

# HUMAN MONOCYTES AND MACROPHAGES

## Interaction with Antigen and Lymphocytes

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### ABSTRACT

PPD-sensitized monocytes and macrophages from tuberculin-positive subjects are both capable of inducing blastogenic transformation of autologous lymphocytes. Incorporation of thymidine-<sup>3</sup>H and morphological transformation were always greater in lymphocyte cultures containing macrophages than in those containing monocytes. More lymphocytes entered the first detectable S phase in cultures containing macrophages. Lymphocyte DNA synthesis occurred as early as 40 hr of culture and always in cells in contact with mononuclear phagocytes. By 120–144 hr, many transformed lymphocytes were free in suspension; at the same time, the “immunological cluster” had increased greatly in size and contained transformed and untransformed lymphocytes. The greater effectiveness of macrophages at induction of lymphocyte transformation may be related to the efficiency of this cell type at trapping antigen and its effectiveness at making contact with and binding lymphocytes.

### INTRODUCTION

The hypothesis that phagocytic mononuclear cells participate in the initial processing of antigen in the immunological response is supported by several observations: (a) Pure populations of lymphocytes containing no mononuclear phagocytes show a considerably reduced response to specific antigens *in vitro*; addition of phagocytes restores the response to normal levels (1); (b) RNA which contains trace amounts of antigen prepared from animal macrophages can induce lymphocytes to produce the corresponding antibody (2–5); (c) human monocytes exposed to antigen *in vitro* and then washed free of extracellular antigen can induce transformation of autologous lymphocytes (6); (d) normal nonimmune lymphoid cells can induce antibody synthesis in X-irradiated recipient animals if living macrophages containing antigen are also transferred (7).

The concept of maturation from the blood monocyte to the larger macrophage and multinucleate giant cell derives from histological ob-

servations dating from the beginning of this century (8). This concept has been strengthened by the detailed cytochemical, morphological, and functional studies of Cohn and his collaborators (9, 10). We have shown that under *in vitro* conditions human macrophages are faster and more efficient phagocytes than are the antecedent monocytes (11). It seemed reasonable to ask whether there are also differences in the interaction of these cells with lymphocytes in the immunological response. In this article, we report a comparison between human monocytes and macrophages in mediating antigen-induced stimulation of autologous lymphocytes.

### MATERIALS AND METHODS

#### *Preparation and Cultivation of Cells*

Leukocytes were obtained from the heparinized venous blood of tuberculin (PPD)-positive human subjects. Lymphocytes were isolated in glass-wool

columns as previously described (6), and were suspended at a final concentration of  $10^6$ /ml in McCoy's medium containing 30% human AB serum (ABM). The purity of the lymphocyte preparations ranged from 74 to 98%, with a mean of 86%.

Monocytes were isolated by a slight modification (6) of the method of Bennett and Cohn (12), and  $1$  or  $3 \times 10^6$  cells were added to Leighton tubes in 1 ml of ABM. Cells cultivated for 11–14 days gave rise to macrophages.

The term "macrophages" is used here to describe cells which have matured from blood monocytes and have the following characteristics: (a) cell diameters of 50–200  $\mu$ ; (b) prominent cytoplasmic extensions, at times several cell diameters in length; (c) single or multiple nuclei, round or oval in shape and having prominent nucleoli; (d) large numbers of phase-dense granules which fill the cytoplasm; (e) ribbon-like mitochondria, frequently seen in the cytoplasmic ruffle; (f) phagocytic capacity for a wide variety of particles.

Macrophages were, in general, cultured for 11–14 days before initiation of experiments. Monocytes were used on the day of isolation. Macrophage and monocyte cultures had, on the average, 99 and 94% phagocytic cells, respectively, with *Candida albicans* as the test particle.

Because macrophages cannot be removed from the glass surface to which they are adherent without seriously impairing their metabolic and functional status, their numbers were approximated by counting cells in a known area of glass; this area was compared to a similar area containing a known number of monocytes. The number of macrophages in a 1-ml Leighton tube averaged  $1 \times 10^5$ , with a range of approximately  $0.5$ – $4 \times 10^5$ . For these studies,  $1 \times 10^6$  monocytes were routinely used; this number produces maximal stimulation of lymphocytes under the conditions employed (6).

