Characterization of Interleukin 5 Receptors on Eosinophilic Sublines from Human Promyelocytic Leukemia (HL-60) Cells

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Summary

The T cell product interleukin 5 (IL-5) has been shown to be a key factor in the development and the maturation of the eosinophilic cell lineage. We report here on the detection of human IL-5 receptors on eosinophilic sublines of the promyelocytic leukemia HL-60. Sodium butyrate, which initiates differentiation to mature eosinophils, also induces the appearance of high affinity (K_d 1-5 $\times$ 10^{-11} M) IL-5 binding sites on these cells. The receptors are specific for IL-5, since binding of radiolabeled ligand can only be inhibited with homologous or murine IL-5 and not by other cytokines. We further show that the receptors are functional, since IL-5 can stimulate the proliferation of these cells. Affinity crosslinking of surface-bound ^{125}I human IL-5 or ^{35}S mouse IL-5 identified two membrane polypeptides of ∼60 and ∼130 kD to which IL-5 is closely associated. The presence of granulocyte/macrophage-colony-stimulating factor or tumor necrosis factor during butyrate induction decreased the expression of IL-5 binding sites compared with control cultures. The identification and characterization of human IL-5 receptors on HL-60 sublines should provide new insight into the role of this cytokine in eosinophil differentiation.

Eosinophils are specialized granulocytes found in blood and peripheral tissues. They are present in relatively low numbers in the circulation of healthy individuals, but their number increases dramatically in certain pathological situations, such as parasitic infections and allergic diseases (reviewed in reference 1). This selective eosinophilia suggests the existence of a specific mechanism controlling eosinophilic growth and/or differentiation. Although IL-3, granulocyte/macrophage CSF (GM-CSF),[1] and IL-5 all induce eosinophil differentiation, only IL-5 appears to be specific for the eosinophil lineage. Both mouse (m) and human (h) IL-5 are potent and specific stimuli for eosinophil colony formation in semisolid cultures (2, 3), and for eosinophil growth and differentiation in liquid cultures (4-6). Furthermore, IL-5 stimulates mouse and human eosinophil function (7, 8). Also, there is evidence pointing to the importance of IL-5 as a mediator of eosinophilia in vivo. For example, antibodies against IL-5 inhibit helminth-induced eosinophilia in mice (9), and neutralize the activity that converts normodense eosinophils to a functionally activated hypodense phenotype in serum of patients with idiopathic hypereosinophilic syndrome (10).

Murine IL-5 was originally described as a T cell–replacing factor (TRF) and as a B cell growth factor (BCGF II), based on its capacity to induce proliferation and Ig secretion by activated murine B cells (reviewed in reference 11). It has been impossible to demonstrate similar activities of IL-5 on human B cells in a wide range of assay systems (12), apart from some early conflicting reports (3, 13). Nevertheless, hIL-5 is active on mouse B cells, but with very low specific activity. The availability of IL-5-responsive B cell leukemic cells and IL-5-dependent early B cell lines facilitated the characterization of IL-5-Rs on murine B cells. IL-5 binding sites displaying both high (K_d 10^{-11} M) and low (10^{-9} M) affinity have been found on a number of such cell lines (14, 15). Crosslinking of bound radiolabeled IL-5 gave evidence for two specifically crosslinked polypeptides, suggesting that the IL-5 associates to more than one polypeptide chain at the cell membrane. The complexity of the IL-5R was further illustrated with the isolation of several mAbs directed against epitopes on the IL-5R (16, 17). Although all these antibodies inhibit IL-5 biological activity and binding of IL-5 to mouse B cells, they do not seem to recognize the same molecules. Some antibodies immunoprecipitate predominantly polypeptides with a molecular weight similar to that of the larger crosslinked chain (16), while others recognize molecules that may correspond to the smaller crosslinked polypeptide (17). Also, the

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[1] *Abbreviations used in this paper:* Con A-sup, Con A supernatant; GM-CSF, granulocyte/macrophage CSF; h, human; m, mouse; RT, room temperature.

cell populations that are surface stained with these different antibodies do not overlap completely.

