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The effect of human monocytes and macrophages on lymphocyte proliferation

J. H. PASSWELL, M. LEVANON, J. DAVIDSOHN, F. KOHEN & B. RAMOT *Departments of Pediatrics and Haematology, Chaim Sheba Medical Center, Sackler School of Medicine, Tel-Aviv University and Department of Hormone Research, Weizmann Institute, Rehovot, Israel*

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Summary. Human monocytes suppressed both the phytohaemagglutin (PHA) or antigen-induced lymphocyte proliferative response, when the monocyte: lymphocyte ratio was increased or when the monocytes were stimulated with zymosan or endotoxin. The effect of monocytes on autologous lymphocyte proliferation was compared with that of macrophages obtained by culturing monocytes *in vitro* for 7 days. The lymphocyte proliferative responses were increased in the presence of macrophages, however, neither increasing their number nor stimulation by zymosan or endotoxin altered the autologous lymphocyte proliferative response to PHA or purified protein derivative (PPD).

The PGE_2 concentration in the medium of both the cultured monocytes or macrophages activated by zymosan and endotoxin rose markedly without a corresponding suppressor effect on lymphocyte proliferation in the presence of macrophages in the culture. Thus it seems that while PGE_2 is a useful marker of mononuclear phagocyte activation, other molecular species are of importance in determining the lymphocyte proliferative response to mitogens and antigens.

Correspondence: Dr J. Passwell Department of Pediatrics, Chaim Sheba Medical Center, Tel-Hashomer, Israel 52 621.

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INTRODUCTION

The cellular cooperation between macrophages and lymphocytes is required for both the induction of the lymphocyte proliferative response and for its suppression (Unanue, 1978). Studies in animals have shown that macrophages are required for the optimal response either to mitogen or antigen stimuli (Seeger & Oppenheim, 1970; Baird & Kaplan, 1977). However, when the ratio of macrophages was increased or when the former were activated, the lymphocyte response was suppressed (Keller, 1975). Furthermore, the type of mitogenic stimulus also determined whether the macrophages had a suppressor function or not (Novogrodsky, Stenzel & Rubin, 1977). Studies of human cells have usually been done on peripheral blood mononuclear cells. Rigorous depletion of monocytes has been shown to decrease the proliferative response to antigens, mitogens and allogeneic lymphocytes (Hersh, & Harris, 1968; Schmidtke & Hatfield, 1976; Rode & Gordon, 1974).

Human monocytes cultured *in vitro*, acquire the morphological characteristics of macrophages. They secrete a diverse range of products including degradative enzymes, prostaglandins and complement components (Karnovsky & Lazdins, 1978). We and others have shown that human monocytes and mouse macrophages can be stimulated *in vitro* to secrete large amounts of prostaglandins (Passwell, Dayer & Merler, 1979; Humes, Banney, Pelus, Dahlgorn, Sadowski,

Kuehl & Davies, 1977; Kurland & Bockman, 1978). We have also shown that monocytes activated with Fc fragments secreted prostaglandin E_2 which inhibited the T-cell proliferative response to tetanus antigen (Passwell, Rosen & Merler, 1980). A suppressor effect of endogenous prostaglandins from adherent cells has been demonstrated when suboptimal dosages of mitogens were used (Goodwin, Messner & Peake, 1978).

In view of the reports that in pathological conditions such as Hodgkin's disease the decreased proliferative response of lymphocytes is due to increased amounts of prostaglandins secreted by monocytes (Goodwin, Messner, Peake, Saiki & Williams, 1977; Schechter & Soehnlen, 1978), it was important to study in detail the monocyte–lymphocyte interactions in normal individuals. In the present study the effect of either human peripheral blood monocytes or cultured macrophages on the lymphocyte proliferative response were compared. In addition the effect of activation of the mononuclear phagocytes on the lymphocyte proliferative response and the role of endogenous PGE_2 in these interactions was studied.