### *Interaction of Lymphocytes and Mononuclear Phagocytes*

Methods for studying the interaction between lymphocytes and mononuclear phagocytes were similar to those previously described for human monocytes (6). In brief, glass-adherent monocytes or macrophages were incubated with either saline or purified protein derivative (PPD, Parke, Davis & Co., Detroit, Mich., 2.5  $\mu$ g/ml final concentration) for 4 hr at 37°C. The cells were washed seven times to remove unattached antigen, and lymphocytes in a volume of 3 ml were added to the mononuclear cells. After incubation for 1–6 days, lymphocyte transformation was quantitated morphologically and by measurement of thymidine- $^3$ H incorporation (6).

Tubes containing no monocytes or macrophages were included as controls to determine adherence of PPD to the glass surface of the containers. In addition, parallel cultures of  $3 \times 10^6$  lymphocytes and various numbers of phagocytes were incubated in continuous contact with PPD (2.5  $\mu$ g/ml) for 1–6 days in order to determine direct lymphocyte transformation by antigen.

### *Uptake of Radioactive PPD by Mononuclear Phagocytes*

PPD was radioiodinated with  $^{125}$ I by the chloramine-T method of Hunter and Greenwood (13). Labeled proteins were separated from unbound  $^{125}$ I by passage through Dowex AG-1-X2 (Dow Chemical Co., Midland, Mich.) ion-exchange columns and diluted with 3% bovine serum albumin in 0.05 M Tris buffer (pH 7.5). The radioactivity of these preparations was 85–95% precipitable with 10% trichloroacetic acid.<sup>1</sup>

To insure that the  $^{125}$ I-labeled PPD was not altered in its antigenicity by the iodination procedure, lymphocytes from a tuberculin-sensitive individual were incubated for 5 days with 0.5  $\mu$ g/ml of iodinated PPD. At 5 days, the degree of morphological transformation was determined; it was similar to that produced by uniodinated PPD (14).

$^{125}$ I-PPD, 25  $\mu$ g (specific activity  $1.64 \times 10^4$  cpm/ $\mu$ g), was added to T-flasks containing  $0.9$ – $2.4 \times 10^6$  monocytes or macrophages and incubated for 30 min. The flasks were then washed by decantation with warm Hanks' solution until no radioactivity was detectable in the wash fluid; the cells were then removed by either of two procedures: (a) Cold saline was added, and the flasks were chilled for 10 min before scraping with a rubber scraper; or (b) EDTA in phosphate-buffered saline was added in a final concentration of 3.3 mg/ml, and the cells were incubated at 40°C for 30 min; the cells were then quantitatively removed by gentle scraping.

The cells were suspended in a known volume of saline, counted in a hemocytometer, and radioactivity was determined in an Auto-Gamma spectrometer (Packard Instrument Co., Downers Grove, Ill.) with Na iodide crystal. Less than 2% of the radioactivity was not bound to cells. Both methods gave similar results.

### *Radioautography*

Radioautographic studies of thymidine- $^3$ H incorporation into lymphocytes and mononuclear phagocytes were performed as previously described (11).

<sup>1</sup> We are grateful to Dr. Sydney Salmon, who supervised the iodination procedure.

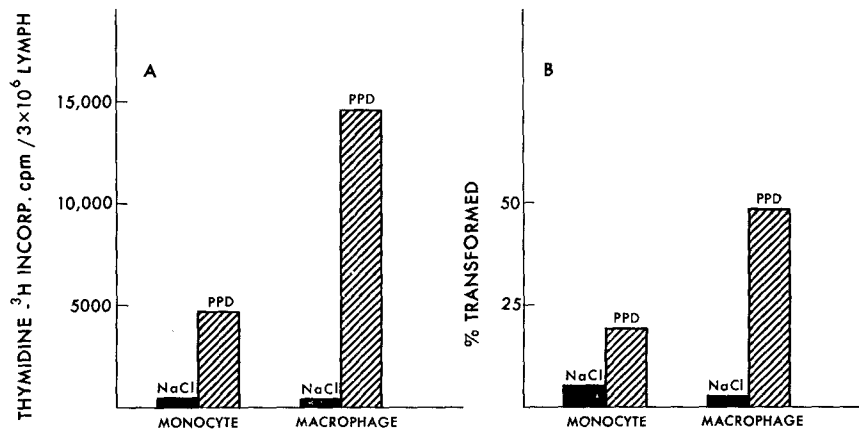


FIGURE 1 Incorporation of thymidine-<sup>3</sup>H (A) and morphological transformation (B) of lymphocytes from a tuberculin-positive subject. Lymphocytes were incubated for 6 days with autologous monocytes or macrophages previously exposed to saline solution (NaCl) or PPD and then washed.

