IDENTIFICATION OF A LYMPHOKINE THAT STIMULATES
EOSINOPHIL DIFFERENTIATION IN VITRO
Its Relationship to Interleukin 3, and Functional Properties of
Eosinophils Produced in Cultures

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Eosinophilia represents an important model for the study of the control of
hemopoiesis because normal animals produce very few eosinophils, although
large numbers are produced under certain conditions. High production is asso-
ciated especially with helminthic infections and allergic responses. Although it is
difficult to attribute a specific role to a single type of cell in a complex disease,
the fact that eosinophils are present in large numbers and can kill parasitic
helminths in vitro, suggests they may be important to the defense system against
these infectious agents.

Based on the demonstration that experimentally induced T cell deficiency
inhibits the development of eosinophilia (1, 2) and reports that athymic (nu/nu)
mice do not develop a significant eosinophilia after infection with a number of
different parasitic helminths (e.g., 3 and 4), it is reasonable to infer that the T
cell system controls the development of eosinophilia. The possibility that this
control is mediated by a lymphokine was first suggested by the ability of
lymphocytes in diffusion chambers to induce eosinophilia (1) and, more recently
(5–8), by observations that supernatants of antigen- or mitogen-stimulated spleen
cells can induce eosinophil differentiation in vitro.

For these reasons we have sought a lymphokine activity in alloantigen- and
parasite antigen–reactive T cell clones isolated from mice undergoing a parasite-
induced eosinophilia. Coordinate analysis of this eosinophil differentiation activ-
ity (EDA) with other lymphokine assays indicated that a lymphokine was being
detected which was different from interleukin 2 (IL-2), IL-3, or colony-stimulat-
ing factors assayed by bone marrow proliferation activity (BMPA), although in
each case T cell clones produced one or more of these lymphokines in addition
to EDA. Since a source of EDA free of other lymphokines was desirable, we have

Abbreviations used in this paper: BMPA, bone marrow proliferation activity; Con A, concanavalin
A; CSF, colony-stimulating factor; CSF-GM, granulocyte-macrophage lineage CSF; CSF-M, macro-
phage lineage CSF; EDA, eosinophil differentiation activity; EDF, eosinophil differentiation factor;
FCS, fetal calf serum; IFN, interferon; IL-2, interleukin 2 (T cell growth factor); IL-3, interleukin 3
(hemopoietic growth factor); LC-CM, L cell–conditioned medium; ML-CM, mouse lung–conditioned
medium; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; SRBC, sheep red blood
cells; SRBC-TNP, trinitrophenyl-coupled SRBC; T2-CM, T cell clone (NIMP-T2)-conditioned me-
dium; TH1-CM, T cell hybrid (NIMP-TH1)-conditioned medium; WE-CM, WEHI-3-conditioned
medium.
produced (9) a series of T hybrids and selected one that produces EDA but none of the other lymphokines so far tested. Fractionation of conditioned media from a T cell clone and the T cell hybrid has enabled us to show that IL-3 has relatively low EDA, and to identify a novel lymphokine provisionally called eosinophil differentiation factor (EDF) of Mr 46,000. This lymphokine is also detectable in serum from mice undergoing a parasite-induced eosinophilia.

This work is based on an assay for eosinophils produced by bone marrow cultures in microplates. To study the production of eosinophils in vitro, we used long term bone marrow cultures in flasks. These provide a model system for the study of eosinophil differentiation and provide a source of eosinophils for functional studies. Our results show that these eosinophils are morphologically normal and functional in that they can lyse antibody-coated sheep red blood cells (SRBC) and Schistosoma mansoni schistosomula.

Materials and Methods

Parasite. Second-stage larvae (tetrathyridia) of the cestode Mesocestoides corti Hoeppli 1925 was used to induce eosinophilia in BALB/c Nimr mice (10). The mice were infected by intraperitoneal injection of 100 µl packed tetrathyridia. Since the larvae reproduce asexually in the peritoneum, the parasite can be maintained by serial passage and large numbers obtained for antigen preparation. Soluble parasite antigen was obtained by disrupting 1 ml packed tetrathyridia in 5 ml phosphate-buffered saline, pH 7.4 (PBS) at 4°C using a Dounce homogenizer. The soluble fraction was removed after centrifugation and filter sterilized before storing at −70°C.

