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The enhancement of eosinophil function by lymphocyte supernatants

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SUMMARY

Supernatants obtained from non-stimulated lymphocytes, lymphocytes stimulated with phytohaemagglutinin and lymphocytes from patients with schistosomiasis that were stimulated with *Schistosomiasis haematobium* ova were shown to enhance a number of eosinophil functions. Eosinophil chemotaxis, phagocytosis, microbicidal activity, Nitro blue tetrazolium reduction, hexose monophosphate shunt activity and glycolysis were increased. Eosinophil iodination was not affected. Only those supernatants obtained from phytohaemagglutinin stimulated lymphocytes and lymphocytes from patients with schistosomiasis that were stimulated with *S. haematobium* ova showed eosinophil chemotactic activity. The active factor was found to be heat stable, and had no effect on cAMP and cGMP metabolism. The most likely mechanism of enhanced eosinophil function is through the increased activity of the hexose monophosphate shunt activity and glycolysis.

INTRODUCTION

A paucity of information exists in our knowledge of human eosinophil–lymphocyte interactions. Kazura *et al.* (1975) and Wadee & Sher (1980a) showed that a lymphokine derived from the lymphocytes of patients with schistosomiasis that were activated either by schistosomal egg antigen or schistosome eggs respectively, enhanced the directed migration of eosinophils from patients with or without schistosomiasis. In the early seventies Basten & Beeson (1970) and McGarry *et al.* (1971) reported a number of experiments in rodents that collectively indicated that thymic derived or thymic-dependent cells, which are present in the thymus, circulating blood or lymphatics, will induce a local accumulation of eosinophils and an increased eosinopoiesis in the presence of antigen. The mechanism of this latter effect was through the production of a diffusible lymphocyte derived factor which augmented the capacity of haemopoietic stem cells to mature into eosinophils.

Subsequent reports by Walls *et al.* (1971) and Schriber & Zucker-Franklin (1975) confirmed the role of lymphocytes in the production of eosinophilia in response to antigen. Furthermore, Ruscetti, Cypess & Cherverick (1976) reported the specific release of neutrophilic and eosinophilic stimulation factors from sensitized lymphocytes. Whether lymphocytes or products elaborated by these cells are capable of influencing other eosinophil functions is not known. The intent of these experiments was to study the effect of supernatants derived from stimulated and non-stimulated lymphocytes *in vitro* on the following eosinophil functions: chemotaxis, phagocytosis, microbicidal

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activity, glycolysis, hexose monophosphate shunt activity (HMPS), iodination, NBT reduction and cyclic nucleotide metabolism.

MATERIALS AND METHODS

Preparation of leucocytes. Eosinophils were obtained from patients with eosinophilias due to a variety of causes and were purified according to the method of Sher & Glover (1976). Neutrophils were derived from healthy donors and were purified as for eosinophils except for the carbonyl iron stage. Populations of eosinophils and neutrophils were over 90% pure. Lymphocytes were obtained from either healthy volunteers or from patients with *Schistosomiasis haematobium* infestation, and were separated on a Hypaque-Ficoll density gradient.

Chemotaxis. Chemotaxis was assessed using a modified Boyden Chamber as previously described (Wadee & Sher, 1980b). Eosinophils were made up to a concentration of 2×10^6/ml in Tris-buffered Hank's balanced salt solution (HBSS). Filters were stained according to the method of Wadee & Sher (1978) and the number of cells which had completely traversed the filter were counted, using high power light microscopy. All experiments were performed in duplicate and the mean recorded. A 1 in 4 dilution of the following lymphocyte supernatants were used as leukoattractants: (1) supernatants derived from the lymphocytes of patients with schistosomiasis that were stimulated with *S. haemotobium* ova, (2) normal lymphocytes stimulated with PHA, (3) unstimulated lymphocytes and (4) heat-inactivated (56°C for 30 min) supernatants from PHA stimulated lymphocytes.

