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The effect of PHA-activated MN-cell supernatants on polymorphonuclear leucocyte function

RUTH LOMNITZER, ANNEMARIE GLOVER & A. R. RABSON *Department of Immunology, School of Pathology of the South African Institute for Medical Research, and the University of the Witwatersrand, Johannesburg, South Africa*

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SUMMARY

The effect of PHA-activated mononuclear-cell (MN) supernatants on various polymorphonuclear-leucocyte (PMN) functions were assessed.

Treatment of PMN with PHA-activated MN-cell supernatants resulted in greater electrophoretic mobility, indicating an increase in the negative surface charge. PMN directional motility was inhibited in the presence of active supernatants but was not affected by a pulse exposure of the PMN to these supernatants. Neither control nor active supernatants were chemotactic for PMN, but treatment of these cells with active supernatants produced an increase in their phagocytic activity, their ability to reduce NBT and in their glucose oxidation through the hexose-monophosphate shunt. Bactericidal capacity of these PMN was unaltered.

Specific loss of leucocyte inhibitory factor (LIF) activity from supernatants of PHA-activated MN cells followed their absorption with PMN cells but not with human MN cells or guinea-pig peritoneal exudate cells. Furthermore, acquired inhibition of migration of the absorbing PMN was observed.

INTRODUCTION

In the cell-mediated immune reaction, lymphocytes produce a number of biologically active products, lymphokines, in response to a sensitizing antigen or non-specific mitogen. The most extensively studied lymphokine is macrophage inhibitory factor (MIF), which not only inhibits guinea-pig macrophages from migrating out of capillary tubes (David, 1966) but has also been found to affect various macrophage functions (Nathan, Karnovsky & David, 1971; Nathan, Remold & David, 1973). Since both macrophages and polymorphonuclear leucocytes (PMN) are involved in the inflammatory reaction, it was of interest to determine whether supernatants from PHA-activated lymphocytes containing leucocyte inhibitory factor (LIF), a lymphokine which has previously been shown to specifically inhibit PMN migration (Rocklin, 1974; Lomnitzer, Rabson & Koornhof, 1975), will affect PMN functions.

MATERIALS AND METHODS

Materials. Human blood was donated by normal laboratory workers. Peritoneal exudate cells were obtained from randomly bred guinea-pigs. Phytohaemagglutinin (PHA) (reagent grade) and foetal calf serum (FCS) were obtained from Wellcome Research Laboratories (Beckenham, England). L-glutamine (Merck, Darmstadt, Germany), 5 μm pore-sized filters (Millipore Corporation, Bedford, Massachusetts), D-glucose (1-^{14}C), 8·64 mCi/mmol (New England Nuclear, Boston, Massachusetts), nitro-blue tetrazolium (NBT) (Sigma Chemical Company, St. Louis, Missouri), Insta gel (Packard, Illinois) and bacterial lipopolysaccharide (*E. Coli* 0127: B8) (Difco Laboratories, Detroit, Michigan) were obtained from the manufacturers.

Production of supernatants. Active and normal control supernatants were obtained as previously described (Lomnitzer *et al.*, 1976a). Briefly, human peripheral blood mononuclear (MN) cells, obtained by separating heparinized whole blood on a Hypaque–Ficoll gradient, were pulsed for 2 hr at 37°C in 5% CO_2 in air. Cell-free supernatants were collected by centrifugation at 200 *g* (active supernatants). Control cultures underwent the same procedure except that PHA was not added (control

Correspondence: Dr Ruth Lomnitzer, P.O. Box 1038, 2000 Johannesburg, South Africa.

supernatants). The supernatants were tested for the presence of LIF activity, using human PMN as indicator cells in the capillary migration system.

Absorption of supernatants with different cell types. Active and control supernatants were absorbed with three cell types: guinea-pig peritoneal exudate cells, human MN cells and human PMN cells. The cells were obtained as previously described (Lomnitzer *et al.*, 1975). Samples of 1·5 ml of supernatant were mixed with $5–6 \times 10^7$ cells in a 15 ml plastic tissue-culture tube, the mixture was incubated in a 37°C water bath for 60 min and was shaken manually every 10 min. At the end of the incubation period the tubes were centrifuged (all centrifuging was done at 200 *g*) and cell-free supernatants collected and stored at −20°C until used.