Tissue Culture. The basic medium consisted of RPMI 1640 containing bicarbonate (Gibco Europe, Paisley, Scotland) supplemented with 10 mM Hepes, 2 mM glutamine, 1 mM sodium pyruvate, and 7.5 × 10⁻⁵ M monothioglycerol (Sigma Chemical Co. Ltd., Poole, Dorset). The bone marrow cultures were further supplemented with 10⁻⁶ M hydrocortisone 21-hemisuccinate sodium salt (Sigma Chemical Co. Ltd.). Preselected fetal calf serum (FCS) (Sera Lab Ltd., Crawley Down, Sussex) was used at 10% for T cell cloning and 15% for bone marrow cultures. Cultures were incubated at 37°C in a humid atmosphere of 5% CO₂ in air.

T Cell Cloning. Spleens were removed 2 wk after infection with M. corti. Cloning was carried out by culturing these spleen cells in limiting dilution, using one plate for each dilution. Parasite antigen–responding clones were obtained by culturing with 2 × 10⁵ irradiated, T cell-depleted, BALB/c spleen cells per well, an optimum dilution of parasite antigen and IL-2 (see below). Alloreactive clones were obtained by culturing with 2 × 10⁵ irradiated, T cell-depleted CBA Nimr spleen cells per well and IL-2. Irradiated cells were obtained by removing the spleens of mice 1 h after irradiation with 1,200 rad from a ⁶⁰Co source. T cell depletion was carried out by treatment with monoclonal anti-Thy-1 (NIM-R1) (11) and guinea pig complement. Cultures were established in 100 µl of medium. After 10 d a further 100 µl of medium containing IL-2 was added. After 14 d the cells in the wells were washed and stimulated as before but without the IL-2. After 24 h clone supernatants were collected for the lymphokine assay and replaced with medium containing IL-2.

Experiments were carried out on clones obtained from plates with <50% positive wells. Two alloreactive clones (NIMP-T1 and NIMP-T2) were selected for high production of EDA and were maintained with IL-2 and restimulation by allogeneic spleen cells every 2 wk.

Bone Marrow Cultures. Femoral bone marrow cells were collected by standard techniques using bicarbonate-free RPMI 1640 (Gibco Europe) containing 20 mM Hepes and 5% newborn calf serum (Sera Lab Ltd.). Cultures were established by a modification of the method described by Dexter and Testa (12). Cells were aliquoted (10⁶ cells/ml) into 24-well cluster plates (1 ml/well; Costar, Data Packaging, Cambridge, MA) or tissue
culture flasks (10 ml/25 cm² flask; Nunc, Roskilde, Denmark). At weekly intervals the cultures were gently shaken and half the medium harvested and replaced with fresh medium. Nonadherent cells were counted with a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL) and differential counts were performed on Giemsa-stained cytocolntrifuge preparations.

Microplate assays were carried out with 10⁴ (100 µl) bone marrow cells per well (round-bottom microplates; Nunc). Test supernatants were added (10 µl) and the plates incubated at 37°C. BMPA was assayed by adding [³²P]thymidine (see below) at 48 h and harvesting the cells at 64–66 h (13). After 5 or 6 d the number of eosinophils was estimated by assaying eosinophil peroxidase using O-phenylenediamine as hydrogen donor, and reading A₄₉₂ in a Titertek Multiskan (Flow Laboratories Ltd., Irvine, Ayrshire, Scotland). Details of this technique and evidence that it is specific for eosinophils are described elsewhere (Strath, Warren, and Sanderson, submitted for publication). Alternatively, total cell counts were made by resuspending the cells in the well and removing 50 µl for counting in a Coulter Counter; differential counts were made by applying 5 µl to the walls of a 15-well multitest slide (Flow Laboratories Ltd.). These slides were prepared by placing 2 µl of 0.1 mg/ml concanavalin A (Con A) in the wells a few minutes before adding the cell suspension. The slides were left at room temperature for 30–45 min to allow the cells to adhere to the Con A–coated glass surface. The slides were then fixed in methanol and stained with Giemsa for differential counts. The number of each cell type produced comprises a myeloid differentiation assay.

**Lymphokine Assays.** IL-2 was assayed on the CTLL cell line (14) by incubating 10 µl of supernatant with 10⁴ cells in 100 µl media for 24 h. During the final 4 h, 0.5 µCi of [methyl-³H]thymidine (5 Ci/mmol; Amersham International plc) was added to each well. The cells were harvested onto glass fiber filter paper and processed for liquid scintillation counting. IL-3 was assayed in the same way on cell line 32D (15). In all these assays, clones were scored positive if higher than the control mean plus three standard deviations.

