ENHANCEMENT OF HUMAN EOSINOPHIL-MEDIATED KILLING
OF SCHISTOSOMA MANSONI LARVAE BY MONONUCLEAR CELL
PRODUCTS IN VITRO

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Previous studies have shown that the human eosinophil is a major effector cell in the complement-independent, antibody-dependent killing of the invasive larval stage (schistosomulum) of Schistosoma mansoni in vitro (1, 2). Early studies were carried out with eosinophils from normal, noneosinophilic individuals. However, helminth infections, including schistosomiasis, are consistently associated with increased levels of eosinophils in the blood. Consequently, a formal comparison of the killing capacity of eosinophils from noneosinophilic and from moderately eosinophilic, helminth-infected patients was made, which showed that eosinophils from moderately eosinophilic individuals were markedly more active in killing schistosomula than were cells from noneosinophilic individuals (3). This suggested that eosinophilia may involve not only an increased number of eosinophils in the blood, but also an increased functional activity of the individual cells.

Since previous work had suggested that eosinophilia is a thymus-dependent phenomenon (4, 5), possibly attributable to soluble mediators (6, 7), the present study was undertaken in an attempt to relate eosinophilia to enhanced eosinophil function, by testing the hypothesis that mononuclear cells from eosinophilic individuals, whose own eosinophils show an increased killing capacity, might produce an activity that could stimulate normal eosinophils and increase their cytotoxic potential. A brief description of our preliminary findings has previously been published (8).

Materials and Methods

Media and Reagents. The following media and reagents were used in these experiments: MEM: 1 Eagle's Minimal Essential Medium (Flow Laboratories), containing 20 mM Hepes, 100 U/ml penicillin, 100 µg/ml streptomycin, and 30 µg/ml DNase, pH 7.3. MEM/FCS: MEM supplemented with 10% fetal calf serum (FCS) (Seralab), heat-inactivated at 56°C for 1 h. PBS: Phosphate-buffered saline, pH 7.3. PBS azide was supplemented with 0.1% sodium azide. Con A: Concanavalin A (Type IV, Sigma Chemical Co.) prepared as a stock solution at 1 mg/ml in MEM and used at 10 µg/ml in either MEM or MEM/FCS. Emetine dihydrochloride

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Abbreviations used in this paper: Ab, antibody; Con A, concanavalin A; ECF-A, eosinophil chemotactic factor of anaphylaxis; E.CSF, eosinophil colony-stimulating factor; Eos, eosinophils; ESP, eosinophil stimulation promoter; FCS, fetal calf serum; HPCM, human placental conditioned medium; IHS, immune human serum; LPS, lipopolysaccharide; MCS, mononuclear cell supernatant; Med, medium; MEM, Eagle's minimal essential medium; Neutros, neutrophils; NHS, normal human serum; PBS, phosphate-buffered saline; TCA, trichloracetic acid.

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(Boehringer) prepared as a stock solution of 100 mM in distilled water and used at $1 \times 10^{-4}$M in MEM/FCS.

*Schistosoma mansoni Life Cycle and Preparation of Schistosomula.* A Puerto Rican isolate of *S. mansoni* is routinely maintained in this laboratory by passage through laboratory-bred * Biomphalaria glabrata* snails and BALB/c mice. Schistosomula were prepared by allowing infective cercariae to penetrate an isolated preparation of rat skin in vitro, according to the method of Clegg and Smithers (9).

**Sera.** Sera taken either from baboons chronically infected with *S. mansoni* or pooled from patients with *S. mansoni* infection were used as a source of antibody. Normal human sera were prepared from noninfected, noneosinophilic laboratory workers. Sera were heat-activated at 56°C for 1 h and were stored in frozen aliquots at -20°C.

**Preparation of Cells.** Mononuclear cells, neutrophils, and eosinophils were purified from heparinized human peripheral blood by dextran sedimentation and centrifugation of the resulting leukocyte-rich supernatant through slightly hypotonic, discontinuous metrizamide gradients, as described by Vadas et al. (10). Preparations containing >90% mononuclear cells, neutrophils, or eosinophils were routinely obtained from the 18%, 22%, and 24% interfaces respectively. Such cells were washed three times in MEM/FCS, counted, and resuspended at an appropriate concentration. Cells in the original peripheral blood and in the purified fractions were counted with the aid of a Coulter counter (Coulter Electronics Limited), and differential counts were carried out on Giemsa-stained blood smears or cytocentrifuge preparations. Cell viability was checked by Trypan Blue exclusion.

In some experiments, mononuclear cells were prepared by an alternative method. Peripheral blood from moderately eosinophilic individuals was defibrinated, diluted 1:1 vol/vol in MEM (without FCS), and layered over an equal volume of Lymphoprep (Nyegaard). After centrifugation for 40 min at 400 g, the overlaying serum was removed and the interface cells were collected, washed four times in MEM, counted, and resuspended at an appropriate concentration.

