Brief Definitive Report

HIGHLY PURIFIED MURINE INTERLEUKIN 5 (IL-5) STIMULATES EOSINOPHIL FUNCTION AND PROLONGS IN VITRO SURVIVAL

IL-5 as an Eosinophil Chemotactic Factor

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A B cell–active factor termed T cell–replacing factor (TRF) derived from the B151 T cell hybridoma has been shown to promote IgM secretion by the BCL_1 B cell line and to induce hapten-specific IgG secretion in vitro by in vivo antigen-primed B cells (1–3). Recently, the gene structure of TRF has been determined, and TRF has been revealed to enhance the production of IgA by mouse B cells (4) and induce IL-2 receptors on B cells (5). It also acts as eosinophil differentiation factor (6). Because of these multiple activities, this TRF was proposed to be called interleukin 5 (IL-5). More recently, we demonstrated that highly purified IL-5 supported the terminal differentiation and proliferation of murine eosinophilic precursors (7). In this study, to elucidate whether IL-5 increases the functional properties of mature eosinophils, we examined its effects on the survival, the superoxide anion production, and the chemotactic activities of murine mature eosinophils.

Materials and Methods

Hemopoietic Factors. We used TRF as rIL-5, which was synthesized as described previously (8). Briefly, pSP6K-mTRF23 was cleaved with Sal I to linearize the plasmid DNA, and mRNAs were synthesized using SP6 RNA polymerase. The synthesized RNAs were injected into Xenopus oocytes, and their conditioned medium was collected after incubation for 36 h at 20°C, and purified using an anti-TRF antibody-coupled affinity column (9). 1 U of IL-5 was defined as the reciprocal of the dilution yielding a response that is 50% of the maximal response to the stimulation activity of BCL_1 cells. Human recombinant granulocyte colony-stimulating factor (rG-CSF) was generously provided by Chugai Pharmaceutical Co. (Tokyo, Japan) and had a specific activity of 2.5–10.0 × 10^7/mg protein (10). Activities and lineage specificities of human G-CSF were not significantly

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different from those of murine G-CSF in murine hemopoietic cells (7). Murine granulocyte/macrophage CSF (GM-CSF) was provided by Sumitomo Pharmaceutical Co. (Osaka, Japan) (11). It had a specific activity of 3.7×10^8 U/mg protein. We used supernatant of COS cells transfected with cDNA of IL-3 which was provided by Dr. T. Yokota (DNAX, Palo Alto, CA) (12). It had a specific activity of 10^5 U/mg protein.

Mice and Cell Preparation. Inbred female BALB/c mice, 8–15 wk old, were purchased from Shizuoka Experimental Animal Center (Shizuoka, Japan). We collected eosinophil-rich peritoneal exudate cells by modifying the method described previously (7). Briefly, mice were intraperitoneally injected with 200 mg/kg cyclophosphamide (Shionogi Co., Osaka, Japan), and 2 d later were infected by 1,000 *Toxocara canis* larvae with oral administration. 12 d after the infection, 1 ml of a 0.2 mg/ml (protein concentration) Anisakis extract was injected intraperitoneally, and the peritoneal exudate cells (PEC) were collected 48 h later. These PEC contained 52% eosinophils, 42% macrophages, 4% lymphocytes, 1% neutrophils, and 1% mast cells. Thereafter, the PEC were isolated by density gradient centrifugation in polyvinylpyrrolidone-coated silica gel (Percoll; Pharmacia Fine Chemicals, Uppsala, Sweden), and enriched eosinophils constituted the layer below 50% Percoll. This preparation always contained >90% eosinophils with an average purity of 90–97%, and was used for the following experiments.

Cell Culture. Liquid cultures of mature eosinophils were performed using 24-well tissue culture plates (Coster, Cambridge, MA), and each well contained 2 ml alpha medium supplemented with 20% FCS, 10^6 eosinophils, a CSF such as purified human rG-CSF, murine rGM-CSF, and murine rIL-3 or murine rIL-5. At various times, viable cells were counted by eosin exclusion, and differential counts were made on cytospin preparations stained with May-Grunwald-Giemsa. PEC were cultured using a methylcellulose culture method (13). 1 ml of culture medium contained 10^5 PEC, alpha medium, 1.2% deionized BSA (Sigma Chemical Co. St. Louis, MO), 0.1 mM mercaptoethanol (Sigma Chemical Co.), and 8 U of IL-5. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Colonies were counted on day 7 of culture.