MATERIALS AND METHODS

Isolation of cells and culture conditions

Heparinized peripheral blood from volunteer donors was first centrifuged at 400 *g* for 5 min at 4° and the plasma and buffy coat removed. The white cell suspension was layered on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, N.J.) centrifuged at 400 *g* for 20 min at room temperature and the mononuclear cell preparation was removed from the interface. These cells were washed three times in Hanks's balanced salt solution (HBSS), resuspended in medium RPMI 1640 (Microbiological Associates, Bethesda, Md) supplemented with 10% pooled heat-inactivated millipore filtered AB positive serum, Penicillin (100 units/ml) and streptomycin (100 μg/ml; GIBCO, Grand Island, N.Y.). The number of monocytes and lymphocytes in each preparation was determined by morphology of Giemsa stained cytocentrifuge preparations and positive staining for non-specific esterase.

Monocytes

Enriched populations of viable and functional monocytes were obtained by layering the mononuclear cell preparation (1×10^7/ml) on plastic Petri dishes (Falcon Chemical Co., Oxnard, Calif) which had been pretreated over night with heat-inactivated foetal calf serum (Gibco). After 1 hr the plates were washed vigourously with HBSS. The adherent monocytes were easily removed by gentle rinsing with 0·2% ethylene diamine tetraacetate (Sigma Chemical Co.) in HBSS. These cells were washed in HBSS resuspended in complete medium and used at the desired concentrations (Kamagai, Itoh, Hinuma & Tada, 1979). The percentage of monocytes in each preparation was determined by morphology. These cells could readhere to plastic surfaces, phagocytosed and killed *Candida albicans*, formed rosettes with complement-coated zymosan particles and secreted lysozyme (Territo & Cline, 1977). Monocyte cultures in complete medium increased in size and were more spread out. After 7 days in culture, they did not stain positively for esterase and were considered macrophages. In order to test the effect of varying monocyte numbers on the lymphocyte proliferative response, monocytes in desired concentrations were added to flat bottom micro wells (96-well microtitre plates, Linbro Labs, New Haven, Conn.) before the addition of the lymphocytes. In experiments where monocytes were kept in culture for 7 days so as to mature into macrophages, an additional blood sample was obtained on day 7. The monocytes were isolated and fresh lymphocytes were used as responder cells with equal numbers of either monocytes or macrophages.

Lymphocytes

Mononuclear cell preparations depleted of monocytes after adhesion to a plastic surface were used. T-cell populations were obtained by E-rosette formation with sheep red cells, separation on Ficoll-Hypaque and lysis of the red cells with 0·87% ammonium chloride. The non-rosetted lymphocytes were considered an enriched B-cell population. Lymphocyte proliferation was assayed by incorporation of [^{3}H]-thymidine (2 μCi/culture of 100,000 cells), added 16–24 hr before the termination of the culture period. Cells were harvested and the radioactivity incorporated by the lymphocytes was counted in a beta scintillation counter. Protein synthesis was measured by incorporation of [^{3}H]-leucine (Amersham) into the lymphocytes. Results are expressed as mean counts/min (c.p.m.) of at least quadruplicate cultures ± standard error (SEM) corrected by subtracting the appropriate control counts. Phytohemagglutinin (PHA, reagent grade, Wellcome Research Laboratories, Beckenham) was diluted in phosphate-buffered saline

(PBS); Tuberculin: purified protein derivative (PPD) 2 μg/culture (Ministry of Agriculture, Weybridge) and Candida antigen (Biological Laboratories, Nes Ziona, Israel) were dialysed extensively in PBS before use.

Monocyte and macrophage stimuli

Zymosan (Sigma Chemical Co. St. Louis, Md) was washed three times in HBSS and sterilised under an ultraviolet light source. Endotoxin (lipopolysacharide B from *Escherichia coli* B6; Difco Laboratories, Detroit, Mich.) was made in appropriate stock solutions of HBSS so that the addition of 20 μl to the respective cell cultures provided the desired concentrations. Both zymosan and endotoxin were added 1 hr before the addition of PHA or PPD and were maintained in the culture until the end of the experiment.