**Hemopoietic Growth Factors.** Conditioned medium from the T hybrid NIMP-TH1 was prepared by stimulating the cells at 5 × 10⁵/ml with 10 ng/ml phorbol myristate acetate (PMA) (Sigma Chemical Co. Ltd.) in medium containing 5% FCS (9). Cultures were incubated at 37°C for 48 h and supernatant harvested by centrifugation. After filtration, conditioned medium was stored at −20°C. BW5147 conditioned medium was prepared in an identical manner. Conditioned media from the alloreactive T cell clones NIMP-T1 and NIMP-T2 were prepared by stimulating the T cells at 10⁵/ml with irradiated CBA spleen cells at 10⁶/ml.

Conditioned medium was also prepared from an EL4 cell line (16) (kindly supplied by Dr. H. R. MacDonald, Ludwig Institute for Cancer Research, Lausanne, Switzerland) by stimulating at 10⁹/ml for 6–8 h with 100 ng/ml of PMA, washing three times, and incubating in fresh medium for a further 24 h. The supernatant was collected, filtered, and stored at −20°C, and was used at 1–2% as a source of IL-2.

Conditioned media from the cell lines WEHI-3 (17) and L-929 (18) were used at 10%. Mouse lung–conditioned medium was prepared and concentrated (19) and used at 0.1%.

**Functional Activity of Eosinophils.** Eosinophils from cultures in 75-cm² flasks were enriched using a two-step gradient of 17.25 and 16% Meterizamide (Nyegaard (U.K.) Ltd., Sheldon), prepared as detailed previously (20). After centrifuging at 1,200 g for 15 min the cells on the lower interface were removed, washed in medium, and resuspended to the required density.

The ability to lyse ⁵¹Cr-labeled, trinitrophenyl-coupled SRBC (SRBC-TNP) in the presence of anti-TNP monoclonal antibodies were determined as described (21); full details of the monoclonal antibodies are given (21). A mouse anti-SRBC antisera was used as a positive control at a dilution of 1:20. The ability to kill S. mansoni schistosomula was determined as described (22) except that RPMI medium containing 10% heat-inactivated FCS was used. We thank Dr. S. R. Smithers for providing the antisera and Fiona Hackett for preparing the schistosomula (both of this institute).

**Fractionation.** Gel filtration was carried out on two separate columns of ~300 ml bed volume of AcA54 (LKB Instruments Ltd., Croydon, Surrey) in PBS (pH 7.4) containing
50 μg/ml polyethylene glycol (PEG 6000; BDH Pharmaceuticals Ltd., Poole, Dorset). A sample volume of 10 or 2 ml was loaded and effluent was collected as 5-ml fractions. Fractions were tested for IL-3 and EDA by adding 10 μl of each fraction (or a dilution of each) to the assay plates. The column was calibrated with: bovine serum albumin, Mr 66,000; ovalbumin, Mr 45,000; carbonic anhydrase, Mr 30,000; and cytochrome c, Mr 12,400 (all from Sigma). The correlation between log Mr and elution volume was calculated by linear regression and the formula used to calculate Mr for EDA and IL-3. The error of Mr determination was very small (r² = 1.00). The Mr range was calculated from the width of the elution profile at 50% maximum activity.

Purified IL-3 was prepared from WEHI-3-conditioned medium (WE-CM) following the procedure described by Ihle et al. (17), which uses the following steps: DEAE cellulose, hydroxyapatite, gel filtration on AcA54, and reverse phase, high pressure liquid chromatography on a u-Bondapak C₁₈ column (Waters Associates, Milford, MA).

**Electron Microscopy.** Samples for transmission electron microscopy were fixed with 2.5% glutaraldehyde in 0.09 M cacodylate buffer, pH 7.2, containing 2 mM CaCl₂, followed by 1% osmium tetroxide in the same buffer, and then 1% uranyl acetate. Samples for scanning electron microscopy were sedimented in serum-free PBS to remove nonadhering cells, and then allowed to settle on coverslips coated with Alcian Blue (Gurr Biological Stains, BDH Pharmaceuticals Ltd.). They were then treated sequentially with 4% paraformaldehyde and 0.25% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2, 2% osmium tetroxide, and 1% uranyl acetate. After dehydrating with absolute alcohol they were critical point dried above Arklone P (ICI Mond Division, Runcorn, Cheshire) and sputter coated with gold.