LIF absorption and migration of the absorbing cells. 1·5 ml of active or control supernatants were mixed with $5–6 \times 10^7$ PMN and incubated. Tubes were removed at various time intervals, centrifuged and supernatants collected and assayed for LIF activity. The remaining pellets were washed twice in MEM and resuspended in complete MEM to a final concentration of 3×10^7/ml. The PMN were packed in capillary tubes which were centrifuged, cut at the cell–medium interface, and stumps containing cells placed in migration chambers. The chambers were filled with complete MEM, sealed and incubated at 37°C in 5% CO_2 in air for 18 hr. Migration patterns were projected, traced and the area measured by planimetry. The migration index (MI) was calculated as follows:

$$MI = \frac{\text{area of migration of PMN used for absorbing active supernatants}}{\text{area of migration of PMN used for absorbing control supernatants}}.$$

The effect of supernatants on PMN electrophoretic mobility. Aliquots of 1 ml pure PMN containing $1·5 \times 10^7$ cells in complete MEM were incubated for 60 min with 1 ml of active or control supernatant. The mixtures were shaken manually every 10 min and at the end of the incubation period the tubes were centrifuged, the cells washed twice and suspended in 3 ml of sucrose and mannitol containing HEPES-buffered MEM. The electrophoretic mobility of the PMN was measured in a Zeiss cytopherometer as previously described (Lomnitzer *et al.*, 1976a).

Preparation of cells for PMN function studies. 25 ml of heparinized whole blood was centrifuged slowly (100 *g*) for 10 min and the plasma, plus about 1 cm of the underlying cells, was collected into 15 ml plastic centrifuge tubes. The cells were allowed to sediment at 37°C for 30–45 min after which the leucocyte-rich plasma was collected and centrifuged. The cells were washed three times in MEM and resuspended to a concentration of 10^7 PMN/ml. 1 ml of cell suspension was incubated with 1 ml of active or control supernatant for 60 min at 37°C. The cells were washed three times and resuspended to 3×10^6 PMN/ml.

Chemotaxis. A modified Boyden chamber (Wilkinson, 1970), which utilizes 0·2 ml of cell suspension in the upper chamber separated from 1 ml of the leuco-attractant solution by a 5 μm pore-sized membrane filter was used. Filters were removed after a 3 hr incubation at 37°C, fixed with methanol and stained with haematoxylin, and the average number of PMN per high-power field (HPF) reaching the lower surface of the filter was determined. Experiments were performed in triplicate. The leuco-attractant was prepared as follows: 1 mg of bacterial endotoxin was incubated for 30 min at 37°C with 2 ml of fresh human serum (EAS) and diluted four-fold with MEM.

Three types of chemotaxis experiment were performed: in the first, chemotaxis of washed cells previously treated with the supernatants (as described above) was assessed. In the second, mixtures of 0·2 ml untreated cells and 0·2 ml control or active supernatants were placed in the upper chamber, and in the third, active or control supernatants were placed in the lower compartment and their ability to attract PMN measured.

Phagocytosis. Phagocytosis of *Candida albicans* by PMN was assessed in two ways: (a) 0·3 ml of cells treated with active or control supernatants, as described above, were incubated with 0·2 ml of 3×10^7/ml *Candida albicans* suspension and 0·5 ml MEM. The non-phagocytic control consisted of 0·2 ml of *Candida albicans* and 0·8 ml MEM. Mixtures were rotated at 37°C and 0·1 ml samples were removed at various time intervals and the percentage phagocytosis calculated. (b) The reaction mixture consisted of 0·3 ml untreated cells, 0·2 ml *Candida albicans* suspension and 0·5 ml of control of active supernatant and phagocytosis was assessed as in (a).

NBT reduction. This was performed according to the method of Sher *et al.* (1974), the reaction mixture consisting of 0·2 ml cell suspension, 0·3 ml NBT solution (1 mg/ml NBT in phosphate-buffered saline) and 0·3 ml active or control supernatants. PMN containing the dark granules of the reduced dye were counted as positive and recorded as a percentage.

The effect of supernatants on hexose-monophosphate shunt (HMPS) activity. The extent of HMPS activity was measured by [^{14}C]carbon dioxide production from glucose radiolabelled with ^{14}C in the C_1 position. A suspension of 2×10^7/ml PMN (obtained from a Hypaque–Ficoll separation of whole blood) was used. Each assay was performed in duplicate and utilized 0·2 ml PMN suspension, 0·6 ml of radiolabelled glucose in phosphate-buffered saline (0·6 μCi) and 0·3 ml control or active supernatants.