**Preparation of Mononuclear Cell Supernatants (MCS).** For routine preparation of MCS, metrizamide-fractionated mononuclear cells from normal or moderately eosinophilic donors (100-1,900 eosinophils/mm$^3$) were cultured in MEM/FCS in a humidified atmosphere of 5% CO$_2$/95% air, at 37°C, at a concentration of $3-10 \times 10^6$ cells/ml, in round-bottomed tubes containing 1-2-ml volumes. Cell-free supernatants were collected after varying periods of time, usually 24 h, and were filtered and stored at -20°C. In some experiments, Lymphoprep-fractionated cells were cultured under similar conditions in serum-containing or serum-free media.

Adherent mononuclear cell supernatants (MCS) were prepared as follows. Aliquots of $1 \times 10^7$ mononuclear cells were incubated in flat-bottomed plastic culture wells (Linbro multiwell plate, Flow Laboratories) in 1 ml MEM/FCS for 1 h at 37°C. Unattached cells were then removed and replated in another well for 1 h, whilst the remaining adherent cells were rinsed several times with MEM/FCS and reincubated for a further 2 h. After this period the adherent cells were washed vigorously with several changes of MEM/FCS, and were finally cultured for 24 h at 37°C, to provide the "adherent" cell supernatant. The "nonadherent" supernatants were obtained by serially replating the original unattached cells three times, to remove any remaining adherent cells, and by culturing the resulting nonadherent cells for 24 h as above.

**Treatment of Mononuclear Cells with an Antilymphocyte Monoclonal Antibody.** The rat monoclonal antilymphocyte antibody, CAMPATH 1, prepared from ascitic fluid by ammonium sulphate precipitation and reconstituted to 5 mg/ml in PBS, was kindly provided by Dr. H. Waldmann (Department of Pathology, University of Cambridge). This antibody kills >99% of all peripheral blood lymphocytes in the presence of human complement, but spares monocytes (Hale, G., S. Bright, G. Chumbley, T. Hoang, D. Metcalf, A. Munro, and H. Waldmann, submitted for publication).

Aliquots of $5 \times 10^6$ mononuclear cells from a moderately eosinophilic individual were incubated in 1 ml of either MEM/FCS or the monoclonal antibody (diluted 1/50 in MEM/FCS) for 30 min at 37°C. The cells were washed once, and resuspended in 0.5 ml of MEM/FCS or of fresh autologous serum (diluted 1/4 in MEM/FCS) as a source of complement, and reincubated for a further 45 min at 37°C. The cells were then washed three times, and resuspended in 1 ml of MEM/FCS. Cell viability and total cell numbers were determined,
before incubation for a further 24 h to obtain the MCS.

Fractionation of Mononuclear Cell Supernatants.

(a) ULTRAFILTRATION: Mononuclear cell supernatants derived from an eosinophilic individual were pooled and passed through a series of Amicon diaflo membranes (Amicon Corp.) at a pressure of either 50 or 70 lb/in² at room temperature. MEM was used to flush the filters and reconstitute the residue.

(b) SEPHADEX CHROMATOGRAPHY: A G-200 Sephadex column, 1.5 × 80 cm, prepared in PBS azide at 4°C, was calibrated with a mixture of protein standards (molecular weight range 21 to 210 × 10⁶), a trace amount of [³⁵S] tyrosine and dextran blue 2000. The column was then equilibrated in sterile MEM to remove the azide, and a pool of MCS from a single eosinophilic individual, previously concentrated 10-fold by ultrafiltration through a UM20E diaflo membrane, was applied. The fractions were collected and stored at -20°C. To test the enhancing capacity of the various fractions, the tubes were thawed and aliquots of each fraction were pooled in series of four, filtered, and then preincubated with eosinophils in the usual manner (below).

Cytotoxicity Assay. Aliquots of 50 μl of eosinophils were mixed and preincubated for 1 h at room temperature with 50 μl of mononuclear cell supernatant, at various dilutions, in 7-mm × 38-mm round-bottomed tubes (LP2, Luckham). Aliquots of 50 μl of antiserum, at an appropriate dilution, and of schistosomula, usually at 2,000/ml, were subsequently added, mixed, and incubated for a further 40–50 h at 37°C in humidified plastic boxes. Control preparations contained equivalent volumes of medium instead of eosinophils and/or antibody. At the end of the incubation period, schistosomulum viability was scored microscopically by toluidine blue exclusion (10).

Cell Protein Synthesis.

(a) [³⁵S] METHIONINE UPTAKE IN THE PRESENCE OR ABSENCE OF EMETINE: Peripheral blood mononuclear cells were washed twice in methionine-free MEM, counted and maintained in methionine-free medium for a further 15 min at 37°C. The cells were adjusted to 1 × 10⁶/ml, and 2-ml aliquots were dispensed into 15-ml conical tubes (Falcon Labware) with or without 1 × 10⁻⁴M emetine dihydrochloride. Immediately thereafter, 40 μCi [³⁵S] methionine was added to both emetine-treated and control tubes (0 h). Triplicate samples of 100 μl were taken from both tubes and were precipitated with 2 ml of 10% iced trichloracetic acid (TCA) in tubes containing 25 μl of 1% bovine serum albumin as a carrier protein. Samples were taken at 0 h and 1 h, whilst the cells remained incubating at 37°C. After 1 h, the cells were washed three times in methionine-free medium, resuspended in 1 ml and counted. More [³⁵S] methionine was added to both tubes and additional samples were collected after a further 1, 3, and 5 h. The resultant TCA precipitates were filtered onto Whatman GF/C filters, dried overnight at 37°C, solubilized, and counted in a liquid scintillation counter (LKB).