Eosinophil Functions. Superoxide anion production by eosinophils was assayed by superoxide dismutase inhibitable cytochrome *c* reduction spectrophotometrically, and the continuous assay was performed in a Hitachi 557 spectrophotometer (a dual wavelength spectrophotometer; Hitachi Ltd., Tokyo, Japan). The reduction of cytochrome *c* was measured at 550 nm with a reference wavelength at 540 nm and the release of superoxide anion was calculated from cytochrome *c* reduced for 3 min after the addition of IL-5.

The in vitro chemotactic activity of IL-5 on eosinophils was measured by a modification by Boyden's chamber technique as described by Ward et al (14). Briefly, the chamber consisted of two compartments separated by a Millipore membrane (pore size of 5 μm in diameter). Eosinophils were placed in the upper compartment and the culture medium containing various concentrations of IL-5 was placed in the lower compartment. To know whether IL-5 is truly an eosinophil chemotactic factor, we carried out the checkerboard assay (15), which is the method to determine if eosinophils could respond to positive and negative gradients of IL-5. Various concentrations of IL-5 were respectively added into the two compartments separated by a Millipore membrane. The chambers were incubated for 4 h at 37°C in 5% CO_2, and chemotactic activity was assessed in terms of the number of cells migrating from the upper surface to the opposite side of the filter. The membranes were stained according to Litt's procedure (16).

Results and Discussion

IL-5 was able to support eosinophil survival up to 10 d, whereas in its absence the number of eosinophils began to decrease and few viable cells could be detected on day 8 (Fig. 1). The length of survival depended on the dose of IL-5. No mitotic figures of eosinophils were observed in the cytospin preparation stained with May-Grunwald-Giemsa. To determine whether the longer eosinophil survival by the addition of IL-5 was due to the recruitment from newly

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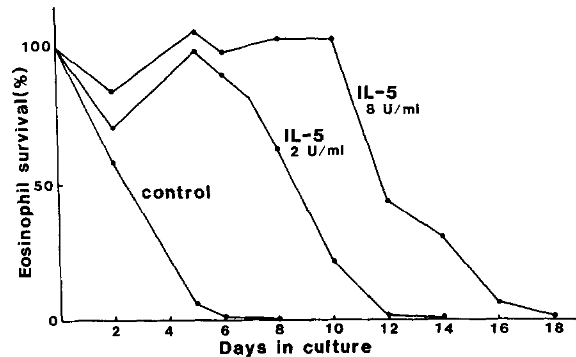

FIGURE 1. In vitro survival of mature eosinophils in the presence of IL-5. At various times, the viable cell number was counted using eosin exclusion, and differential counts were carried out by examination of cytocentrifuge preparations stained with May-Grunwald-Giemsa. Each value is the mean of data of an experiment performed in duplicate cultures. Control means the absence of CSF.

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proliferating eosinophils by IL-5, we tested whether PEC contain hemopoietic precursor cells by methylcellulose culture. No colony formation was observed by 4×10^5 PECs in the presence of 8 U/ml IL-5.

Fig. 2 shows the effects of various CSF on the survival of mature eosinophils. G-CSF and IL-3 did not maintain the viability of eosinophils. GM-CSF supported eosinophil survival only for the first 4 d of culture. On the other hand, in the presence of IL-5 the eosinophils remained viable for 16 days.

The proliferation and differentiation of eosinophilic precursors are stimulated by GM-CSF, IL-3, and IL-5; however, the effects on mature eosinophils differed among them. From our previous (7) and present results, it could be concluded that IL-5 is a lymphokine that acts preferentially on terminal differentiation of eosinophils and maintains their survival. By contrast, IL-3 supports the differentiation of early hemopoietic precursor cells to form the eosinophils, until they are morphologically recognizable.

We examined the effects of IL-5 on superoxide anion production by eosinophils. IL-5 dose dependently induced superoxide anion production, with a plateau being reached at a concentration of 16 U/ml IL-5 (Fig. 3). The grade of this superoxide anion production of eosinophils was comparable for that upon stimulation with 0.16 nM PMA (Sigma Chemical Co.). Lopez et al. (17) recently reported that cloned gibbon IL-3 stimulated the function of purified human mature eosinophils (17). It remains to be clarified whether this effect was comparable to IL-5 or GM-CSF.

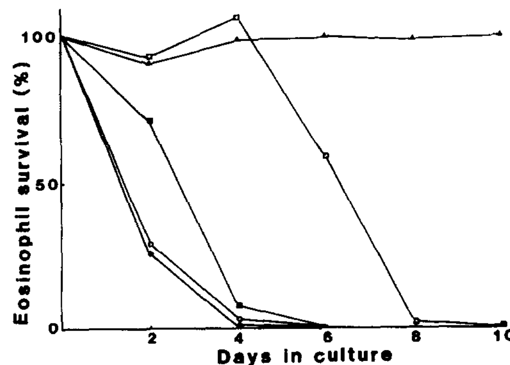

FIGURE 2. In vitro survival of mature eosinophils when cultured in the absence of CSF *(open circle)* or in the presence of 20 ng/ml G-CSF *(closed circle)*, 100 U/ml IL-3 *(closed square)*, 100 U/ml GM-CSF *(open square)*, or 8 U/ml IL-5 *(open triangle)*. The number of viable cells was counted at various times using eosin exclusion. Each value is the mean of duplicate cultures.