The test apparatus consisted of a tightly stoppered glass scintillation vial containing a 1 ml autoanalyser cup. The radiolabelled glucose and the supernatants to be tested were placed in the vial and 0·6 ml of 1 M KOH was placed in the inner cup. The reaction was initiated by the injection of the cell suspension into the vial through the cap, and was terminated at various time intervals with 2 ml of 2 N HCl. The chambers were allowed to stand for 60 min to permit release of $^{14}CO_2$, and allow its absorption by the KOH in the inner cup. 0·5 ml of the KOH was then transferred to scintillation vials containing 10 ml of acidified Insta gel and the activity counted in a Tri-carb liquid scintillation counter. Results were expressed as corrected mean counts per minute (background control values being subtracted from the corresponding experimental values).

Bactericidal ability. Cells treated with active or control supernatants as described above were washed six times in antibiotic-free Gey's solution. 5×10^6 cells in 0·8 ml of Gey's solution was mixed with 0·1 ml fresh autologous serum and 0·1 ml Gey's containing 10^7 *Staphylococcus aureus*. The mixture was rotated at 37°C, 0·1 ml aliquots removed at various time intervals into 9·9 ml of sterile distilled water and the total number of viable bacteria determined by colony counts.

Statistical analysis. Experiments were repeated four to ten times. The Student's *t*-test was used to determine the significance of the results and a *P* value higher than 0·05 was considered non-significant.

RESULTS

Absorption of LIF-containing supernatants

The absorption of active supernatants with three different cell types, namely guinea-pig peritoneal exudate cells, human MN cells and human PMN cells, resulted in the removal of LIF activity only when supernatants were absorbed with PMN cells (Fig. 1). Experiments, in which both the inhibitory activity of the supernatants and the migrating capacity of the absorbing PMN in the presence of complete MEM were assessed, showed that removal of LIF activity from the supernatants correlated with increasing migration inhibition of the absorbing PMN (Fig. 2).

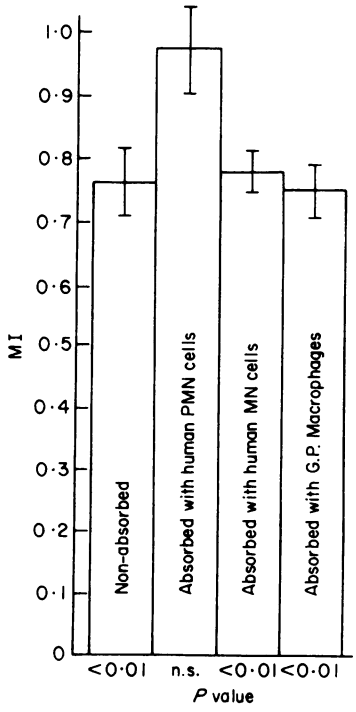

FIG. 1.

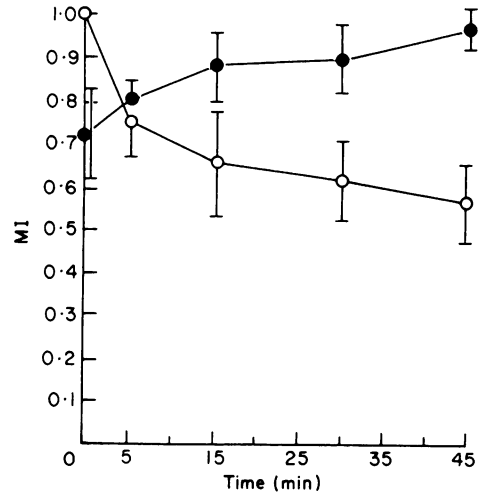

FIG. 2.

FIG. 1. PMN migration in the presence of LIF containing supernatants previously absorbed with different cell types (mean ± s.e.m.), n.s. = not significant.

FIG. 2. The inhibitory activity of active supernatants after absorption with PMN for varying time intervals (●) and migration of the PMN used for absorption (○) (mean ± s.e.m.).

The effect of supernatants on PMN electrophoretic mobility

The electrophoretic mobility of PMN treated with active supernatants was greater than that of PMN treated with control supernatants, indicating an increase in the surface negative charge. In six out of nine experiments the difference in velocity was significant (Table 1).