**Results**

**Assays for Hemopoietic Growth Factors.** To illustrate the effect of different hemopoietic growth factors on the liquid culture system, and to justify the use of bone marrow from parasitized mice in the assay for differentiation factors, we tested different sources of hemopoietic growth factors in different assays (Fig. 1). In a myeloid differentiation assay, bone marrow from parasitized mice gave a higher number of eosinophils than normal bone marrow. This presumably reflects the relative paucity of eosinophil precursors in normal bone marrow, and makes the assay for EDA more sensitive. The assay for eosinophil peroxidase is a sensitive assay for the number of eosinophils in cultures. It is important to note that the use of bone marrow from parasitized mice did not materially affect the ability to detect neutrophil or macrophage differentiation activities present in mouse lung– (ML-CM) and L cell– (LC-CM) conditioned media, respectively.

These data show that the different biological activities present in these sources of hemopoietic growth factors can be distinguished using the liquid culture systems. For example, LC-CM known to contain CSF-M (macrophage lineage colony-stimulating factor) (CSF1) had strong BMPA, no IL-3, and stimulated macrophage differentiation. ML-CM known to contain granulocyte-macrophage CSF (CSF-GM) and granulocyte CSF (CSF-G) had BMPA, low IL-3 activity, and stimulated mainly neutrophils. Purified IL-3 had activity in the BMPA assay and stimulated both neutrophil and macrophage differentiation with low levels of EDA (detectable only by peroxidase assay in this experiment). The T clone (T2-CM) produced IL-3, had strong EDA, and was positive for BMPA. The T hybrid (TH1-CM) was positive for EDA, had low activity in the BMPA, and was negative for IL-3.

To investigate the relationship between IL-3 and EDA in more detail, we tested dilutions of conditioned media for both activities. The relative activity of
Eosinophil Peroxidase, $A_{492}$

**Figure 1.** A comparison of different sources of hemopoietic growth factors in different assays. (Left) Production of different cell types in a 7 d liquid culture system in cluster plates using normal bone marrow. Total cells were determined in a Coulter Counter; the proportion of the different cell types was based on differential counts (error, 1 SD). (II) Eosinophils; (III) neutrophils; (I) mononuclear cells. Assay for eosinophil peroxidase ($A_{492}$) is shown as a small box; errors in this assay are smaller than can be represented by symbols. (Middle) Parallel assay using bone marrow from parasitized mice (14 d infection). Details as above. (Right) Assay of the same sources of hemopoietic growth factors for IL-3 ($\Delta$) and BMPA using normal (○) and parasitized (●) bone marrow. Values to the left of the dotted line are not significantly different from control; values to the right are significant at the 0.1% level or greater.

**Table I**

| Conditioned Media | IL-3 units* | Calculated IL-3 units | EDA due to IL-3 | EDA units$^\dagger$ | Estimated EDF units |
|-------------------|-------------|-----------------------|----------------|---------------------|---------------------|
| Purified IL-3     | 1,749       | 622                   | 622            | 0                   |
| WE-CM             | 65          | 23                    | 41             | 18                  |
| TH1-CM            | None        | 0                     | 1,353          | 1,353               |
| T2-CM             | 89          | 32                    | 1,576          | 1,544               |

* IL-3 units determined graphically as the dilution of the preparation giving 30% of the maximum counts in the IL-3 assay.

$^\dagger$ EDA was assumed to be due to IL-3 in this purified preparation of IL-3. Thus 1 U of IL-3 produces 0.36 U of EDA. Other values were calculated from the IL-3 activity using this constant.

$^\ddagger$ EDA units were determined graphically as the dilution of the proportion giving 30% of maximum $A_{492}$ in the EDA assay.

$^\S$ Putative EDF units after subtracting the calculated EDA units due to IL-3 from the EDA units determined in the assay.

Each conditioned medium was estimated graphically (Table I). Thus, purified IL-3 had 1,749 U of IL-3 and 622 U of EDA. Using this as a basis, the EDA due to IL-3 in each sample was calculated. As seen in Table I, WE-CM contained little or no EDA that could not be accounted for by the IL-3, whereas TH1-CM and T2-CM contained more EDA than would be expected from their IL-3 activity.

**T Cell Clones.** A series of 53 parasite antigen-reactive T clones and 31
alloantigen-reactive T clones were isolated from the spleen of a mouse infected with *M. corti*. After 2 wk growth, these were restimulated in the absence of exogeneous IL-2 and supernatants were collected after 24 h. These supernatants were assayed for IL-2, IL-3, BMPA, and EDA (Fig. 2).

