-stimulated cultures was greater than that from cultures stimulated with PHA. Concentrations of antigen too low to stimulate blast transformation would not induce interferon production.

The observations presented here raise two major questions of general biologic interest. The first is whether the macrophage-lymphocyte interaction to produce interferon is in any way related to the better-known and well studied macrophage-lymphocyte interactions in cellular immunity and antibody production. Macrophage-lymphocyte interactions are known to occur in certain in vitro model systems. Highly purified, immune lymphocyte preparations show little blastogenic response to antigen; addition of macrophages enhances the blastogenic response (8, 12). Also, surface-adherent cells, presumably macrophages, are required for antigen-stimulated antibody production in clusters of spleen cells in vitro (13). Furthermore, interferon production itself, induced either by viruses or by other agents, has been related in several ways to the immune response (for review see DeClercq and Merigan, reference 14). For example, nonviral antigens—PPD, tetanus toxoid, and diphtheria toxoid—can induce interferon production in in vitro leukocyte cultures prepared from immune donors (7). Glasgow (15) demonstrated that peritoneal leukocytes from an animal immune to a given virus produced more interferon when incubated with that virus in vitro than did cells from a nonimmune animal. The possibility of an immune-type "recall" phenomenon for interferon is suggested by the latter study.

The second and more important question of general biologic interest is whether interferon production which occurs in association with the transformation of lymphocytes in vitro is a significant defense mechanism in vivo. Transformation-associated interferon production can occur in vivo.[1] Low levels of interferon were observed in mice for several days after intravenous injection of PHA. The kinetics of response roughly paralleled the transformation of lymphocytes noted in lymph nodes of these animals (16). More recently, Stinebring and Absher have observed immune-specific interferon production in vivo (17). Mice infected with *Mycobacterium tuberculosis,* strain BCG, produced interferon which reached a peak 6 hr after intravenous challenge with tuberculin.

If transformation-associated interferon production is an important mechanism of host defense, then an explanation might be provided for the unusual severity and increased frequency of viral infections in patients with impairment of lymphocyte transformation and delayed hypersensitivity (18).

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