Biochemical assays

Culture fluids were assayed for their content of prostaglandin E_2 by a radioimmunoassay (Miles Yeda, Rehovot, Israel; Jaffe & Behrman, 1974). Aliquots of the medium were removed at the time of pulsing the culture with [^{3}H]-thymidine. Lysozyme was assayed by determining the initial rate of lysis of a suspension of micrococcus lysodeikticus (Sigma) in 1 M acetate buffer pH 6·2 at room temperature with a Gilford spectrophotometer fitted with a recorder. Egg white lysozyme (Sigma) was used as a standard (Gordon, Todd & Cohn, 1974).

RESULTS

Effect of alteration of monocyte numbers on the lymphocyte proliferative response

The addition of increasing numbers of autologous monocytes to 1×10^5 lymphocytes resulted in a decrease in thymidine incorporation into DNA following stimulation with suboptimal doses of PHA. No effect of increasing the number of monocytes was apparent when optimal concentrations of PHA were used (Fig. 1). The lymphocyte proliferative response to both candida antigen and PPD was also decreased when increasing numbers of monocytes were added to the lymphocyte cultures (Table 1). A similar degree of suppression of lymphocyte protein synthesis was observed when radiolabelled leucine rather than thymidine was used (results not shown).

In order to test the effect of depleting monocytes on the lymphocyte proliferative response, the mononuclear cell preparation was allowed to adhere to a plastic surface for an hour and thereafter the proliferative response of the non-adherent cells and mononuclear cell preparations were compared. Depletion of monocytes from the mononuclear cell population resulted in a decreased proliferative response to PHA which was

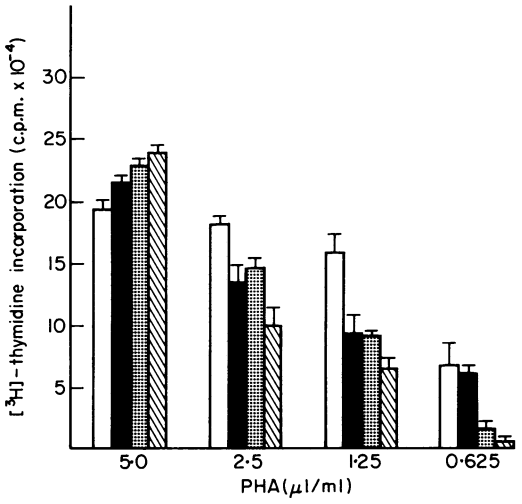

Figure 1. The effect of increasing numbers of monocytes on the lymphocyte proliferative response (1×10^5 lymphocytes/culture) at various dosages of phytohaemagglutinin. Each bar represents the additional number of monocytes added to mononuclear cell cultures. [^{3}H]-thymidine incorporation (c.p.m. $\times 10^{-4}$) of the lymphocytes of the mononuclear cell preparation at the varying doses of PHA was $19{\cdot}0 \pm 0{\cdot}55$; $18{\cdot}25 \pm 0{\cdot}33$; $13{\cdot}58 \pm 1{\cdot}57$ and $6{\cdot}75 \pm 2{\cdot}01$. Results are the mean ± SEM of quadruplicate cultures. (□) 1×10^4 Monocytes; (■) 2×10^4 monocytes; (▤) 5×10^4 monocytes; (■) 1×10^5 monocytes.

Table 1. Effect of increasing numbers of monocytes on lymphocyte proliferative response to PPD and Candida antigens

Antigen	No. of monocytes	[^{3}H]-TdR Uptake (c.p.m. ± SE)	Suppression (%)
PPD	—	15,467 ± 789	—
	1×10^4	4233 ± 537	73
	$2{\cdot}5 \times 10^4$	823 ± 239	95
	5×10^4	107 ± 7	99
Candida	—	2533 ± 537	
	1×10^4	583 ± 164	77
	$2{\cdot}5 \pm 10^4$	183 ± 61	93
	5×10^4	58 ± 16	

Results are mean ± SEM of quadruplicate cultures. Lymphocytes (1×10^5) were present in all cultures.