(b) MCS PRODUCTION IN THE PRESENCE OR ABSENCE OF EMETINE: Mononuclear cells from a moderately eosinophilic individual were incubated at 5 × 10⁶/ml, for 1 h or 3 h, at 37°C, with 1 × 10⁻⁴M emetine dihydrochloride. The cells were then washed three times and recultured for a further 3 or 5 h. Control preparations without emetine were treated identically. Viability of cells at all stages was >89%. The final cell concentrations ranged from 3 to 3.3 × 10⁶/ml. Supernatants were collected after the 3 or 5 h culture periods, filtered, and stored at -20°C before testing for enhancing activity.

Analysis of Data. Groups of replicate samples in the cytotoxicity and other assays were compared by the Student's t test.

Results

Eosinophils from Eosinophilic Individuals Demonstrate Increased Killing Activity. Previous studies have shown a significant correlation between peripheral blood eosinophil counts and the schistosomulum-killing activity of individual eosinophils (3). Eosinophils from moderately eosinophilic and normal individuals were then compared in more detail for their capacity to kill schistosomula in the presence of varying concentrations of antischistosomular antibody or at varying effector to target ratios (Fig. 1). Eosinophils from some eosinophilic individuals demonstrated markedly
increased levels of killing at all antibody concentrations tested, at a fixed effector to target ratio, and at all ratios tested, at a fixed antibody dilution.

Enhancement of Eosinophil-mediated Killing by Mononuclear Cell Supernatants (MCS). Supernatants of mononuclear cell cultures from eosinophilic individuals, whose own eosinophils showed high killing activity, significantly enhanced the antibody-dependent, complement-independent capacity of eosinophils to kill schistosomula in vitro. Such supernatants were active at dilutions of up to 1/400. 1 out of 60 experiments in which such enhancement was demonstrated is shown in Fig. 2. Immune human or immune baboon serum could be used equally effectively. In some experiments a slight enhancement of antibody-independent killing was observed, but this was a less pronounced and less consistent finding. The supernatants, although present throughout the assay, were not directly toxic to the schistosomula.

Comparison of the Effects of MCS from Normal and Eosinophilic Individuals. Undiluted supernatants from mononuclear cell cultures of both eosinophilic and normal individuals demonstrated an ability to enhance eosinophil-mediated killing (Table I, Expt. 1), but the capacity of supernatants from normal individuals to enhance killing, when detectable, tended to titrate out more acutely (Table I, Expts. 3, 4, and 5). Routinely, the MCS from four eosinophilic and four noneosinophilic individuals have been used, but similar results have been obtained with supernatants prepared from 16 Kenyan patients (data not shown). Eosinophils both from normal individuals (Table I, Expts. 4–6) and also from eosinophilic individuals (Table I, Expts. 1–3), could be stimulated by these supernatants. Eosinophils from eosinophilic individuals could also be further
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Fig. 2. Enhancement of eosinophil-mediated killing of schistosomula by the mononuclear cell supernatant of an eosinophilic individual. Schistosomula were incubated for 40 h in the presence or absence of eosinophils (Eos) and immune baboon serum (Ab) at 1/480. The eosinophils had been preincubated for 1 h either in control medium or in 24 h MCS (at a final dilution of 1/4 or 1/40) derived from the mononuclear cells of an eosinophilic individual, and were added to the assay, without washing, at an effector to target ratio of 2,000:1. Each point represents the mean ± 1 SD of three replicate tubes. Clear bar, medium; speckled bar, supernatant 1/40; cross-hatched bar, supernatant 1/4.

stimulated by MCS prepared from the same individual (Table I, Expts. 1–3).

MCS Do Not Affect Neutrophil-mediated Killing. Eosinophils and neutrophils were prepared under identical conditions from the same peripheral blood. When tested in the cytotoxicity assay, eosinophils at an effector to target ratio of 1,000:1 showed a low level of killing in the unstimulated controls (Fig. 3), but demonstrated a marked enhancement following preincubation for 1 h in MCS prepared from an eosinophilic individual. In contrast, neutrophils at a ratio of 4,000:1 showed no demonstrable killing in the unstimulated controls, nor was any enhancement of any previously undetectable killing evident after similar preincubation in MCS.