Phagocytosis. Phagocytosis was assessed by the ingestion of *Candida albicans.* The reaction mixtures contained two organisms per cell with 10% fresh AB serum. The reaction mixture was incubated at 37°C on a turntable and aliquots were removed every 5 min. Phagocytosis was expressed according to the formula:

$$\% \text{ phagocytosis} = 100 - \frac{\text{No. of extracellular organisms}}{\text{No. of organisms in the non-phagocytic control}} \times 100.$$

Prior to the reaction mixture being constituted the cell suspensions were incubated with equal volumes of the particular lymphocyte supernatants to be tested for 30 min at 37°C. The cells were then centrifuged gently and half the volume of supernatant was removed and the cells resuspended.

Bactericidal ability. This was assessed using the method of Quie *et al.* (1967). The difference in counts between test and control systems was assessed and expressed as the percentage of bacteria killed according to the following formula:

$$\% \text{ killing} = 100 - \frac{\text{No. of colonies in the test system}}{\text{No. of colonies in the control}} \times 100.$$

Prior to constituting the reaction mixtures, eosinophils were incubated with various lymphocyte supernatants for 30 min at 37°C, centrifuged and the pellet washed twice with Gey's medium. To ensure that no antibiotics were present in the cell suspensions after two washings with Gey's medium, aliquots of the medium derived after two washings were incubated with bacteria and compared to the normal controls. A close correlation was always found.

METABOLIC ACTIVITIES

Assay for hexose monophosphate shunt activity

HMPS activity was assessed by the production of $^{14}CO_2$ from glucose-1-^{14}C D-Glucose-1-^{14}C; (New England Nuclear, Boston, Massachusetts, USA) by the method of Wood, Katz & Landau (1963). The cells were incubated initially with equal volumes of lymphocytes supernatants for 30 min at 37°C and then washed twice with PBS to remove glucose present in the supernatants. All tests were performed in duplicate and expressed as mean counts per minute (c.p.m.). Due to serum inhibition of HMPS activity, presumably due to a competitive effect of serum glucose, results obtained from

experiments involving the use of serum were corrected for this factor by subtracting serum control counts from the experimental values.

Assay for glycolytic activity

The extent of glycolysis was measured by lactate production as described by Hohorst (1962) using recognized procedures (Boehringer, Mannheim, Biochemical Test Combination). Eosinophil suspensions were incubated with equal volumes of various lymphocyte supernatants for 30 min at 37°C, washed twice with PBS and resuspended in 0·15 M PBS containing 10 mM glucose. Results were expressed as μg lactate/6×10^6/ml/unit time.

Iodination

Iodination was determined by the ability of cells to incorporate ^{125}I into trichloroacetic acid (TCA) precipitable material according to the method of Pincus & Klebanoff (1971).

Nitroblue tetrazolium reduction (NBT)

NBT reduction was measured according to the method of Sher *et al.* (1974). Stimulation of cells was achieved by the addition of equal volumes of endotoxin activated serum and lymphocyte supernatants to cell suspensions. Cells containing the blue formazan precipitate were counted as positive and expressed as a percentage.

In all the metabolic studies the various cell suspensions were incubated at 37°C for 30 min with equal volumes of the various supernatants, centrifuged and washed twice with the medium used for the various tests and then reconstituted to the required concentration.

Lymphocyte supernatants

Mononuclear cells (MN) were separated from heparinised peripheral blood on Ficoll-Hypaque gradients and resuspended in MEM plus 10% heat-inactivated AB serum.

The MN cells were divided into two aliquots. The first was pulsed with 10 μg/ml PHA (reagent grade) at 37°C in 5% CO_2 in air for 2 hr, washed twice with MEM and reconstituted with complete MEM to the original volume (active supernatants or PHA-S). The second was pulsed with medium alone without PHA (control-S). Both aliquots were incubated in plastic tissue culture flasks for 72 hr at 37°C in 5% CO_2 in air, after which cell free supernatants were obtained by centrifugation of the cultures at 250*g* for 10 min. The supernatants were stored at −20°C until required.