The data for the alloreactive clones indicate that there is no correlation between EDA and BMPA, although the slope for the correlation between IL-3 and EDA is significant. This correlation probably derives from the fact that all the clones are positive for IL-3. There is a cluster of points negative for EDA and low for IL-3. However, some clones high for IL-3 are negative for EDA, making it extremely unlikely that IL-3 is solely responsible for the EDA. None of the parasite antigen-reactive clones produced detectable IL-2. All gave significant EDA, but the correlation with IL-3 or BMPA is not significant. Several

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**Figure 2.** Comparison of different lymphokine activities by individual T cell clones. (a) Alloreactive clones showing IL-2 and EDA. \( r = 0.065; \) slope, 0.42 ± 1.99 (not significant). There is clearly no correlation between these activities. (b) Antigen-reactive clones showing BMPA and EDA (control mean ± 3 SD; \( A_{\text{ref}} = 0.3 \)). All the clones were positive for EDA. \( r = 0.28; \) slope, 0.07 ± 0.06 (not significant); thus there was no correlation between these two activities. (c) Alloreactive clones showing IL-3 and EDA. All the clones were positive for IL-3. \( r = 0.66; \) slope, 0.11 ± 0.04 (significant at 1% level, see text). (d) Antigen-reactive clones showing IL-3 and EDA. All the clones were positive in both assays. \( r = 0.14; \) slope, 39.5 ± 59.5 (not significant); thus there was no correlation between these two activities.
of the alloreactive T clones have been expanded as sources of EDA, and the high activity of two of these and the T hybrid is shown in Fig. 3. Note also that serum from *M. corti* mice shows high levels of EDA compared with uninfected mice.

**Colony Formation in Agar.** When tested in an agar colony assay using the same medium as used in liquid culture (data not shown), the T clone supernatants produced granulocyte/macrophage colonies with a few large mixed colonies, but small eosinophil colonies were rarely seen. This was comparable to colonies found with WE-CM. ML-CM produced only neutrophil and neutrophil/macrophage colonies, LC-CM produced largely macrophage colonies, and TH1-CM produced no colonies of any type.

**Fractionation of Samples Containing EDA.** Fractions tested after passing conditioned medium through a gel filtration column confirm the existence of a novel factor controlling eosinophil differentiation (Fig. 4). First, passing a 20-fold-concentrated sample of WE-CM indicated that EDA from this source corresponded to the elution profile of IL-3, with a mean *M* of 38,000 (Table II). In contrast, T2-CM and TH1-CM contain EDA corresponding to a mean *M* of 46,000. T2-CM also contain IL-3, with an *M* of 26,000, but this is significantly smaller in size than IL-3 from WE-CM. Thus, these two lymphokines in T2-CM are readily separated by gel filtration. The fractions of TH1-CM contain no detectable IL-3.

It should be noted that fractions of WE-CM gave significant EDA only when they contained high activity IL-3. Fractions giving <1,000 cps in Fig. 4 had only low levels of EDA. Thus, the amount of IL-3 in T2-CM would be virtually undetectable in the EDA assay. The relatively low ability of purified IL-3 to induce eosinophil differentiation was also noted in Fig. 1; however, at higher concentrations this material had significant EDA.

**Long-term Bone Marrow Cultures.** Preliminary results showed that bone mar-
Conditioned medium was fractionated by gel filtration and the fractions assayed for IL-3 and EDA. (Top) WE-CM fractions tested for IL-3 at 10% (○) and 1% (●) final concentration. Fractions tested for EDA at 10% (△). (Middle) TH1-CM fractions assayed for IL-3 at 10% (○) and EDA at 10% (▼) and 1% (●). (Bottom) T2-CM fractions assayed for IL-3 at 10% (○) and EDA at 10% (▼) and 1% (■). In each case, fractions were assayed over the total elution volume of the column, but no activities were detected outside the range shown.

row from parasitized animals did not maintain eosinophil production in the absence of added factors. Eosinophils disappeared within a week, although neutrophil production continued for many weeks, as in bone marrow cultures from normal animals (8). However, in the presence of TH1-CM, eosinophil numbers were maintained for several weeks (Fig. 5), although neutrophil numbers were significantly lower than in control flasks. When addition of conditioned medium was delayed for 1 wk so that eosinophils in the original inoculum disappear, eosinophils reappeared and were produced for at least 6 wk. In this experiment eosinophils represented 90% or greater of the nonadherent cells released into the medium at peak production. In a series of different experiments, eosinophils consistently represented >60% of the nonadherent cells. When addition of conditioned medium was delayed beyond 2 wk, fewer eosinophils were produced; furthermore, the difference between flasks was very large, with
TABLE II