Much less is known about IL-5Rs on mouse or human eosinophils. One explanation for this is the lack of established IL-5-responsive eosinophilic cell lines. To our knowledge, no such cell lines have been described so far. The human promyelocytic leukemia cell line HL-60 has been an important model of myelocytic cellular differentiation (18). By the addition of specific chemical agents to the culture medium, HL-60 can be induced to mature to cells with many of the functional, morphological, and biochemical features of macrophages, neutrophils, or eosinophils (19, 20). Under normal culture conditions, few HL-60 cells differentiate to eosinophils (21, 22). However, after passage in alkaline medium, HL-60 becomes committed to differentiate primarily along the eosinophilic lineage, when they are subsequently cultured in presence of butyric acid (23). We hypothesized that such cells, by analogy with normal eosinophils, were capable of expressing IL-5Rs. Here, we describe that HL-60, committed to eosinophil differentiation, start to express specific high affinity binding sites for IL-5 upon maturation induced by butyrate. We further demonstrate that these receptors are functional and that cell-bound IL-5 is associated with two membrane proteins with molecular weights of ∼130,000 and ∼60,000.

Materials and Methods

Cell Lines. HL-60 cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Gibco Laboratories) and gentamycin (25 μg/ml). Clone 18.16 is a subclone of HL-60 that was selected for IL-5 binding. Briefly, cells were cloned at 100–300 cells/ml in RPMI 1640, 10% FCS, 20 ng/ml human GM-CSF, and 0.92 methyl cellulose (24). After 2 wk, colonies were randomly picked and expanded. Different clones were then passaged in alkaline medium (pH 7.6, 25 mM Tricine) and induced to differentiate with butyrate as described (20). One of the clones that showed specific binding of IL-5 (clone 18) was subjected to a second round of subcloning. Clone 18.16 was selected on the basis of highest IL-5 binding. Clone 15 and clone 2 cells are sublines of HL-60 with a tendency toward eosinophilic differentiation. Their isolation and characterization have been described elsewhere (25). Aliquots of fresh cells were thawed every month. To induce IL-5Rs, the cells were incubated at 2 × 10⁵ cells/ml with 0.4 mM sodium butyrate for 7 d. B13 is an IL-3- or IL-5-dependent Ly-1⁺ murine Pro-B lymphocyte clone (26), and was kindly given to us by R. Palacios (Basel Institute for Immunology, Basel, Switzerland). These cells were cultured in DMEM supplemented with 5% FCS, 25 μg/ml gentamycin, 50 μM 2-ME, and 100 U/ml recombinant mIL-5.

Cytokines and mAbs. rhIL-5 was obtained from transformed *Saccharomyces cerevisiae* and recombinant baculovirus-infected Sf9 cells, as described elsewhere (27). Baculovirus-derived IL-5 was purified from the supernatant of the cells to homogeneity using single-step immunoaffinity chromatography with an anti-hIL-5 mAb 5A5 (J. Van der Heyden, unpublished results, and see below). rmIL-5 was also produced in the baculovirus system (27) and purified to homogeneity. Briefly, the 50–80% (NH₄)₂SO₄ precipitation fraction of the Sf9 culture medium was dialyzed against 20 mM Tris HCl, pH 7.0, and loaded on a Mono-Q column (Pharmacia Fine

Chemicals, Uppsala, Sweden). Under these conditions, mIL-5 does not bind to the matrix, in contrast to the majority of proteins. The flow trough was brought to pH 9.0 with ethanolamine and reloaded on Mono-Q. The mIL-5 that was bound to the matrix this time was eluted at 65 mM NaCl using a linear gradient. Purity (>95%) was evaluated by SDS-PAGE according to Laemmli (28). The biological activity of hIL-5 and mIL-5 was 5 × 10⁴ and 5 × 10⁷ U/mg, respectively, as measured in the B13 proliferation assay (see below). 1 U of IL-5 is defined as the activity that causes 50% of the maximal incorporation. Recombinant human GM-CSF and G-CSF were generous gifts of Dr. J. DeLamarter, and hIL-1β was obtained from Dr. A. Shaw (both from Glaxo IMB, Geneva, Switzerland). rhIL-3 was kindly given by Dr. Y. Furuichi (Nippon Roche Research Center, Kamakura, Japan). Y. Guisez (State University of Ghent) provided us with rhIL-6. Production and purification of rhTNF-α have been described elsewhere (29). Supernatant of Con A-stimulated human PBL (Con A-sup) was prepared by standard procedures. 5A5 is a mouse mAb (IgG1 class) against hIL-5 that neutralizes biological activity, as measured in the B13 proliferation assay (J. Van der Heyden, unpublished results). The rat anti-IL-5R mAb R52.120 inhibits the biological activity of mIL-5 and the binding to its receptor (16), and was kindly given to us by Dr. A. Rolink (Basel Institute for Immunology). Human IgE was obtained from Allergene, Dublin, Ireland.