Stimulation of Eosinophils by MCS is Not Reversed by Washing. Experiments were undertaken to test whether the continued presence of MCS was required for enhanced killing. Eosinophils were preincubated for 24 h in control medium or in MCS from an eosinophilic individual. Thereafter, they were washed three times to remove the stimulating activity, before addition to the usual cytotoxicity assay in the presence or absence of further MCS. Additional preparations were tested without washing. Eosinophils that had been previously preincubated in MCS, then washed and resuspended without further stimulation, still showed a significantly (P < 0.05) enhanced killing activity during the subsequent 40-h assay (59 ± 4%), in comparison
| Expt. | Eo donor | MCS dilution | Eosinophil | MC donor | Medium | MV | MB | CR | AB | JC | JH | BR | NM | DT | % Dead schistosomula* |
|-------|----------|--------------|-------------|----------|--------|----|----|----|----|----|----|----|----|----|---------------------|
|       |          |              |             |          |        |    |    |    |    |    |    |    |    |    |                     |
| 1     | NM       | 1/1          |             |           | 27 ± 2 | 41 ± 3* | 40 ± 1 | 50 ± 1 | 83 ± 2* | 90 ± 1* | 70 ± 1* | 85 ± 3* | 92 ± 1* |
|       |          |              |             |           |        |    |    |    |    |    |    |    |    |    |                     |
| 2     | DT       | 1/20         |             |           | 17 ± 2 | 41 ± 3* | 23 ± 7 | ---  | ---  | ---  | ---  | 23 ± 2* | 56 ± 8* |
| (950-1,885) |       |              |             |           |        |    |    |    |    |    |    |    |    |                     |
| 3     | DT       | 1/40         |             |           | 25 ± 3 | 37 ± 3* | ---  | ---  | 27 ± 4 | ---  | ---  | 75 ± 6* |
| (950-1,885) |       |              |             |           |        |    |    |    |    |    |    |    |    |                     |
| 4     | AB       | 1/12         |             |           | 6 ± 1  | 13 ± 2* | 4 ± 0  | ---  | ---  | ---  | ---  | 9 ± 1*  | 33 ± 3* |
| (146) |          |              |             |           |        |    |    |    |    |    |    |    |    |                     |
| 5     | AB       | 1/12         |             |           | 3 ± 3  | 55 ± 9* | ---  | ---  | ---  | ---  | 67 ± 5* | 64 ± 19* |
| (187) |          |              |             |           |        |    |    |    |    |    |    |    |    |                     |
| 6     | AB       | 1/12         |             |           | 20 ± 7 | 25 ± 8 | ---  | 21 ± 4 | 68 ± 4* | 65 ± 10* | 48 ± 12* | ---  |
| (49)  |          |              |             |           |        |    |    |    |    |    |    |    |    |                     |

*Schistosomula were incubated for 40 h in the presence or absence of eosinophils and heat-inactivated immune human serum (Expts. 1, 2, and 4-6) or immune baboon serum (Expt. 3).

1 The eosinophils used in the killing assay were derived from eosinophilic or normal donors: range of peripheral blood eosinophil counts shown in parentheses.

2 Eosinophils were preincubated for 1 h with various dilutions of MCS prepared from the mononuclear cells of different donors whose range of peripheral blood eosinophil counts, or percentage eosinophils, are shown in parentheses. Effector to target ratios: Expt. 1, 1,500:1; Expt. 2, 340:1; Expt. 3, 500:1; Expts. 4-6, 1,200:1.

3 Mean ± 1 SD, three replicate tubes. Spontaneous death of schistosomula (medium only): Expt. 1, 4%; Expt. 2, 6%; Expt. 3, 2%; Expts. 4 and 6, 1%; Expt. 5, 5%.

** Represents not tested.

Values differ from control preparations containing eosinophils preincubated in medium alone at P < 0.05 or less.
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Fig. 3. Neutrophil-mediated killing of schistosomula is not enhanced by the mononuclear cell supernatant (MCS) of an eosinophilic individual. Schistosomula were incubated for 40 h in the presence of neutrophils (NEUTROS), eosinophils (EOS), or medium (MED), and immune human serum at 1/20. The cells had been preincubated for 1 h either in medium (MED) or in supernatant (MCS) from a 24-h culture of mononuclear cells from an eosinophilic individual, and were added to the assay without washing. Neutrophils were tested at an effector to target ratio of 4,000:1; eosinophils were tested at 1,000:1. Each point represents the mean ± 1 SD of three replicate tubes.

with unstimulated preparations preincubated in medium alone (42 ± 9%). Control cells preincubated in medium, and washed in the same way, could still be stimulated when MCS was added during the cytotoxicity assay (69 ± 5%), whereas the prestimulated and washed eosinophils could not be further stimulated (60 ± 11%).

Properties of the Stimulating Activity in MCS. When 24-h MCS from an eosinophilic individual were heated at 56°C, 80°C, or 100°C for 30 min, the capacity to enhance killing was not significantly affected by heating at 56°C (Table II). Above this temperature, a progressive loss was noted, although some activity still remained following heating at 100°C.