Molecular Weight Estimates from Gel Filtration

| Assay | Column | Source |
|-------|--------|--------|
|       |        | WE-CM  | TH1-CM | T2-CM  |
| IL-3  | A      | NT     | None   | 25 (19-34) |
|       | B      | 38 (31-44) | None | 27 (23-32) |
| Mean, 26 |
| EDA   | A      | NT     | 44 (32-58) | 45 (34-72) |
|       | B      | 35 (28-45) | 47 (37-60) | 49 (39-62) |
| Mean, 46 |

Values represent the mean $M_r (\times 10^{-5})$ and range estimated from the width of the elution peak. NT, not tested. Two columns of similar dimensions were used, and samples were applied as a 10 ml vol in column A and a 2 ml vol in column B. Elution profiles from column B are shown in Fig. 4.

some flasks producing almost no eosinophils and other producing large numbers. Essentially similar results were obtained with T2-CM (data not shown), except that, as shown in Fig. 3, this conditioned medium can be used at a lower concentration.

Functional Activity of Eosinophils Produced In Vitro. Eosinophils were produced in bulk culture with TH1-CM. The pooled cells contained 75% eosinophils; these were separated from neutrophils by centrifugation on Metrizamide. They were tested for cytotoxic activity towards SRBC-TNP in the presence of monoclonal antibodies of different isotypes (Table III). Eosinophils recovered from the peritoneal exudate of *M. corti*-infected mice were tested in parallel. Eosinophils from both sources lysed SRBC in the presence of IgG1, IgG2a, and IgG2b, but not IgM, IgA, or IgE. As further evidence of functional activity, these eosinophils killed schistosomula in the presence of antibody or mouse complement, with highest levels of killing in the presence of both antibody and complement (Table IV).
TABLE III

Cytotoxic Activity of Eosinophils Produced In Vitro and In Vivo Against SRBC-TNP in the Presence of Monoclonal Antibodies of Different Isotypes

| Antibody | Source of eosinophils |
|----------|-----------------------|
|          | In vitro | In vivo |
| IgG1     | 14.7*    | 16.8*   |
| IgG2a    | 10.6     | 18.7    |
| IgG2b    | 22.8     | 6.8     |
| IgM      | 2.2 (NS) | 0.5 (NS) |
| IgA      | 4.0 (NS) | 0.0 (NS) |
| IgE      | 5.1 (NS) | 0.8 (NS) |
| Anti-SRBC| 35.1     | 20.9    |
| Control  | 5.1      | 0.2     |

* Percentage specific 51Cr release calculated as 100 x (100 - test)/(100 - medium) where medium is the release in the absence of antibody to effectors (7.7%). NS, Not significantly different from control (eosinophils but no antibody). All other values are significant at the 1% level or greater.

Eosinophils produced in vitro consisted of 89% eosinophils, 8% neutrophils, 3% mononuclear cells. Eosinophils produced in vivo consisted of 92% eosinophils, 7% neutrophils, 1% mononuclear cells. Eosinophils were used at a ratio of 8:1 SRBC and incubated for 6 h at 37°C.

TABLE IV

Ability of Cultured Eosinophils to Kill Schistosomula In Vitro

|                  | Percent dead schistosomula* |
|------------------|-----------------------------|
|                  | Eosinophils | No eosinophils |
| Antiserum†       | 30 ± 5      | 2 ± 1          |
| Complement‡      | 15 ± 4      | 0              |
| Antiserum + complement | 41 ± 1 | 2 ± 1          |
| Control (normal serum) | 3 ± 2   | 2 ± 1          |

* Percentage dead schistosomula determined by methylene blue uptake after 16 h incubation (±1 SD). The eosinophil preparation was used at a ratio of 1,800:1 schistosomula and consisted of 95% eosinophils, 2% neutrophils, and 3% mononuclear cells.

† Heat-inactivated antiserum from mice 11 wk after infection with S. mansoni at a final dilution of 1:100.
‡ Fresh serum from A strain mice (C5 deficient) at a dilution of 1:10. Control tubes contained heat-inactivated normal mouse serum at a dilution of 1:100.