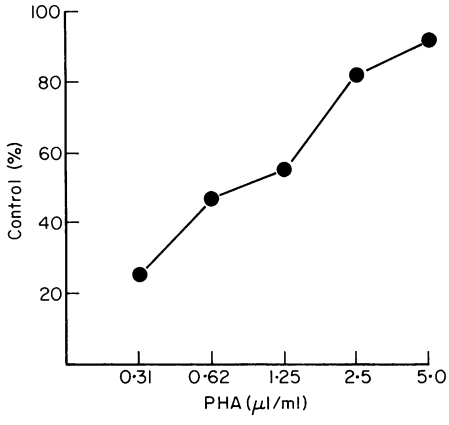

Figure 2. Effect of monocyte depletion on lymphocyte proliferative response at various dosages of phytohaemagglutinin (percentage control is the lymphocyte proliferative response of the monocyte depleted mononuclear cells divided by the lymphocyte proliferative response of the mononuclear cells).

most marked at suboptimal dosages of PHA (Fig. 2). Results are expressed as a percentage of the lymphocyte proliferative response of the monocyte-depleted cell suspension divided by the lymphocyte proliferative response of the mononuclear cells. The effect of alteration of monocyte numbers on the lymphocyte proliferative response to PHA was observed only on T cells and no proliferation of the B-cell population was discerned. Therefore subsequent studies were done on unseparated lymphocyte populations.

Effect of endotoxin and zymosan on the monocyte-mediated lymphocyte proliferative response

The prior addition of endotoxin or zymosan to an enriched monocyte population resulted in suppression of the lymphocyte proliferative response to both PPD (Table 2) and PHA (Table 5). Addition of endotoxin resulted in a dose-dependent increase of PGE_2 concentration in the extracellular medium from both cultures with either monocytes or macrophages. However, increased PGE_2 concentrations corresponded with suppression of the lymphocyte proliferative response only when monocytes were present in the cultures (Table 5). The levels of PGE_2 in the extracellular medium were increased following addition of endotoxin or zymosan and were higher when the numbers of monocytes were increased (Table 2).

Comparison of the effects of monocytes or macrophages on the lymphocyte proliferative response

The lymphocyte proliferative response to both PHA and PPD was increased when cultured in the presence of macrophages compared to monocytes (Tables 3 and 4). Increasing the macrophage:lymphocyte ratio from 1:10 to 1:4 did not alter the amount of lymphocyte proliferation to either PHA or PPD. Neither the addition of endotoxin (Table 5) nor zymosan (results not shown) to macrophages caused any suppression of the lymphocyte proliferative response to PHA or antigen.

The basal secretion of PGE_2 and lysozyme was

Table 2. Effect of endotoxin and zymosan on the proliferative response to PPD and PGE_2 production

Culture conditions	No. of monocytes culture	[^{3}H]-TdR uptake (c.p.m. ± SE)	Control (%)	PGE_2 concentration (ng/ml/culture)
Control	5×10^4	10,381 ± 1454	100	2·5
Endotoxin	5×10^4	6609 ± 283	64	> 10·0
Zymosan	5×10^4	1503 ± 534	14	4·6
Control	1×10^4	23,157 ± 2626	100	0·6
Endotoxin	1×10^4	8003 ± 1138	35	1·26
Zymosan	1×10^4	8555 ± 1062	37	0·46

Results are the mean of at least quadruplicate cultures. PGE_2 is the mean of at least two determinations of extracellar medium removed at the time of thymidine pulse of the cultures.

Endotoxin was used at final concentration of 20 μg/ml and Zymosan (1×10^7 particles/ml).

Table 3. Lymphocyte proliferative response to PHA in the presence of monocytes or macrophages

PHA (μg/ml)	No. of monocytes or macrophages	[^{3}H]-TdR Uptake (c.p.m. ± SE)	
		Macrophages	Monocytes
0·5	5×10^4	72,136 ± 1817	43,333 ± 3169
	1×10^4	73,911 ± 3594	58,882 ± 1263
1·0	5×10^4	127,570 ± 1880	66,924 ± 6954
	1×10^4	125,800 ± 3374	73,030 ± 4706

Lymphocytes (1×10^5) were added at the same time to the monocyte or macrophage monolayers and harvested after 72 hr.