For the preparation of supernatants obtained from immune MN cells stimulated with *S. haematobium* ova, the procedure followed has been previously described (Wadee & Sher, 1980a). In a further series of experiments the supernatants derived from PHA stimulated and non-stimulated lymphocytes were harvested after 24, 48 and 72 hr. To demonstrate that lymphocytes were the source of the soluble factors, monocytes were removed from the MN cells by allowing them to adhere to petri dishes for 18 hr after which time the non-adherent cells were harvested by washing with warm (37°C) MEM. The purity of the lymphocyte population was determined by non-specific esterase activity. Lastly, lymphocytes were separated into enriched T and B cell populations using rosetting techniques as described previously (Wadee, Sher & Rabson, 1980).

Characterization of the active factor present in the lymphocyte supernatants

The lymphocyte derived factor was characterized by heat inactivation (56°C for 1 hr), dialysis (48 hr at 4°C against MEM) and separated by molecular sieve chromatography (Sephadex G-75). Samples were pooled into 6 ml volumes and absorbance at 280 nm was measured with an u.v. spectrophotometer (Unicam SP1800). Fractions were tested by their ability to enhance phagocytosis.

Estimation of cyclic nucleotides (cAMP, cGMP)

Purified eosinophils were resuspended to a concentration of 2×10^7/ml in Tris-HBSS. Equal volumes (0·5 ml) of cell suspension and lymphocyte supernatants were incubated at 37°C in a shaking waterbath. The reaction was terminated by the addition of an equal volume of 2% perchloric acid at 0,1,3,5,10 and 20 min. Control systems contained equivalent volumes of

Tris-HBSS. Eosinophils were disrupted in a MSE ultrasonic disintegrator. Specimens were subsequently centrifuged at 5000 *g* for 10 min at 4°C, the supernatants harvested and restored to pH 7 by the addition of 0·3 N KOH and aliquots were used for cAMP and cGMP estimations. Radioimmunoassay kits (Schwarz/Mann) were used for the estimations. Samples were assayed in duplicate and the final results expressed as pmoles/10^7 eosinophils.

RESULTS

Chemotaxis

Four different preparations of lymphocyte supernatants were tested for their effect on eosinophil chemotaxis (Table 1). The greatest effect was exerted by the supernatant derived from lymphocytes from patients with schistosomiasis that were incubated with *S. haematobium* ova. Both normal and inactivated PHA-S possessed chemotactic ability but this was less than that of the immune lymphocytes stimulated by *S. haematobium* ova. Control-S had no chemotactic effect on eosinophils.

Phagocytosis

Comparative phagocytosis of *C. albicans* by neutrophils and eosinophils is shown in Fig. 1. Phagocytosis by neutrophils was always greater than that of eosinophils and this was significant at 10 min ($P < 0{\cdot}005$), 15 min ($P < 0{\cdot}005$) and 20 min ($P < 0{\cdot}05$). Prior incubation of eosinophils with

Table 1. The effect of various lymphocyte supernatants on eosinophil chemotaxis

Types of supernatants	Number of cells/HPF
Control medium	17
Immune lymphocytes + *S. haematobium* ova	75
PHA stimulated lymphocytes	35
Heat-inactivated PHA stimulated lymphocytes	31
Control supernatants (non-activated)	16

Supernatants were used in dilutions of 1:4.