Electron Microscopy. Transmission electron microscopy showed the presence of characteristic eosinophil granules with a crystalline core (Fig. 6, left), some immature granules lacking this core were also present. Most cells contained polyribosomes, endoplasmic reticulum, and well-developed Golgi apparatus. Scanning electron microscopy was carried out after 5 min incubation of schistosomula with cultured eosinophils in the presence of immune serum. Fig. 6, right shows that even in this short time period a significant number of eosinophils had adhered to the parasite surface. After about half an hour the parasites would be completely obscured by adhering eosinophils (not shown).
Discussion

The basis of this work has been the ability to produce eosinophils in bone marrow cultures (8) and the adaptation of this to microculture assays for detecting eosinophil differentiation. In this system eosinophils can be detected either morphologically or by assay for peroxidase. This assay is a simple and specific method for detecting eosinophils, in which in vivo and in vitro eosinophils show comparable levels of the enzyme (8). The specificity is due to the low level of peroxidase in mouse neutrophils, and possibly also to the low sensitivity of the assay for myeloperoxidase. The assay is more sensitive if bone marrow cells from parasitized mice are used, but the results in Fig. 1 indicate that other hemopoietic factors can be detected, as with normal bone marrow. These data confirm that the BMPA assay detects CSF and IL-3, and that purified IL-3 has very low EDA. Furthermore, LC-CM containing CSF-M had no significant EDA, and ML-CM containing CSF-GM and CSF-G contained only low EDA. It is important that TH1-CM produced no colonies in agar, indicating that EDF is distinct from the characterized CSF. The significant but low BMPA stimulated by TH1-CM agrees with the concept that only cells in the eosinophil lineage are stimulated.

By a comparison of the EDA and IL-3 activity in purified IL-3, the EDA due to IL-3 in different samples could be calculated. These results confirmed that purified IL-3 does have EDA, and indicated that the WE-CM probably contains only IL-3. The high levels of EDA in TH1-CM and T2-CM could not be accounted for by IL-3, suggesting the presence of a novel factor. Also, analysis of the lymphokine activity of a panel of 55 parasite antigen–reactive and 31 alloantigen–reactive T cell clones from M. cortii–infected mice indicate that EDA is not correlated with the production of IL-2, IL-3, or BMPA. The close relationship between the development of eosinophils and helminthic infections is well known; it is interesting that all of the parasite antigen–reactive clones produced EDA. This raises the question whether the development of eosinophilia is controlled by a specific subset of T cells capable of producing EDA, which is only activated by parasite infections. Experiments are being carried out to test for EDA in other infections to test this possibility.
The relative molecular weight of IL-3 from WE-CM is known to be heterogeneous, with $M_r$ values of 28,000 (28 K), 32.5 K, and 41 K (17, 23), although the purified material appears to be the lower end of this range. In this study IL-3 from WE-CM appeared to peak at 38 K. However, the IL-3 activity in T2-CM had a mean $M_r$ of 26 K. Thus it appears that the larger species of IL-3 are not produced by this T cell clone.

The results with the T clones and the fact that TH1 produces no IL-2, IL-3, interferon (IFN), or CSF (9) indicate that a unique lymphokine is involved in eosinophil differentiation. The hybrid NIMP-TH1 is a unique source of this lymphokine, because it does not produce significant amounts of other lymphokines and can be grown in bulk cultures. Control experiments have shown that the PMA used to stimulate the hybrid has no effect on the EDA assay (9). Fractionation by gel filtration indicates that the activity in TH1-CM is due to a factor of $M_r$ 46 K, which is provisionally called eosinophil differentiation factor (EDF). EDF can be separated from IL-3 ($M_r$ 26 K) although high concentrations of IL-3 have some EDA. We were unable to detect a factor of $M_r$ ~5 K as described by Bartelmez et al. (7).

In long-term bone marrow cultures both T2-CM and TH1-CM stimulate the production of eosinophils, which can be used in functional assays. The results with TH1-CM are particularly significant, as this contains no CSF or IL-3. The time course of eosinophil production has several interesting features: (a) As discussed above, more eosinophils are produced in cultures from $M. corti$-infected mice than those from normal mice. This suggests that EDF is acting on precursors present in greater number in bone marrow from parasitized mice than in normal bone marrow. (b) Eosinophil production is transient compared with neutrophil production (8), suggesting that, unlike neutrophil precursors produced in these cultures (24), eosinophil precursors are not being produced de novo from stem cells. (c) If the addition of EDF is delayed, allowing eosinophils in the inoculum to disappear, they reappear within $\sim 1$ wk of adding EDF. This indicates that differentiation rather than prolonged survival is taking place. (d) However, if addition is delayed beyond 2 wk, the variation in eosinophil production in different flasks is very great, suggesting that only a small proportion of precursors have survived. These surviving precursors have considerable potential to divide, because in some flasks relatively large numbers of eosinophils are produced.