In addition, it was found that the activity was retained by ultrafiltration through
TABLE II

Heat Stability of MCS Activity

| Dilution of MCS: | 1/1  | 1/3.2 | 1/10 | 1/32 | 1/100 |
|------------------|------|-------|------|------|-------|
| Eos preincubated in MCS: |      |       |      |      |       |
| Unheated         | 75 ± 3\(^a\) | 60 ± 1 | 33 ± 10 | 11 ± 4 | 8 ± 3 |
| Heated 56°C, 30 min | 78 ± 1 | 53 ± 3 | 25 ± 3 | 18 ± 7 | 12 ± 5 |
| 80°C, 30 min     | 65 ± 9 | 37 ± 2 | 20 ± 4 | 15 ± 3 | 14 ± 6 |
| 100°C, 30 min    | 62 ± 8 | 24 ± 6 | 20 ± 1 | 12 ± 5 | 12 ± 5 |
| Medium control   | 6 ± 3 | —     | —    | —    | —     |
| Antibody only    | 3 ± 3 |       |      |      |       |
| Unheated MCS only| 2 ± 2 |       |      |      |       |

\(^a\) MCS from an eosinophilic individual were heated to either 56°C, 80°C, or 100°C for 30 min and were subsequently tested for enhancing activity. Eosinophils were preincubated for 1 h in MCS at various dilutions and were added, unwashed, to schistosomula with immune human serum (1/120) at an effector to target ratio of 500:1.

\(^b\) Mean ± 1 SD, three replicate tubes.

Production of Eosinophil-stimulating Activity by Mononuclear Cells. When supernatants of mononuclear cell cultures were harvested after various intervals and tested for enhancing activity, significant (P < 0.05) stimulatory capacity was detectable as early as 1 h of culture (Table III). A gradual increase in activity was evident up to 6 h of culture, after which the activity reached a plateau level. Mononuclear cells cultured for 24 h, washed three times and recultured for a further 24 h still produced significant (P < 0.01) enhancing activity (T 24 + 24, Table III).

In early experiments, it was assumed that some mitogenic stimulant might be required for the production of enhancing activity. Initial MCS were therefore prepared in the presence or absence of 10 \(\mu g/ml\) concanavalin A (Con A). At the end of the culture period, the supernatant fluids were collected and the unstimulated supernatants were reconstituted with Con A. When these MCS were tested, no differences were observed in the capacity of stimulated and reconstituted supernatants, respectively, to enhance eosinophil-mediated killing (data not shown). In all subsequent experiments therefore, no Con A was added. In separate experiments mononuclear cells were purified by defibrination of peripheral blood and centrifugation over Lymphoprep, with serum-free medium being used throughout the procedure. The cells from the interface were then cultured with or without serum supplement. It was found that the production of significant enhancing activity was possible in the absence of serum, but that such MCS required a subsequent addition of serum to minimize the loss of activity upon filtration (data not shown).

To ascertain whether production of the stimulatory activity depended on protein synthesis, mononuclear cells from an eosinophilic individual were cultured in the...
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Figure 4. Sephadex G-200 fractionation of the mononuclear cell supernatant of an eosinophilic individual. A column of Sephadex G-200 (1.5 x 80 cm) was prepared in PBS azide, pH 7.3 at 4°C, and calibrated with molecular weight marker proteins. The column was then equilibrated in sterile MEM under identical conditions and a concentrated (x 10) sample of MCS (<1% of bed volume) was applied and eluted by reverse flow at a rate of 3.8 ml/h. Fractions of 1.3 ml were collected, and aliquots of these were pooled in series of four and tested for enhancing activity in an antibody-dependent eosinophil-mediated cytotoxicity assay (Materials and Methods). Each point represents the mean ± 1 SD of three replicate tubes. * These values differ significantly (P < 0.001) from the control (eosinophils preincubated in medium alone).

Presence or absence of emetine dihydrochloride. To test whether emetine inhibited protein synthesis under conditions appropriate for the production of MCS, the incorporation of [35S]methionine into mononuclear cells was measured. There was no increase in [35S]methionine incorporation by emetine-treated cells between 0 h and 1 h of culture (27,000 ± 11,000 to 22,000 ± 1,000 cpm). Similarly there was no increase in incorporation from 1 h to 5 h, following the removal of emetine (8,000 ± 650 to 7,000 ± 300 cpm). In contrast, untreated control cells doubled their [35S]methionine counts between 0 h and 1 h (24,000 ± 12,000 to 42,000 ± 9,000 cpm) and showed a progressively increased incorporation after the cells were washed, between 1 h and 5 h (25,000 ± 3,000 to 47,000 ± 4,000 cpm). These data showed that emetine, under these conditions, inhibited protein synthesis and that it could be removed after 1 h without allowing a resurgence of protein synthesis within the following 5-h culture period.