Results are the mean ± SE of at least quadruplicate cultures.

larger from the macrophage cultures compared with equal numbers of monocytes (Table 6). In addition the endotoxin-induced secretion of PGE_2 was greater from the macrophage cultures compared with the monocyte cultures (Table 5).

DISCUSSION

These studies were designed to re-evaluate in humans the role of cells of the monocyte-macrophage lineage on the lymphocyte proliferative response to PHA and antigens. The simple technique of adherence of monocytes to a plastic surface pretreated with foetal calf serum made possible the recovery of large numbers of viable functional monocytes, so that accurate

Table 4. Lymphocyte proliferative response to PPD in the presence of monocytes or macrophages

Experiment	No. of monocytes or macrophages	[^{3}H]TdR Uptake (c.p.m. ± SE) macrophages	monocytes	Increase (%)
1	5×10^4	35,623 ± 716	6992 ± 1351	409
	1×10^4	37,967 ± 2036	8779 ± 1904	332
2	5×10^4	34,752 ± 3582	12,506 ± 932	178
	1×10^4	42,372 ± 5310	23,157 ± 2626	83

Lymphocytes (1×10^5) were added at the same time to autologous monocyte or macrophage monolayers and harvested after 144 hr.

Results are the mean of at least quadruplicate cultures.

Table 5. The effect of endotoxin on the lymphocyte proliferative response to PHA and PGE_2 production in the presence of monocytes or macrophages

Endotoxin (μg/ml)	Monocytes [^{3}H]-TdR uptake (c.p.m. ± SEM)	Control (%)	PGE_2 (ng/ml/culture)	Macrophages [^{3}H]-TdR uptake (c.p.m. ± SEM)	Control (%)	PGE_2 (ng/ml/culture)
=	117,000 ± 8350	100	3·25	214,840 ± 14,900	100	5·8
0·2	149,750 ± 3500	128	2·05	182,580 ± 13,480	85	14·7
2	39,290 ± 2000	34	5·05	177,750 ± 4390	83	31·5
20	41,800 ± 3450	36	44·00	190,400 ± 15,924	89	122·0

Monocytes (5×10^4) or macrophages were present in each culture.

Extracellular medium for PGE_2 determinations was harvested at the time of pulsing with thymidine.

Results are the mean ± SEM of at least quadruplicate cultures.

PGE_2 determinations are the average of duplicate assays.

Table 6. Comparison of lysozyme and PGE_2 concentration in the extracellular medium of monocytes and macrophages

No. of Cells		PGE_2 (ng/ml/culture)	Lysozyme (μg/ml/culture)
5×10^4	Monocytes	$0{\cdot}53 \pm 0{\cdot}10$	$2{\cdot}18 \pm 0{\cdot}18$
5×10^4	Macrophages	$0{\cdot}95 \pm 0{\cdot}32$	$2{\cdot}76 \pm 0{\cdot}20$
$2{\cdot}5 \times 10^4$	Monocytes	$0{\cdot}28 \pm 0{\cdot}05$	$1{\cdot}16 \pm 0{\cdot}09$
$2{\cdot}5 \times 10^4$	Macrophages	$0{\cdot}52 \pm 0{\cdot}12$	$2{\cdot}07 \pm 0{\cdot}26$
1×10^4	Monocytes	$0{\cdot}05 \pm 0{\cdot}03$	$1{\cdot}07 \pm 0{\cdot}44$
1×10^4	Macrophages	$0{\cdot}26 \pm 0{\cdot}04$	$1{\cdot}16 \pm 0{\cdot}14$

The results represent the means $\pm$ SEM of quadriplicate determinations from each of four different experiments.

quantification of monocyte numbers was easily accomplished. We have confirmed that depletion of monocytes resulted in a decreased proliferative response to PHA. This was most marked at suboptimal dosages of PHA. However, as the monocyte:lymphocyte ratio was increased a dose-dependent suppressor effect was apparent on lymphocytes stimulated with antigen or suboptimal concentrations of PHA. In addition *in vitro* activation of monocytes by endotoxin or zymosan resulted in suppression of proliferation. The increased suppression observed when the numbers of monocytes were increased in the culture suggests that the suppressor activity of endotoxin and zymosan was indeed mediated via monocytes.