At present, we do not know why NIMP-TH1 does not stimulate eosinophil colonies in semisolid media, when the number of eosinophils produced from bone marrow in liquid culture suggests that $\sim 100$ colonies of 300 cells each should be produced per $10^5$ bone marrow cells. This result confirms previous work (6) which indicated that eosinophil colonies could not be detected in agar but that eosinophils were produced in liquid cultures. Clearly, the EDF described in the present report could not have been identified if a colony assay rather than a liquid culture system had been used.

Two functional assays have been carried out on eosinophils produced by stimulation of bone marrow cultures with NIMP-TH1-conditioned medium. First, using monoclonal antibody-coated SRBC, we found that these eosinophils can lyse SRBC coated with IgG1, IgG2a, and IgG2b, but not with IgA, IgM, or
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The level of lytic activity suggests that, at least in this assay, they are as active as eosinophils taken from mice. Secondly, these eosinophils can kill *S. mansoni* schistosomula in the presence of an antiserum or fresh A strain mouse serum (C5 deficient) as a source of complement. A combination of antisera and complement enhanced killing (Table IV). In comparison with previously published data on mouse eosinophils, eosinophils produced in culture were at least as active against schistosomula as were eosinophils in peritoneal exudates (25) or liver granulomata of *S. mansoni*-infected mice (26).

Electron microscopy of eosinophils produced in culture showed the characteristic eosinophil granules with crystalline bar. The presence of polyribosomes, endoplasmic reticulum, and Golgi apparatus, as well as some immature granules, and the preponderance of cells with ring-shaped nuclei, suggest that most of the eosinophils are not fully mature. However, the scanning electron microscopy and functional assays indicate that fully functional eosinophils are produced in the presence of TH1-CM.

Although we cannot rule out the possibility that NIMP-TH1 produces other factors stimulating eosinophil differentiation or maturation, preliminary purification steps suggest that a single factor is involved. It seems likely that EDF controls the differentiation of eosinophils from committed precursor cells to functional eosinophils, but, at least in vitro, it does not appear to control the production of committed precursors from stem cells.

Summary

Factors stimulating eosinophil differentiation in vitro have been studied by means of a liquid bone marrow culture system in which the number of eosinophils is estimated directly by morphology or indirectly by assay for eosinophil peroxidase. The results show that eosinophil colonies are not formed in agar, emphasizing the importance of the liquid culture system.

Three types of evidence identify a novel lymphokine, eosinophil-differentiating factor (EDF). (a) Coordinate analysis of lymphokine activity in media conditioned by a panel of parasite antigen and another panel of alloantigen-reactive T cell clones indicates that EDF is distinct from interleukin 2 (IL-2), IL-3, and bone marrow proliferation activity (BMPA). (b) A T hybrid (NIMP-TH1) produces EDF but no IL-2, IL-3, interferon, or colony-stimulating factor. (c) Gel filtration of conditioned media (CM) indicates that NIMP-TH1 and a T clone (NIMP-T2) produce EDF (*M*, 46,000). NIMP-T2 also produced IL-3 (*M*, 26,000) but this was easily separated from EDF. IL-3 is also shown to have eosinophil differentiation activity (EDA) but this represents a very small proportion of the EDA in T2-CM. Fractionation of WEHI-3-CM indicates that EDA from this source has a similar elution profile to IL-3 (*M*, 35–36,000). Furthermore, a comparison of the relative activities in purified IL-3 and WEHI-3-CM indicates that all the EDA can be attributed to the IL-3 in the latter.

EDF is shown to stimulate production of eosinophils in long-term bone marrow cultures; the kinetics of eosinophil production suggests that EDF is acting on committed precursors in the bone marrow. The transient nature of eosinophil production suggests that precursors from multipotential stem cells are not produced. The eosinophils produced in these cultures are morphologically...
normal and functional in that they lysed sheep red blood cells coated with IgG1, IgG2a, and IgG2b, but not with IgM, IgA, or IgE. In addition, they were capable of adhering to and killing Schistosoma mansoni schistosomula.

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