Mononuclear cells from an eosinophilic individual were then cultured in the presence or absence of emetine (1 x 10^{-4} M) for 1 or 3 h, washed, and recultured for a further 3 or 5 h, and the resulting supernatants were tested for their enhancing potential (Table IV). MCS prepared from cells cultured without emetine significantly
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TABLE III

Time Course of Production of Enhancing Activity

| Eos preincubated in: | +Eos | +Eos | +Medium |
|----------------------|------|------|---------|
|                      | +IHS | +NHS | +Medium |
| Medium               | 8 ± 3 | 2 ± 1 | 1 ± 1 |
| MCS 1 h              | 15 ± 3 | 2 ± 1 | 2 ± 1 |
| 2 h                  | 21 ± 3 | 1 ± 2 | 0 ± 0 |
| 4 h                  | 41 ± 3 | 5 ± 1 | 1 ± 2 |
| 6 h                  | 53 ± 9 | 10 ± 4 | 0 |
| 8 h                  | 52 ± 3 | 8 ± 6 | 0 |
| 24 h                 | 57 ± 4 | 25 ± 8 | 0 |
| 24 h + 24 h          | 28 ± 8 | 4 ± 3 | 2 ± 1 |

* Schistosomula were incubated for 40 h in the presence or absence of eosinophils (Eos) and heat-inactivated immune human serum (IHS) or normal human serum (NHS) at 1/120. The eosinophils had been preincubated for 1 h either in control medium or in 1/4 dilutions of MCS harvested from mononuclear cells from an eosinophilic individual after 1, 2, 4, 6, 8, and 24 h of culture. The mononuclear cells which had been cultured for 24 h were washed three times, checked for viability, and recultured for a further 24 h (24 h + 24 h).

The eosinophils were added to the cytotoxicity assay after washing.

§ Mean ± 1 SD, replicate tubes.

I Values differ from the medium controls at P < 0.05 or less.

(P < 0.005) enhanced eosinophil-mediated killing of schistosomula, whereas MCS prepared from cells preincubated with emetine for 1 or 3 h, and cultured for 3 or 5 h thereafter, demonstrated no enhancement.

Nature of the Cell Producing the Eosinophil-stimulating Activity. When mononuclear cells, eosinophils or neutrophils, purified from the peripheral blood of an eosinophilic individual, were cultured under identical conditions for 24 h, only the supernatant of the mononuclear cell culture demonstrated significant stimulation of eosinophil-mediated killing (8). The mononuclear cell population consisted of a mixture of monocytes and lymphocytes, usually contaminated with <5% neutrophils. In further experiments populations of predominantly adherent or nonadherent mononuclear cells were prepared. When supernatants were harvested from these two populations and tested for enhancing activity, there was a significant (P < 0.001) difference in their ability to stimulate eosinophil-mediated killing (Fig. 5). The MCS prepared from the adherent population demonstrated significant (P < 0.001) enhancing activity, comparable to that produced by unfractionated cells. In contrast, the MCS from the nonadherent population showed some enhancement, but less than the MCS from unfractionated or from adherent cells. In a further experiment, it was found that the adherent population, which produced significant enhancing activity, was composed of cells 92% of which stained positively for nonspecific esterase (11).

To test the possibility that an adherent T lymphocyte was producing the activity, a monoclonal antilymphocyte antibody and complement were used to kill both T and B lymphocytes (Table V). When mononuclear cells were treated with either medium, antibody, or complement only, there was no loss of cell viability and in each case the resulting MCS demonstrated significant enhancing activity. When mono-
TABLE IV

Emetine Inhibits the Production of Enhancing Activity by Mononuclear Cells

| Eos preincubated in MCS from mononuclear cells incubated | Culture period after removal of Eos | % Dead schistosomula* |
|----------------------------------------------------------|------------------------------------|-----------------------|
|                                                           | Experiment 1                       |                       |
|                                                           | +Eos IHS -Eos IHS                  |                       |
| Without emetine for:                                      |                                    |                       |
| 1 h                                                       | 3 h                                | 41 ± 4**              |
|                                                           |                                    | 1 ± 0                 |
|                                                           |                                    | 81 ± 3**              |
|                                                           | 10                                 |                       |
| 1 h                                                       | 5 h                                | 51 ± 7**              |
|                                                           |                                    | 2 ± 2                 |
|                                                           |                                    | 80 ± 4**              |
|                                                           | 11 ± 4                             |                       |
| 3 h                                                       | 3 h                                | 48 ± 15**             |
|                                                           |                                    | 2 ± 2                 |
|                                                           |                                    | NT                   |
|                                                           | 11 ± 4                             |                       |
| 3 h                                                       | 5 h                                | 52 ± 6**              |
|                                                           |                                    | 1 ± 2                 |
|                                                           |                                    | NT                   |
|                                                           | 11 ± 4                             |                       |
| With emetine for:                                         |                                    |                       |
| 1 h                                                       | 3 h                                | 8 ± 4                 |
|                                                           |                                    | 3 ± 0                 |
|                                                           |                                    | 22 ± 1                |
|                                                           | 13 ± 4                             |                       |
| 1 h                                                       | 5 h                                | 3 ± 1                 |
|                                                           |                                    | 4 ± 2                 |
|                                                           |                                    | 30 ± 6                |
|                                                           | 14 ± 3                             |                       |
| 3 h                                                       | 3 h                                | 5 ± 1                 |
|                                                           |                                    | 9 ± 3                 |
|                                                           |                                    | NT                   |
|                                                           | 14 ± 3                             |                       |
| 3 h                                                       | 5 h                                | 5 ± 3                 |
|                                                           |                                    | 3 ± 1                 |
|                                                           |                                    | NT                   |
|                                                           | 14 ± 3                             |                       |
| Eos preincubated in medium                                 |                                    |                       |
|                                                           | 6 ± 5                              | 4 ± 0                 |
|                                                           |                                    | 29 ± 2                |
|                                                           | 9 ± 3                              |                       |