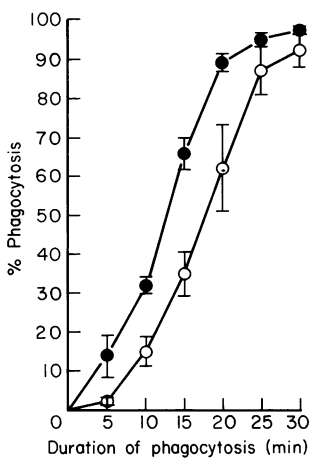

Fig. 1. Comparative phagocytosis of *C. albicans* by neutrophils (●——●) and eosinophils (○——○). Mean ± s.e.m. of six experiments. Significant differences in phagocytosis was found at 10 min ($P < 0{\cdot}005$), 15 min ($P < 0{\cdot}005$) and 20 min ($P < 0{\cdot}005$).

supernatants obtained from PHA stimulated lymphocytes resulted in enhanced phagocytosis of *C. albicans* by eosinophils to levels almost equivalent to that of neutrophils (Fig. 2). When compared to non-stimulated eosinophils, stimulated eosinophils showed increased phagocytosis (Fig. 3). Both PHA-S and control-S enhanced the phagocytic ability of eosinophils compared to non-stimulated eosinophils. The enhancing effect of both supernatants were similar and was significant at 5 and 10 min ($P < 0{\cdot}05$ and $P < 0{\cdot}02$) respectively. The medium itself was found to have no enhancing effect. Absorption of lymphocyte supernatants with neutrophils failed to reduce the enhancing effect of the supernatants. Inactivation (56°C for 30 min) of PHA-S and control-S also failed to alter the enhancing capacity of the supernatants.

Table 2 shows the results obtained from experiments in which supernatants were derived from various preparations. Results obtained from supernatants harvested at different times suggest that

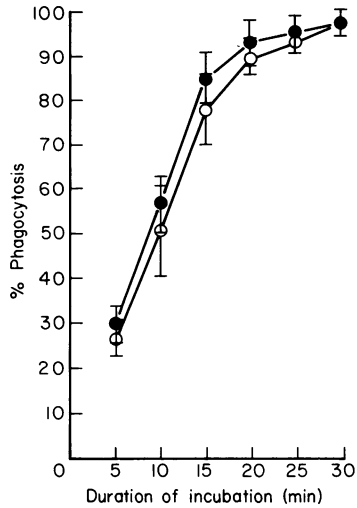

Fig. 2. Time kinetic studies of neutrophil ●——● and eosinophil ○——○ (pre-incubated with lymphocyte supernatants) phagocytosis of *C. albicans*. No significant difference was found. Mean ± s.e. of four experiments.

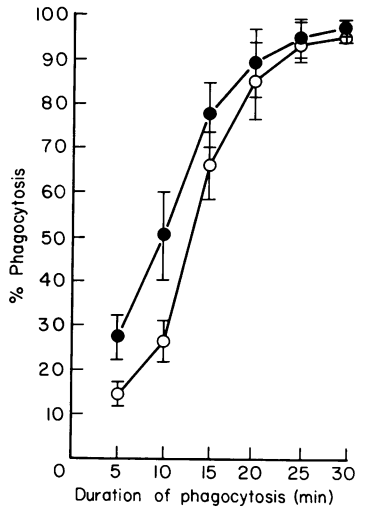

Fig. 3. Time kinetic studies of phagocytosis of *C. albicans* by eosinophils pre-incubated with PHA-S ●——● and untreated eosinophils ○——○. There was a significant difference at 5, 10 and 15 min. $P < 0{\cdot}05$, $P < 0{\cdot}05$ and $P < 0{\cdot}01$ respectively. Mean ± s.e. of four experiments.

the factor responsible for this effect was produced within the first 24 hr. Furthermore, results obtained from the different cell sources suggest that the factor is most likely derived from cells of the T lymphocyte lineage.

When various supernatants were removed from the eosinophil environment by washing with medium, the enhancing effect was lost. This procedure in fact resulted in an impairment of eosinophil phagocytic ability. In experiments in which supernatants were added to neutrophils, their effects on phagocytosis were inconsistent. In six out of 11 experiments lymphocyte supernatants potentiated neutrophil phagocytosis. In the remaining five experiments, however, no enhancement was evident (results not shown). In those experiments where enhancement of neutrophil phagocytosis occurred, removal of the supernatants by washing resulted in decreased phagocytosis.