## RESULTS

### *Transformation of Lymphocytes by Antigen-Sensitized Monocytes and Macrophages*

We have shown previously that monocytes from tuberculin-positive subjects, when exposed to PPD and washed free of extracellular antigen, can induce "blastogenic" transformation of autologous lymphocytes (6). Lymphocytes from tuberculin-negative subjects do not transform. Neutrophils cannot substitute for monocytes. Other glass-adherent cells in culture could not be examined in this way because of antigenic differences from the test lymphocytes. This technique, when applied to macrophages grown from blood monocytes, produced the results shown in Figs. 1 and 2. The incorporation of thymidine-<sup>3</sup>H and the number of morphologically transformed lymphocytes was increased above control values in cultures containing antigen-sensitized mononuclear phagocytes. This stimulation was greater in 6-day cell cultures containing antigen-sensitized macrophages than in those containing sensitized monocytes. The difference between monocytes and macrophages was independent of the concentration of PPD in the range of 0.5–10  $\mu$ g/ml.

In order to exclude the possibility that macrophages or monocytes had incorporated thymidine-<sup>3</sup>H, radioautographs were prepared 8 hr after the addition of isotope to cultures in the 2nd through the 6th day after addition of lymphocytes. In every instance, grains were localized over the nuclei of cells morphologically identifiable as

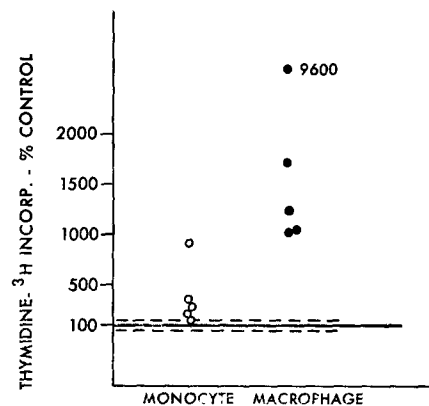


FIGURE 2 Transformation of lymphocytes from five tuberculin-positive subjects induced by autologous PPD-sensitized monocytes or macrophages. Thymidine-<sup>3</sup>H incorporation is expressed as a percentage of saline-treated controls. The broken lines define the range of the controls.

transformed lymphocytes, and characterized by round nuclei with prominent, large, blue nucleoli and deep blue cytoplasm (Fig. 3). Less than 0.2% of macrophages had demonstrable uptake of the radioactive label. Radioautography was, therefore, used to study the interaction between lymphocytes and antigen-sensitized macrophages. This interaction had the following characteristics.

**TIME COURSE OF THYMIDINE-<sup>3</sup>H INCORPORATION:** Incorporation of thymidine-<sup>3</sup>H by lymphocytes was first detectable after 36–48 hr of culture with sensitized macrophages and increased progressively thereafter through day 6 (Table I).