* Schistosomula were incubated for 40 h in the presence or absence of eosinophils (Eos) and heat-inactivated immune human serum (IHS) at 1/120. The eosinophils had been preincubated for 1 h either in medium or in the undiluted supernatants prepared from mononuclear cells of an eosinophilic individual. The mononuclear cells had been incubated for 1 or 3 h with emetine (1 × 10^{-4}M), and washed three times, and the supernatants were harvested after a further 3 or 5 h. Control MCS were prepared from cells not treated with emetine.

** Effector to target ratio was 1,600:1 (Expt. 1); 1,500:1 (Expt. 2).

§ Mean ± 1 SD, three replicate tubes.

NT, not tested.

nuclear cells were treated with both antibody and complement, 76% of the cells were killed. The MCS derived from the remaining cells showed no loss of enhancing ability.

Discussion

The results presented here are consistent with the interpretation that a nonlymphocytic, nonspecific esterase-containing, adherent mononuclear cell is responsible for the production of a relatively heat-stable activity, with an estimated molecular weight of 35-45,000, which can enhance the antibody-dependent, and to some extent the antibody-independent killing of schistosomula by eosinophils.

It has been known for some time that the functional properties of mature eosinophils can be altered by incubation in the presence of various mediators. For example, eosinophil stimulation promoter (ESP), a T cell mediator, stimulates the migration of eosinophils out of agarose droplets (12), and an ESP-like activity permits the eosinophil-mediated destruction of schistosome eggs in vitro (13). Likewise eosinophil chemotactic factor of anaphylaxis (ECF-A) not only is selectively chemotactic for eosinophils, but also enhances the expression of human eosinophil receptors for Fc, C3b, and C4 (14, 15), and increases both eosinophil-mediated, antibody-dependent and eosinophil-mediated, complement-dependent killing of schistosomula in vitro (16, 17).

Since schistosomiasis is classically associated with eosinophilia, a study has been
previously undertaken to compare the capacity of eosinophils from eosinophilic and normal individuals to kill schistosomula in vitro (3). This study showed that eosinophils from eosinophilic individuals demonstrate an increased killing activity, and may be in an "activated" state in vivo. In various systems, the induction of eosinophilia has been found to be a T lymphocyte-dependent phenomenon (4, 5) that may be attributable to the release of soluble mediators (6, 7). Murine lymphocytes can release colony-stimulating factors (E.CSF) that will specifically accelerate the growth of eosinophil colonies in bone marrow cultures in vitro (18, 19). In addition, CSF-α partially purified from human placenta-conditioned medium (HPCM) (20), not only demonstrates eosinophil colony-stimulating activity (21), but can also enhance antibody-dependent eosinophil-mediated killing in vitro (22).

In the present study, in order to test in more detail the relationship between eosinophilia and eosinophil activation, we asked whether mononuclear cells from eosinophilic individuals would produce mediators that would affect the function of mature eosinophils. When mononuclear cells from an eosinophilic individual were cultured with or without Con A, and the resulting supernatants tested for their capacity to enhance eosinophil-mediated killing, a marked enhancement was noted in both cases. The production of enhancing activity was independent of mitogenic or antigenic stimulation, and did not require serum in the culture medium.
Effect of MCS Prepared from Cells Pretreated with an Antilymphocyte Monoclonal Antibody and Complement

| Pretreatment of mononuclear cells* | Final cell viability | % Dead schistosomula§ | +Eos | -Eos |
|-----------------------------------|---------------------|-----------------------|------|------|
| Medium only                       | 100                 | 60 ± 6†                | 0 ± 0|      |
| Antibody only                     | 100                 | 68 ± 3                 | 0 ± 0|      |
| complement only                   | 100                 | 78 ± 6                 | 0 ± 0|      |
| Antibody + complement             | 24                  | 62 ± 6                 | 1 ± 1|      |
| Controls:                         |                     |                       |      |      |
| Unstimulated EOS. Preincubated in medium | 18 ± 15             | 0 ± 0                  |      |      |

* Mononuclear cells from an eosinophilic individual were incubated for 30 min at 37°C with or without antibody (monoclonal CAMPATH 1 at 1/50), washed, and subsequently incubated for 45 min at 37°C with or without complement (fresh autologous serum at 1/4).
† Treated cells were washed three times and the viability determined by Trypan Blue exclusion. Supernatants were recovered after a further 24 h of culture.
§ Schistosomula were incubated for 40 h in the presence or absence of eosinophils (EOS) and immune human serum (Ab) at 1/120. The eosinophils had been preincubated for 1 h either in control medium or in one of the 24 h MCS described above, and were added to the assay, without washing, at an effector to target ratio of 1,000:1. Killing in the presence of antibody alone was 0 ± 0.