Microbicidal activity of eosinophils

Eosinophils stimulated with various lymphocyte supernatants were found to have enhanced killing ability against *S. aureus* and *E. coli* (Figs 4 & 5 respectively).

Table 2. The effect of various types of supernatants on eosinophil phagocytosis of *C. albicans**

Nature of supernatants	Number of experiments	Duration of incubation (min) 5	10	15	20	25	30
Harvested after 24 hr†	3	2·43±0·56	1·54±0·19	1·12±0·01	1·09±0·05	1·02	1·02
Harvested after 48 hr	3	2·46±0·59	1·61±0·26	1·12±0·01	1·04±0·01	1·02±0·01	1·01±0·01
Harvested after 72 hr	3	2·30±0·48	1·57±0·48	1·12±0·01	1·04±0·01	1·02	1·01±0·01
Derived from enriched lymphocytes	3	1·94±0·45	1·8±0·18	1·17±0·06	1·04±0·02	1·02±0·01	1·01
Derived from enriched T cells	2	3·37±0·31	1·45±0·01	1·27±0·22	1·03±0·02	1·02±0·01	1·00
Derived from enriched B cells	2	1·64±0·14	0·94±0·07	1·1±0·09	0·98±0·02	1·00	1·00
Following removal of supernatants by two washings with medium	3	1·23	0·92	0·06	0·71	0·11	0·81	0·13	0·57	0·26	0·88±0·06

*Results are expressed as the ratio $\left(\frac{\%\text{ phagocytosis of stimulated eosinophils}}{\%\text{ phagocytosis of non-stimulated eosinophils}}\right)$ mean ± s.e.

†Supernatants derived from non-stimulated lymphocytes (control supernatants).

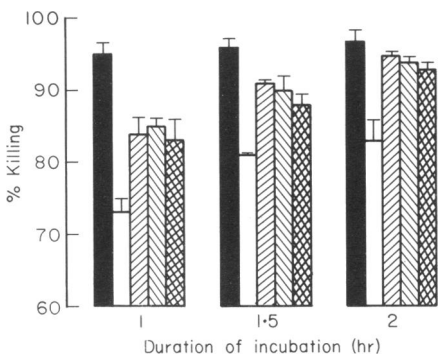

Fig. 4. Killing of *S. aureus* by (a) neutrophils (■), (b) eosinophils (□), (c) eosinophils stimulated with PHA-S (▨), (d) eosinophils stimulated with control-S (▩) and (e) eosinophils stimulated with heat-inactivated PHA-S (▩). Eosinophils treated with the three types of supernatants showed significant increases in killing ability compared to untreated eosinophils. Results expressed as the mean ± s.e. of four experiments.

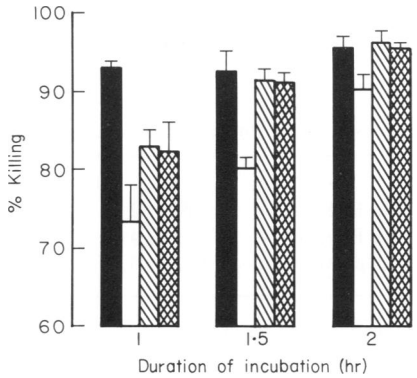

Fig. 5. Killing of *E. coli* by various cell preparations (a) neutrophils (■), (b) untreated eosinophils (□), (c) eosinophils stimulated with PHA-S (▨) and (d) eosinophils stimulated with control-S (▩). Neutrophils showed significantly greater killing than untreated eosinophils. Although PHA-S treated eosinophils showed greater killing capacity than untreated eosinophils these were not always significant (NS, $P<0{\cdot}01$, NS) respectively. Control-S treated eosinophils similarly showed increased microbicidal capacity (NS, $P<0{\cdot}001$, $P<0{\cdot}05$) respectively. Results expressed as the mean ± s.e. of four experiments.