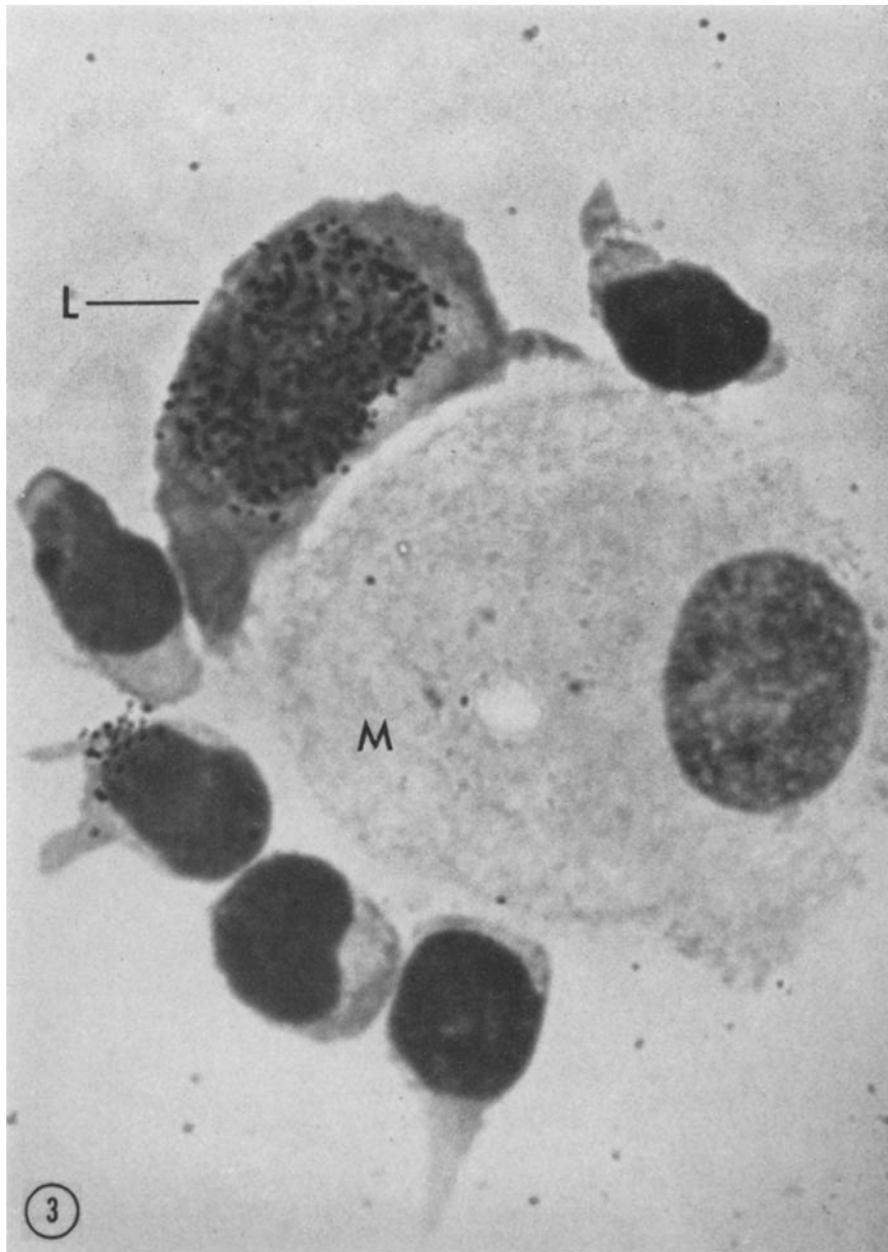


FIGURE 3 Radioautograph of thymidine-labeled, transformed lymphocytes (*L*) and untransformed lymphocytes surrounding a PPD-sensitized macrophage (*M*). Grains are localized to lymphocyte nuclei. Giemsa stain. Original magnification,  $\times 1250$ .

TIME COURSE OF MORPHOLOGICAL TRANSFORMATION: Morphologically identifiable transformation of Giemsa-stained lymphocytes did not occur before 60–72 hr of culture.

ATTACHMENT OF LYMPHOCYTES TO MAC-

ROPHAGES: During the early periods of culture (36–92 hr), only lymphocytes in direct contact with macrophages were labeled; free lymphocytes were unlabeled. By the late stages (120–144 hr), many labeled and morphologically

TABLE I  
Incorporation of Thymidine-<sup>3</sup>H by Lymphocytes Incubated with PPD-Sensitized Macrophages\*

Time of thymidine- <sup>3</sup> H pulse after addition of lymphocytes	Lymphocytes with labeled nuclei	
	Adherent to macrophages on glass	In suspension
hr	%	%
24-36	<0.1	<0.1
40-48	3.0 ± 0.3	<0.1
64-72	4.5 ± 1.0	0.5 ± 0.4
72-92	18.0 ± 4.0	2.7 ± 1.3
126-144		19.0 ± 10

\* The percentage of labeled nuclei among lymphocytes cultured with unsensitized macrophages was less than 0.3%.

transformed lymphocytes were free and unattached.

**SIZE OF CLUSTERS:** During the 1st and 2nd days of culture, one or two or, rarely, three lymphocytes were attached to a single macrophage. By the 5th day, the number of lymphocytes surrounding a single macrophage was usually greater than four and included both transformed and untransformed cells. At days 5-6, when the lymphocyte transformation was maximal, many of the cells were collected in "immunological islands" consisting of a central core of one or more macrophages and a periphery of thymidine-<sup>3</sup>H-labeled, transformed lymphocytes and unlabeled, untransformed cells (Fig. 4).