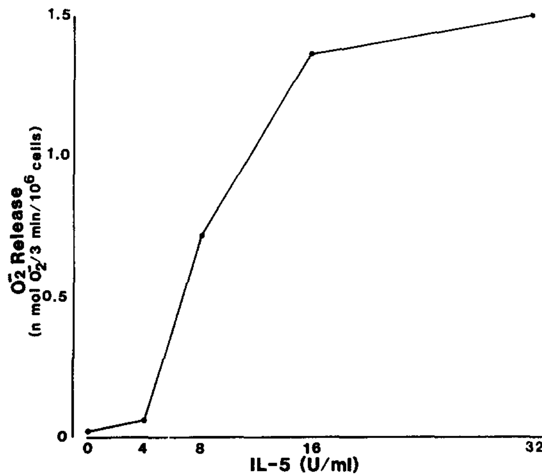

FIGURE 3. Effect of IL-5 on the superoxide anion production of eosinophils. Mature eosinophils were prepared as described in the text. Superoxide anion production was assayed spectrophotometrically by the reduction of cytochrome *c*. Each value is the mean of duplicate determinations.

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Table I shows that IL-5 had a highly chemokinetic effect on eosinophils in a dose-dependent manner when it was added only in the lower compartment of the chambers at concentration from 2 to 32 U/ml. As shown in Fig. 4, the results of the checkerboard assay indicate that IL-5 enhanced migration in a positive gradient and that it is an eosinophil chemotactic factor.

Several investigators have studied the specific stimuli for eosinophil chemotaxis and migration. Among them, eosinophil stimulation promotor (ESP) is a lymphokine that stimulates eosinophil migration (18). The eosinophil chemotactic factor of anaphylaxis (ECF-A) is a mast cell–derived factor that enhances the eosinophil's capacity to kill worms (19). However, no factors supporting both proliferation of eosinophilic precursors and stimulating eosinophil functions have been reported.

We found that rIL-5 maintained eosinophil viability, induced superoxide anion production by eosinophils, and had eosinophil chemotactic activity. IL-5 may be produced locally at inflammatory sites and then may mobilize eosinophils to inflammatory foci from circulating blood, and stimulate phagocytosis by enhancing superoxide release. These eosinophils could be recruited by IL-5, which specifically stimulates the terminal differentiation and amplification of eosinophils.

TABLE I

In Vitro Chemokinetic Activity of IL-5 for Eosinophils

Materials	Chemokinetic effect*
Buffer (1X Hanks' BSS)	26 ± 2.3
IL-5 (U/ml):	
2	31 ± 3.5
4	48 ± 7.9
8	55 ± 5.0
16	100 ± 13
32	148 ± 62

* This activity is expressed as the total count of migrated cells on the 10 high power fields of micropore filter. Data are represented as the mean ± SD of three experiments.

Concentration of IL-5 above filter (columns); Concentration of IL-5 below filter (rows)

U/ml	0	4	8	16
0	25	30	24	25
4	57	27	31	34
8	60	54	35	31
16	115	77	54	29

FIGURE 4. Checkerboard assay of IL-5 on eosinophils. Values represent the mean of duplicate filters.

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Summary

The recent molecular cloning of the complementary DNA encoding T cell–replacing factor (TRF) has demonstrated that a single molecule is responsible for B cell growth factor II (BCGF-II) activity and eosinophil differentiation activity. It has been proposed that this molecule be called interleukin 5 (IL-5). We previously reported that purified rIL-5 supports the terminal differentiation and proliferation of eosinophilic precursors. In this study, we examined the effects of IL-5 on functional activities of mature eosinophils. IL-5 maintained the viability of mature eosinophils obtained from peritoneal exudate cells of mice infected with parasites. It also induced superoxide anion production in a dose-dependent manner. The Boyden's chamber Millipore assay revealed that IL-5 had a marked chemokinetic effect on eosinophils in a dose-dependent manner. Moreover, IL-5 was found to be an eosinophil chemotactic factor by the checkerboard assay. In conclusion, IL-5 is suggested to play an important role in increasing the functional activities of eosinophils as well as their production in allergic and parasitic diseases.

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