TABLE 1. The effect of control and active supernatants on PMN electrophoretic motility

Experiment no.	PMN treatment	Migration time (sec) ±s.e.m.	Enhancement (%)	*P* value
1	Control supernatant	4·16±0·09	13	< 0·05
	Active supernatant	3·66±0·10		
2	Control supernatant	3·84±0·08	10	< 0·05
	Active supernatant	3·49±0·06		
3	Control supernatant	4·08±0·15	20	< 0·01
	Active supernatant	3·39±0·09		
4	Control supernatant	3·18±0·06	28	< 0·001
	Active supernatant	2·47±0·06		
5	Control supernatant	3·71±0·08	2	n.s.
	Active supernatant	3·65±0·06		
6	Control supernatant	4·48±0·10	17	< 0·001
	Active supernatant	3·82±0·10		
7	Control supernatant	4·77±0·16	22	n.s.
	Active supernatant	3·89±0·09		
8	Control supernatant	3·51±0·06	6	n.s.
	Active supernatant	3·30±0·08		
9	Control supernatant	3·72±0·11	12	< 0·05
	Active supernatant	3·30±0·10		

n.s. = Not significant.

The effect of supernatants on PMN chemotactic activity

Pulsing PMN with active or control supernatants did not significantly effect their directional mobility. Although in all experiments the number of active supernatant-treated cells moving through the filters was less than the number treated with control supernatant, the difference was not statistically significant. However, when supernatants were present in the upper chamber during the entire test period a significant reduction in the number of PMN reacting to the chemotactic stimulus was noted in active supernatants (Table 2).

Chemotactic activity of supernatants

When the chemotactic activity of control and active supernatant was assessed, no significant difference could be detected (Table 3).

TABLE 2. Chemotactic ability of PMN after pulse exposure with supernatants (a) and in the presence of supernatants (b)

PMN treatment	Cells/HPF (±s.e.m.)	*P* value
(a) Control supernatant	46·5±5·2	n.s.
Active supernatant	38·1±3·6	
(b) Control supernatant	92·1±6·5	< 0·05
Active supernatant	60·3±5·6	

n.s. = Not significant.

TABLE 3. The ability of supernatants to attract PMN

Chemoattractant	Cells/HPF (±s.e.m.)	*P* value
Control supernatants	49·20±5·5	n.s.
Active supernatants	56·25±4·9	n.s.

The effect of supernatants on bactericidal capacity of PMN

As can be seen in Fig. 3, the bactericidal capacity of PMN treated with active supernatants was the same as that of PMN treated with control supernatants.

The effect of supernatants on PMN phagocytosis

The effect of active and control supernatants on the ability of PMN to phagocytose *Candida albicans* is indicated in Fig. 4. Phagocytosis was markedly enhanced in the presence of active supernatants and also when PMN were pulsed with active supernatants prior to assaying their phagocytic capacity.

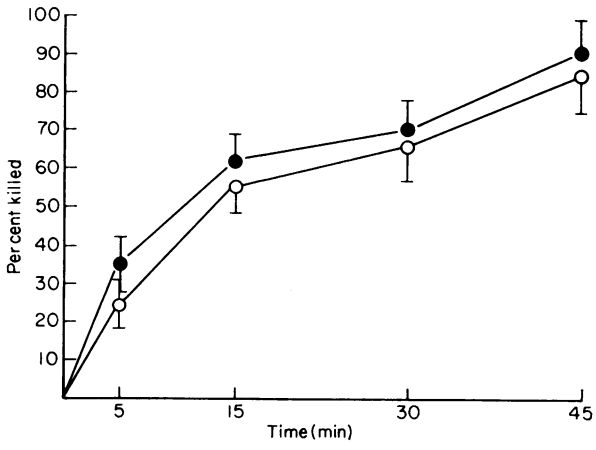

FIG.3.