**GLASS ADHERENCE OF MACROPHAGES:** During the early stages of lymphocyte transformation, macrophages and associated lymphocytes were adherent to glass; by the 5th-6th day many of the macrophages had lost their cytoplasmic extensions, had become round, and had left the glass still associated with a cuff of lymphocytes (Figs. 3 and 4). A similar phenomenon was observed in cultures of lymphocytes with monocytes. By the 6th day of culture, most of the monocytes associated with transformed lymphocytes had left the glass surface of the incubation vessel. By this time, the monocytes had the morphological appearance of macrophages.

#### *Uptake of Antigen by Monocytes and Macrophages*

From a number of studies, including our own (6, 14), it is apparent that the transformation of

tuberculin-positive lymphocytes is dependent upon the concentration of PPD in the culture medium. Under the conditions of our experiments, when lymphocyte populations containing few phagocytes are continuously incubated in the presence of antigen, little enhancement of thymidine-<sup>3</sup>H incorporation occurs until the concentration of PPD exceeds 0.1  $\mu$ g/ml. Maximal stimulation is achieved at concentrations between 1 and 2.5  $\mu$ g/ml. Since the local concentration of PPD in the immediate vicinity of a sensitized lymphocyte may be important in the induction of transformation, it was of interest to compare the uptake of labeled PPD by monocytes and macrophages. Experiments were performed using leukocytes from three different subjects. All showed an approximately sixfold greater uptake of <sup>125</sup>I-PPD per cell by macrophages than by monocytes (Table II). In radioautographic studies of localization of label, grains were observed in macrophages over the nuclei, at the cell surface, and within vacuoles. The resolution provided by this technique was not sufficiently good to permit a statement about quantitative distribution of label.

#### *Analysis of Differences between Monocytes and Macrophages*

The increased lymphocyte transformation associated with antigen-sensitized macrophages (relative to that associated with monocytes) could have several possible explanations: (a) Lymphocytes adhere in greater numbers to macrophages than to monocytes; (b) lymphocytes adherent to macrophages begin DNA synthesis earlier than those associated with monocytes; (c) a greater number of lymphocytes enter DNA synthesis in cultures containing sensitized macrophages; (d) the generation time of transformed lymphocytes associated with macrophages is shorter than that of lymphocytes associated with monocytes. Each, or a combination of these explanations, could account for a greater absolute number of transformed lymphocytes after 5-6 days of culture in association with macrophages.

The percentage of macrophages with adherent lymphocytes was greater than the percentage of monocytes with adherent lymphocytes and appeared to be independent of the presence of antigen (Table III). Because the numbers of mononuclear phagocytes attached to glass were not known, the absolute number of lymphocytes adherent to monocytes or macrophages was indeter-