In vitro incubation of monocytes for 7 days indicated by virtue of morphological changes, lack of peroxidase staining and increase in secretion of lysozyme and prostaglandins that these cells had matured into macrophages. These macrophages enhanced the lymphocyte proliferative response to PHA two-fold by comparison with equal numbers of monocytes. This effect was even more marked when antigen was used as the lymphocyte stimulus. The enhancing activity was independent of the lymphocyte:macrophage ratio used suggesting that a qualitative change occurred in these macrophages by comparison with monocytes.

In animal models resident peritoneal macrophages result in enhanced proliferation, while activation of the macrophages *in vivo* results in suppression of the lymphocyte proliferative response. However, Wing & Remington recently showed that incubation of macrophages activated by *Toxoplasma gondii* for longer than 12 hr enhanced rather than suppressed lymphocyte proliferation (Wing & Remington, 1980). It is possible that suppressor molecules may be inactivated or not produced during this period of culture or that in fact enhancing factors are produced later and negate the effects of the suppressor substances. In only one other study has the effect of human monocytes and macrophages on lymphocyte responses been compared (Rinehart, Orser & Kaplan, 1979). Diametrically opposite results were obtained in their studies. Monocytes enhanced while macrophages suppressed the proliferative response. Several explanations may account for these differences. Firstly isolation of monocytes was achieved with Xylocaine, which has been shown to have direct effects on monocyte membrane activities; secondly enhanced lymphocyte responses with monocytes were only present when monocyte: lymphocyte ratios were greater than 1:1 and were tested with optimal concentrations of PHA; thirdly, macrophages were cultured for 14 rather than 7 days, but, perhaps more importantly, 80% of macrophages were lost in culture suggesting that selection of a particular subpopulation may have occurred.

The presence of both inhibitory and enhancing factors in macrophage supernatants has been described (Calderon & Unanue, 1975). Considerable attention has been devoted to the inhibitory action of PGE_2 on several *in vitro* correlates of immune function particularly inhibition of lymphocyte proliferation (Passwell *et al.*, 1980; Goodwin *et al.*, 1978). Increasing levels of PGE_2 were found in both the monocyte and macrophage cultures by increasing the number of cells. In addition, endotoxin resulted in a dose-dependent increase of PGE_2 secretion in both monocyte and macrophage cultures. Thus increased PGE_2 levels correlated with the decreased lymphocyte responses, when monocytes were used; however, no inhibitory effect was observed when macrophages were used. The concentrations of PGE_2 found in these cultures were 100-fold less than those required to decrease lymphocyte responses by exogenous PGE (Passwell *et al.*, 1980). However, differences in time course and mechanism of action of endogenous and exogenous prostaglandins may account for this apparent discrepancy. In addition higher concentrations of prostaglandins may be present in localized areas of inflammation *in vivo* or other prostaglandins such as thromboxanes and other mediators may exert suppressor activity (Goldstein, Malmsten, Kindhal, Kaplan, Rodmark, Samuelsson & Weissmann, 1978). It has also been demonstrated recently that monocyte prostaglandins exert their suppressor effects by preferentially activating T-suppressor cells (Stobo, 1977;

Fischer, Durandy & Griscelli, 1981). We were not able to investigate these possibilities in this study. Nevertheless, PGE_2 which is easily assayed does provide a useful measure of monocyte or macrophage activation.

These studies have shown that varying lymphocyte proliferative responses may be obtained in human mononuclear cell preparations depending on the monocyte lymphocyte ratio in the culture or the degree of 'activation' of the monocytes. Human macrophages at the same concentration as monocytes did not have the same suppressor effects on the proliferative response. These findings emphasize that the routine study of peripheral blood mononuclear proliferative responses may not be a true reflection of these responses *in vivo*.

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