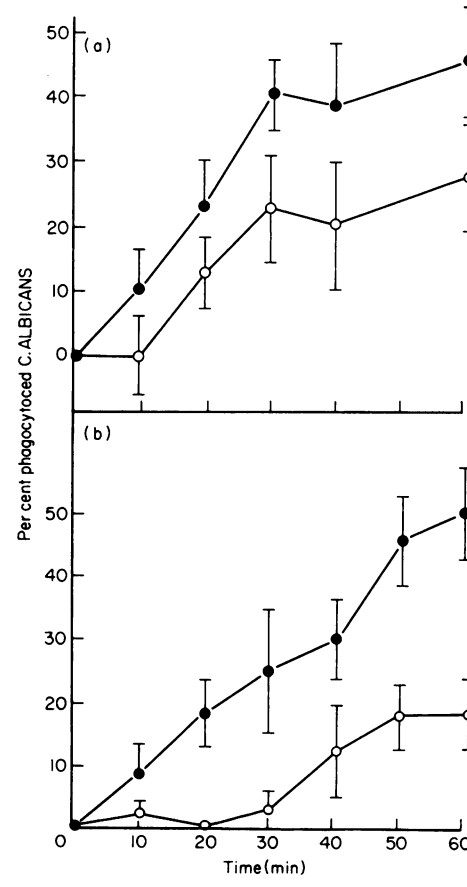

FIG. 4

FIG. 3. The effect of active (●) or control (○) supernatants on PMN bactericidal ability (mean±s.e.m.).
FIG. 4. PMN phagocytosis (a) in the presence of active (●) or control (○) supernatants and (b) after treatment with the supernatants (mean±s.e.m.).

The effect of supernatants on NBT reduction by PMN

A significantly higher percentage of NBT-positive cells was noted in the presence of active supernatants (Table 4).

The effect of supernatants on glucose oxidation

As can be seen from Fig. 5, oxidation of glucose to CO_2 through the HMPS was markedly enhanced when the reaction was performed in the presence of active supernatants. This effect was apparent at 30 min and became even more marked with time.

TABLE 4. The effect of control or active supernatants on NBT reduction

PMN treatment	NBT-reducing cells (±s.e.m.) (%)	*P* value
Control supernatant	5·6±0·6	< 0·001
Active supernatant	17·8±1·5	

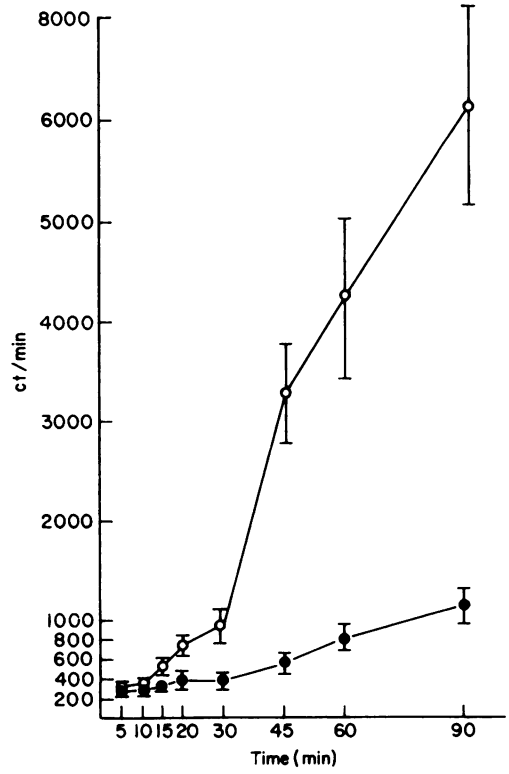

FIG. 5. The effect of active (○) or control (●) supernatants on PMN HMPS activity (mean±s.e.m.).

DISCUSSION

Results presented here indicate specific removal of LIF activity from PHA-activated lymphocyte supernatants by absorption with pure PMN, while absorption with human MN cells or guinea-pig peritoneal exudate cells fail to produce a similar effect. Such removal might be due either to specific

interaction between the mediator and the PMN, or to the inactivation or breakdown of LIF by the absorbing PMN. The direct correlation between the removal of LIF activity from the supernatants and the increased inhibition of the absorbing cells favours a mediator–receptor interaction and a receptor model for LIF has been suggested by Bendtzen (1975) and Rocklin (1976). A similar model has been suggested for the interaction between guinea-pig macrophages and MIF. Leu *et al.* (1972) reported absorption of MIF by peritoneal macrophages (but not by alveolar macrophages) resulting in MIF depletion from the active supernatant, corresponding with acquired inhibition of the absorbing cells.