Metabolic studies

All four types of lymphocyte supernatants were found to increase the HMPS activity of eosinophils both at rest and following phagocytosis (Table 3). The incubation of eosinophils with PHA-S and control supernatants resulted in increased glycolysis when compared to the resting state of the cells. However, these supernatants had no effect on eosinophil iodination. Both control-S and PHA-S increased NBT reduction of resting eosinophils (Table 4). These supernatants also showed increased NBT reduction by neutrophils.

Molecular weight

Using molecular sieve chromatography (Sephadex G-75) the molecular weight of the active component of the supernatants appears to lie in the range 55,000–98,000.

The effect of lymphocyte supernatants on cAMP and cGMP

The various lymphocyte supernatants had no effect on the levels of cAMP and cGMP.

Table 3. The effect of various lymphocyte supernatants on the hexose monophosphate activity during the resting state and phagocytosis*

	Hexose monophosphate shunt activity	
Type of supernatant	Resting state	After phagocytosis
PHA stimulated lymphocytes	1·97 ± 0·19†	1·29 ± 0·13
Non-stimulated lymphocytes	3·08 ± 0·79	1·11 ± 0·01
Immune Lymphocytes stimulated with *S. haematobium* ova	2·33 ± 0·56	1·41 ± 0·37
Inactivated PHA stimulated lymphocytes	2·52 ± 0·45	1·14 ± 0·07

Results expressed as the ratio of: $\frac{\text{resting or phagocytozing cells stimulated by various supernatants}}{\text{non-stimulated resting or phagocytosing cells}}$.

†Mean ± s.e. of three experiments.

Table 4. Nitro blue tetrazolium reduction by resting and activated polymorphonuclear cells and eosinophils

State of the cells	Cell types	
	Polymorphonuclear cells	Eosinophils
Resting	3·75±0·48†	1·8±0·66
Activated with EAS*	39·7±2·03	15·75±1·65
Activated with PHA-S	31±4·6	10±2·65
Activated with control-S	17·33±3·53	8·67±1·45

*Endotoxin activated serum.
†Mean±s.e. of four experiments.

DISCUSSION

This study has demonstrated that a number of eosinophil functions such as chemotaxis, phagocytosis, microbicidal activity, glycolysis, hexose monophosphate shunt activity and NBT reduction were enhanced in the presence of supernatants obtained from various lymphocyte cultures. Experiments involving eosinophil chemotaxis revealed that the enhancing factor could only be produced from lymphocytes that had been stimulated by antigens or mitogens. The greatest effect was achieved by supernatants stimulated by *S. haematobium* ova. This chemotactic enhancement is similar to that of Colley's eosinophil stimulation promoter (ESP) obtained from mouse lymphocytes stimulated with antigens or mitogens (Colley, 1973). Other workers (Kazura *et al.* 1975; Wadee & Sher 1980b) have reported that lymphocytes previously stimulated released a factor that showed an enhancing capacity for eosinophil chemotaxis. The finding of the present study demonstrating that supernatants from non-stimulated lymphocytes failed to induce eosinophil chemotaxis, but enhanced other eosinophil functions, suggests that at least two different factors were present in the supernatants. By definition (Dumonde *et al.*, 1969; Pick, 1972) the factor liberated by stimulated lymphocytes in culture that enhanced eosinophil chemotaxis is most likely a lymphokine. Results obtained by molecular sieve column chromatography showed that the molecular weight of the chemotactic enhancing factor was in the range of 23,000–56,000 whilst the molecular weight of the factor responsible for enhanced phagocytosis was in the range 55,000–98,000. This indicates that two separate mediators were present in the supernatants. Whether the factor found in control-S is a lymphokine is controversial. It has been shown that T lymphocytes can be stimulated by non-T (B or K) lymphocytes in the autologous mixed lymphocyte reaction (Opelz *et al.*, 1975; Kuntz, Innes & Weksler, 1976). Furthermore, separation techniques or the presence of foetal calf serum have been shown to be capable of inducing lymphocytes to produce lymphokines (Arvilommi *et al.*, 1978). Previous studies (Arvilommi & Räsänen, 1975) showed that lymphocytes in culture appeared to produce a lymphokine like inhibitory substance without any stimulus. Similar findings have also been reported by Anders & Natvig (1976). In subsequent studies Arvilommi *et al.* (1978) demonstrated that it was the B lymphocyte that elaborated the lymphokine. Our experiments with enriched populations of T and B lymphocytes suggest that the active factor(s) were derived mainly from T lymphocytes. Experimental evidence suggests that T lymphocytes in the control cultures may well have been activated during culture and that the supernatants from these cultures contained effector molecules that could be defined as lymphokines. A further mechanism whereby control-S could enhance certain eosinophil functions is that during culture, cell surface antigens could be released into the supernatants either from dead or live cells and that these macromolecules could, by perturbating the eosinophil plasma membrane, cause increased metabolic activity.