Mean ± 1 SD, three replicate tubes.

When the mononuclear cells were fractionated by adherence to plastic, the adherent cells produced significant enhancing activity. To test for the possible involvement of an adherent lymphocyte, a monoclonal antilymphocyte antibody was used to kill both T and B lymphocytes. The remaining cells still produced the enhancing activity. Thus, although eosinophilia and eosinopoiesis are associated with T lymphocyte involvement, the enhancing activity we have described here appears to be produced by an adherent, macrophagelike cell. This is not surprising, since macrophages are known to produce various biologically active mediators, including colony-stimulating factors (23), interleukin I (a 13–16,000 mol wt protein which leads to the proliferation and activation of T lymphocytes) (24), interferon, fibroblast-activating factor (a 40–60,000 mol wt protein inducing proliferation of fibroblasts) (25), prostaglandins, and various complement components (26).

Several properties of the MCS have been investigated. The enhancing activity was relatively heat stable (50% loss after heating at 100°C for 30 min), with a molecular weight of approximately 35–45,000. ESP is also relatively heat stable with a molecular weight range of 24–56,000 (27, 28), but is produced by sensitized T cells only upon exposure to the specific antigen or mitogen. The activity of ESP is lost at dilutions of 1/4 to 1/8, whereas the enhancing activity described here is more comparable with CSF-α, being effective at dilutions up to 1/400. The MCS activity is also distinguishable from eosinophilopoietin, found in the serum of eosinophil-depleted mice (29), and from the mast cell-derived ECF-A, both being peptides of low molecular weight. Enhancing activity was produced early, being detectable in MCS after 1 h of culture, and required protein synthesis for its production or release. Interleukin I activity, on the other hand, is only detectable after 6 to 12 h of culture, even after induction of the cells with lipopolysaccharide (LPS), immune complexes, or lymphokines (24): unstimulated macrophages produce little, if any, activity. Fibroblast-activating factor is
found in macrophage culture supernatants after 1–4 h of culture but, again, the cells required stimulation with LPS (25).

Although the enhancing activity failed to reveal any previously undetectable neutrophil-mediated killing, it would be premature to conclude that it is selective for eosinophils, since alternative conditions or other neutrophil functions were not tested.

The mechanism of increased killing is not yet known. Although the enhancement is primarily evident in antibody-dependent killing, and although an increased adherence of eosinophils to larvae has been noted under such conditions, there appears to be little effect on the proportion of eosinophils bearing Fc receptors for IgG (unpublished observations). We have confirmed the finding that HPCM increases protein synthesis in eosinophils (30) but under the same conditions the enhancing activity in MCS fails to do so.

Possibly the most interesting feature of this study was that, although mononuclear cells from most individuals produced detectable enhancing activity when the MCS were tested undiluted, the MCS that showed most activity and that titrated out to the greatest extent were derived from mononuclear cells from eosinophilic individuals. This suggests the possibility that there may be some association between enhanced eosinophil activity and eosinophilia. However, a more extensive investigation to test larger numbers of normal and eosinophilic individuals, and to examine whether the enhancing activity also shows any selective eosinophil colony-stimulating capacity, is required to provide a firmer foundation for future speculation.

Summary

Previous studies have shown that eosinophils from eosinophilic individuals differ functionally from those of normal individuals. In order to test whether agents that might induce eosinophilia could also affect eosinophil function, we have compared the capacity of culture supernatants from mononuclear cells of eosinophilic or normal individuals to enhance eosinophil activity, as reflected by an increased killing of schistosomula of Schistosoma mansoni in vitro. An enhancing activity was detected, which increased both the antibody-dependent, and to some extent the antibody-independent killing of schistosomula by eosinophils, in the absence of complement. Under similar conditions, the supernatants failed to stimulate an otherwise undetectable neutrophil-mediated killing. The activity could be removed from the assay by washing, without reversing previous eosinophil stimulation, and was not directly toxic to the schistosomula.

Preliminary characterization of the activity indicated that it was relatively heat-stable at 100°C for 30 min, and had an estimated molecular weight of 35,000–45,000 as judged by G-200 Sephadex fractionation. The activity was produced by a nonlymphocytic, nonspecific esterase-containing adherent mononuclear cell in the absence of either Con A or antigenic stimulation. Significant enhancing activity was detectable after 1 h of culture and continued for at least 25 h. Protein synthesis was required for its production or release.

Although the activity was detectable in supernatants from both eosinophilic and normal individuals, the supernatants that demonstrated highest activity and that could be titrated out furthest were generally derived from eosinophilic individuals, suggesting that there might be some association between eosinophilia and enhanced eosinophil function.
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