The finding that a specific LIF–PMN interaction occurs, and the previous finding (Lomnitzer *et al.*, 1976b) that LIF may act by affecting the microfilament system, which is thought to be attached to the cell membrane (Reaven & Axline, 1973), led us to investigate the effect of active supernatants containing LIF activity on PMN surface properties and functions.

Treatment of PMN with active supernatants resulted in increased electrophoretic mobility, indicating an increase of the cell-surface negative charge. The exact mechanism responsible for this alteration in surface charge is unknown. It could be due to the direct effect of attachment of a charged molecule (?LIF) onto the membrane, and proteolytic activity attributed to LIF (Rocklin, 1975; Bendtzen 1976) could cause an exposure and/or removal of charged groups on the cell surface.

The effect of the supernatants on PMN directional motility towards endotoxin-activated serum was studied in two ways: by a pulse exposure of the PMN to the supernatants prior to assaying their chemotactic mobility, and by assessing their directional motility in the presence of supernatants. The pulse-exposure experiments did not show a significant difference between the directional motility of PMN treated with control or active supernatants. However, the chemotactic mobility of PMN was diminished in the presence of active supernatants. This decreased directional mobility could be due to increased adherence of PMN in the upper chamber, but the failure to demonstrate decreased chemotaxis after a 1 hr pulse exposure of the PMN to the supernatant (a procedure which produced substantial inhibition in the capillary migration system) raises the possibility that a different mechanism, perhaps reversible, may be responsible. As yet, it is not known whether this effect can be directly attributable to LIF, or whether it is due to some other factor present in the supernatant. The above supernatants did not contain chemotactic activity for PMN, although such activity was demonstrated in antigen-activated guinea-pig supernatants (Ward, Remold & David, 1970) and human lymphoid cell supernatants (Yoshida *et al.*, 1976).

Three PMN functions, phagocytosis of *Candida albicans*, NBT reduction and glucose oxidation through the HMPS were markedly stimulated in the presence of active supernatants. In the case of phagocytosis, stimulation was also evident after a 1 hr pulse exposure. Enhanced phagocytosis by guinea-pig macrophage monolayers in the presence of MIF-containing supernatants has been described by Nathan *et al.* (1971), and similar findings were reported by Thrasher *et al.* (1973). Phagocytosis is most probably a microfilament-dependent activity (Malawista, 1971; Tizard & Holmes, 1974), and its stimulation in the presence of active supernatant might well be due to the action of LIF which has previously been shown to affect microfilaments (Lomnitzer *et al.*, 1976b). It is possible, however, that the increased phagocytosis was due to the marked stimulation of cell metabolism by active supernatants.

A higher percentage of NBT-reducing cells was obtained in the presence of active supernatants, which also markedly enhanced glucose oxidation by PMN through the HMPS. Preliminary data showing that active supernatants lost their capacity to effect NBT reduction after absorption with PMN (unpublished observation) suggests that LIF may be responsible for the activity. Similar findings of the effect of MIF on macrophage NBT-reducing capacity (Krueger, Ogden & Weston, 1976) and HMPS activity (Nathan *et al.*, 1971) have been reported. Since oxidated nicotinamide adenine dinucleotide phosphate (NADPH), which is produced by the HMPS, is involved in synthesis of membrane lipids (Oren *et al.*, 1963), stimulation of HMPS in the presence of active supernatants may reflect increased turnover or even net synthesis of membranes involved in locomotion and adherence, as suggested by Nathan *et al.* (1971).

The oxidation of reduced pyridine nucleotide has been shown to take part in the generation of hydrogen peroxide (Iyar, Islam & Quastel, 1961), which plays an important rôle in bactericidal activities of

PMN (Klebanoff, 1967). In the experiments described here, however, an increase in bactericidal capacity of PMN treated with active supernatants could not be detected and similar results in a MIF–macrophage system were obtained by Simon & Sheagren (1972).

Lymphokines, factors with various biological activities, affect numerous cells involved in the inflammatory response such as macrophages, neutrophils, eosinophils and basophils (Cohen, 1976). Results presented in this paper indicate that active supernatants affect a number of PMN functions including chemotaxis, phagocytosis, NBT reduction and HMPS activity. The *in vivo* significance of these effects is still obscure, but they might modulate both early steps in inflammation, such as margination and adherence to the vascular endothelium, and later steps involving the directional movement of the cells to the inflammatory site and their activities at that site.

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