The broad spectrum of enhanced eosinophil function brought about by lymphocyte supernatants is probably best explained by the stimulation of a common denominator underlying most of these functions, namely certain metabolic functions. This study has shown that lymphocyte

supernatants were capable of enhancing the hexose monophosphate shunt activity, glycolysis and NBT reduction. The stimulation of metabolic functions of granulocytic cells by a number of membrane active agents such as EAS (Cline *et al.*, 1968; Anderson, Glover & Rabson, 1978), phorbol myrisate (De Chatelet, *et al.*, 1977) and phagocytosis (De Chatelet, 1978; Cline, Hanifin & Lehrer, 1968) has been previously reported. It would thus seem that one of the mechanisms of enhanced eosinophil function may be due to the stimulation of glycolysis and hexose monophosphate shunt activity. Iodination of micro-organisms by PMN is thought to result from myeloperoxidase associated incorporation of iodide into the tyrosine residues of microbial protein (Klebanoff & Clark, 1977). In disease states such as chronic granulomatous disease and myeloperoxidase deficiency, defects in the iodinating capacity of PMN correlate well with defects in microbicidal activity (Pincus & Klebanoff, 1971). In the present study, no enhancement of eosinophil iodination by lymphocyte supernatants could be demonstrated even though the microbicidal capacity was increased. This might reflect an enhancement of myeloperoxidase independent mechanisms of killing in the eosinophil. Lehrer (1972, 1975) demonstrated that myeloperoxidase deficient monocytes and neutrophils could kill certain Candida species more effectively than normal monocytes.

The finding that lymphocyte supernatants do not alter cylic nucleotide metabolism to any significant degree suggests that there may be an alternative mechanism of activation. De Chatelet (1978) has suggested that stimulation could occur via the activation of an oxidative enzyme in the nature of an 'ectoenzyme' localized on the exterior of the cell membrane.

As a result of the experiments conducted in this study it cannot be concluded that these enhancing factors were specific for eosinophils. Although absorption of the supernatant with neutrophils failed to eliminate the enhancing effect on eosinophils, the addition of these supernatants to neutrophils showed an enhancing effect in 50% of the experiments.

The finding of diminished phagocytosis of both eosinophils and neutrophils following the removal of the supernatant by washing was an unexpected phenomenon which is difficult to explain. A possible mechanism for this diminished phagocytosis is that these soluble molecules formed complexes with certain plasma membrane structures, such as opsonic receptors and that during the washing procedures these 'complexes' were shed resulting in a decreased number of receptors for opsonins.

Enhancement of eosinophil microbicidal activity by lymphocyte supernatants has not been previously reported. This is most likely not due to accelerated phagocytosis as the technique employed requires the removal of the supernatants by washing, a procedure which has been shown to be associated with decreased phagocytosis in this study. The increased microbicidal activity is more likely due to the augmented metabolic processes such as HMPS, glycolysis and NBT reduction. These processes are associated with phagocytosis and the intracellular killing ability of phagocytic cells.

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