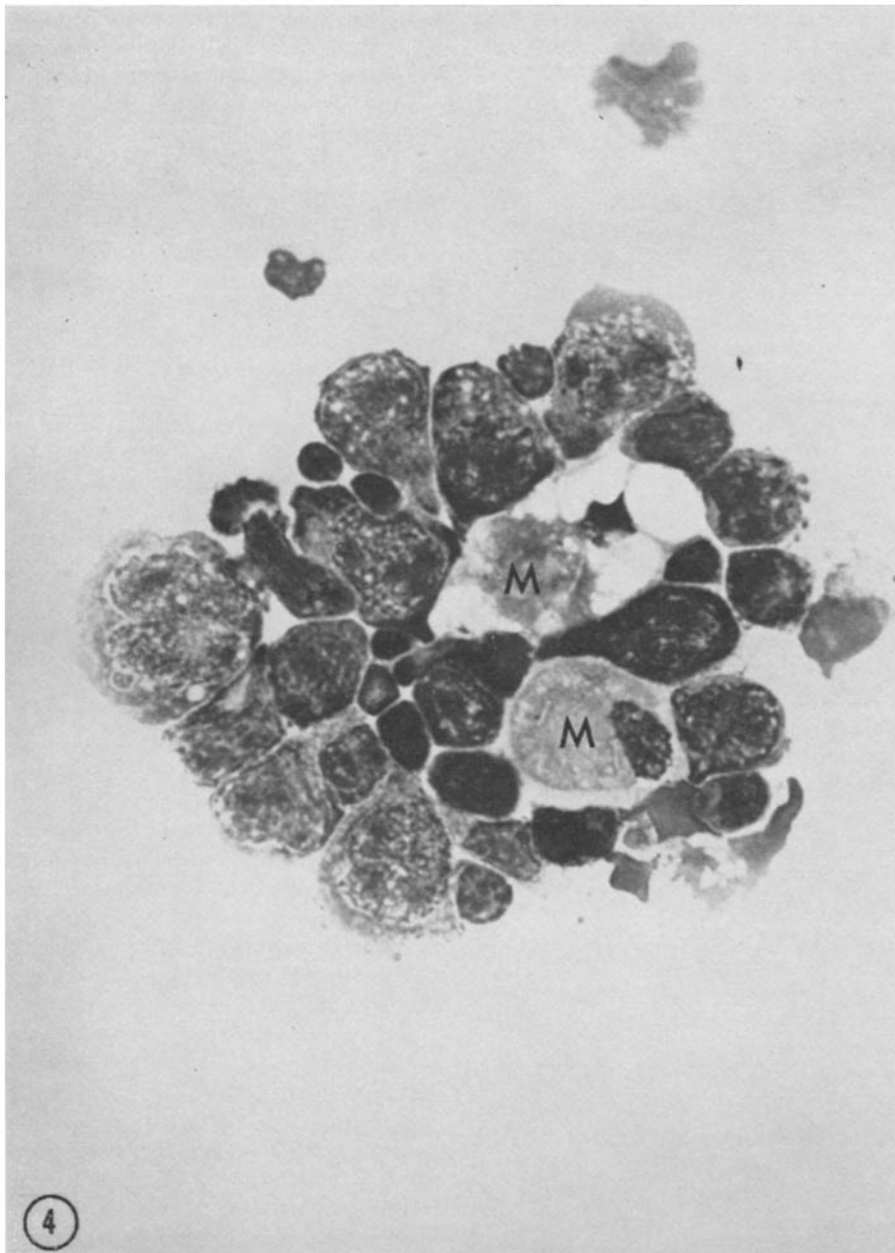


FIGURE 4 An "island" of transformed and untransformed lymphocytes around central macrophages (M). Giemsa stain. Original magnification,  $\times 500$ .

minate. Therefore, an estimate of the numbers of adherent lymphocytes was made by determining the total number of lymphocytes free in suspension at a time when there was no increase in the total lymphocyte population. At 72 hr of culture, approximately 20-30% of lymphocytes were ad-

herent to PPD-sensitized monocytes and approximately 60-70% were adherent to macrophages.

Time of onset of transformation of lymphocytes was investigated in a series of four experiments in which thymidine- $^3\text{H}$  was added in pulses of between 8 and 20 hr duration. The first labeled

lymphocytes were always associated with glass-adherent mononuclear cells. No labeled lymphocyte nuclei, either in cultures containing sensitized monocytes or in those containing macrophages, could be detected before 36 hr of incubation. Labeled lymphocyte nuclei were detectable in both monocyte and macrophage cultures (Table I) by 40–48 hr. It was inferred that, under the conditions used, the onset of the first detectable S phase of the transforming lymphocytes was similar whether the lymphocytes were in contact with monocytes or with macrophages.

In five experiments, the percentage of labeled lymphocytes was greater in cultures containing macrophages at all time periods after 40–48 hr. At this earliest time period,  $3 \pm 0.3\%$  of macrophage-associated lymphocytes were labeled and only  $0.75 \pm 0.25\%$  of monocyte-associated lymphocytes ( $P < 0.01$ ).

These data on cell numbers combined with those obtained from radioautographic studies indicated that in cultures containing PPD-sensitized macrophages, a greater number of the original population of lymphocytes entered the first detectable period of DNA synthesis than in cultures containing monocytes.

TABLE II  
Uptake of  $^{125}\text{I}$ -PPD by Monocytes and Macrophages

Sample	Cell-bound $^{125}\text{I}$ -PPD ( $\mu\text{g}/\text{cell}$ )	
	Monocytes	Macrophages
1	$2.36 \times 10^{-8}$	$8.74 \times 10^{-8}$
2	$4.98 \times 10^{-8}$	$30.6 \times 10^{-8}$
3	$1.92 \times 10^{-8}$	$7.74 \times 10^{-8}$

TABLE III  
Percentage of Glass-Attached Mononuclear Phagocytes with Adherent Lymphocytes

Day of culture	Cells with adherent lymphocytes			
	Monocytes		Macrophages	
	Saline	PPD	Saline	PPD
	%	%	%	%
1	$20 \pm 4$	$24 \pm 8$	$44 \pm 10$	$62 \pm 8$
2	$13 \pm 4$	$12 \pm 4$	$27 \pm 8$	$42 \pm 6$
3	$21 \pm 8$	$14 \pm 6$	$55 \pm 6$	$59 \pm 11$
4	$22 \pm 6$	$16 \pm 6$	$39 \pm 9$	$41 \pm 8$
5				
6	$30 \pm 4$	$30 \pm 8$	$45 \pm 5$	$59 \pm 10$

It was not possible to measure lymphocyte generation time because of the technical difficulties of obtaining sufficient blood from a single PPD-positive donor.

## DISCUSSION

The steps involved in the processing of antigen by mononuclear phagocytes and the subsequent interaction with lymphocytes are not definitely known. It seems clear that intimate contact between the surfaces of the mononuclear cell and the lymphocyte is necessary for the maximal lymphocyte response *in vitro* (6, 15). Close contact and cytoplasmic connections have been demonstrated between lymphocytes and macrophages *in vivo* (16, 17). *In vitro*, the central macrophage and surrounding lymphocytes form an "immunological island;" the formation of such clusters is required for continued division of antibody-forming cells (15). A metabolically intact monocyte or macrophage is necessary for this process (6). It is not definitely known whether the antigen is bound to the surface of the phagocyte or is distributed internally, nor is it known whether the antigen is modified or in its native configuration (7, 18). A recent report provides evidence that a portion of exogenous antigen escapes localization within phagocytic vacuoles and becomes associated with the rough-surfaced endoplasmic reticulum of the macrophage (19). Small amounts of antigen are retained in immunogenic form for long periods of time, although the major portion is quickly degraded within the macrophage. After administration of a radioactive antigen to an intact animal, the label is found associated with medullary macrophages as well as with surface processes of dendritic reticular cells within the cortical areas of lymph nodes (20, 21). The reticular cells are in intimate contact with the surfaces of surrounding lymphocytes.

The studies reported here indicate that, *in vitro*, antigen-sensitized macrophages are more efficient than monocytes at inducing blastogenic transformation of lymphocytes. It appears likely that in the presence of sensitized macrophages a higher proportion of the lymphocyte population enters the first detectable phase of DNA synthesis. Two possible explanations for the greater effectiveness of macrophages are suggested by the reported data. Macrophages are more efficient than monocytes at making contact with autologous lymphocytes and are capable of binding and/or internalizing more

antigen. Both characteristics may simply be a function of the greater surface area of the macrophage. Since the amounts of antigen associated with monocytes and macrophages is far less than that necessary to stimulate directly populations of lymphocytes containing few mononuclear phagocytes, it is likely that, at least *in vitro*, the phagocytes serve to concentrate antigen and, perhaps, to amplify its effects. If surface contact and local concentration of antigen are important to the induction of lymphocyte transformation, then the macrophage should be superior to the monocyte. It is possible that monocytes can initiate lymphocyte transformation only when they themselves are in the process of differentiation toward macrophages. Thus, even in cultures initiated with monocytes, only cells identifiable as macrophages appeared at the center of immunological clusters.

Early in the *in vitro* interaction of lymphocytes with mononuclear phagocytes, the involved cells were always in close contact and were few in number: one to three lymphocytes associated with a single mononuclear cell. When lymphocyte blastogenic transformation was well advanced, many of the transformed cells were free. In addition, the cell clusters had grown in size and con-

tained many untransformed, as well as transformed, lymphocytes. The untransformed lymphocytes showed no nuclear labeling and, therefore, probably were cells initially added to the culture tubes rather than those arising from division of transformed lymphocytes. If these events observed *in vitro* reflect similar processes in the intact animal, they suggest that dispersion of immunologically stimulated lymphocytes and attachment of unstimulated lymphocytes to a cluster of reactive cells may be simultaneous phenomena.

The series of events described in this paper have so far been demonstrated only with the macrophage-lymphocyte-PPD system. Using a similar system in which phytohemagglutinin is substituted for PPD, we have obtained no evidence of macrophage-induced lymphocyte transformation.

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