Radiolabeling of IL-5. ¹²⁵I-labeled hIL-5 and mIL-5 were prepared from purified IL-5 with the IODO-GEN (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) iodination reagent (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instruction. Briefly, 40 μg of hIL-5 or mIL-5 was incubated with 1 mCi Na ¹²⁵I (sp act, 2,000 Ci/mmol; Amersham International, Amersham, UK) in phosphate buffer, pH 7.6, for 10 min at 4°C in a glass tube that was previously coated with 2 μg iodogen. Radiolabeled IL-5 was separated on a gel filtration column (PD-10; Pharmacia Fine Chemicals) in PBS containing 0.25% gelatin. The specific radioactivity of these preparations was routinely between 3 and 10 μCi/μg protein. After iodination, 10–50% of the IL-5 remained biologically active, as determined in the B13 bio-assay (see below). The purity of the labeled hIL-5 was checked by SDS-PAGE and autoradiography (Fig. 1). It consisted of a dimer with the expected molecular mass of 30 kD. Biosynthetically labeled ³⁵S mIL-5 was produced by injection in *Xenopus laevis* oocytes of SP6-RNA polymerase transcripts made using a pSP64T-mIL5 plasmid (27). Batches of five oocytes were incubated for 3 d at 18°C in 50 μl oocyte medium with 5 μl ³⁵S-methionine (>1,000 Ci/mmol; Amersham International). Supernatants were collected and passed over a Sephadex-G25 column to remove unincorporated ³⁵S-methionine. mIL-5 was found to be the major labeled protein upon SDS-PAGE analysis (not shown). The specific activity of the ³⁵S-mIL-5 was calculated to be between 100 and 300 μCi/μg.

Binding Assay. Binding was performed according to the protocol described by Robb et al. (30) with minor modifications. 2.5 × 10⁶ or 5 × 10⁶ cells were incubated at 4°C or at room temperature in 250 μl binding medium (RPMI 1640 with 20% FCS) with a constant amount of ¹²⁵I-hIL-5, or with serial dilutions of ¹²⁵I-labeled human or mouse IL-5. To measure nonspecific binding, a parallel series of tubes containing a 100-fold excess of cold IL-5 was included. To assess equilibrium binding of IL-5 to HL-60 cells, the samples were incubated for 2 h at 4°C. During incubation, the tubes were placed on a rotation wheel to avoid aggregation of the cells. Subsequently, the suspension was layered onto a 0.5-ml phthalate oil (di-*n*-butylphthalate/dinonylphthalate, 2:1 [vol/vol], density, 1.02 g/ml) mixture (31) in a 1.5-ml Eppendorf tube and centrifuged at 12,000 rpm for 5 min. The tips of the tubes were

cut off with a glowing razor blade, placed in vials, and counted in a gamma counter (minigamma LKB; LKB Instruments Inc., Turku, Finland). Specific binding was determined as the difference between total binding and nonspecific binding in the presence of 100-fold molar excess of cold ligand. The dissociation constant (K_d) and number of binding sites per cell were calculated by Scatchard analysis of the saturation binding data (32) with the computer program LIGAND (33). The number of binding sites was calculated assuming that dimeric IL-5 bound to the receptor, since the biologically active form of IL-5 is a dimer (27, 34).

IL-5 Biological Assays. We measured the biological activity of both mIL-5 and hIL-5 preparates by testing their capacity to stimulate proliferation of HL-60 clone 15 and B13 cells. Butyrate-induced HL-60 clone 15 cells grow independently of exogenous added growth factors, but proliferation can be increased twofold by addition of IL-5 or GM-CSF (see Results). HL-60 clone 15 cells were induced to differentiate with 0.4 mM sodium butyrate for 4 d before usage. Murine B13 cells are completely dependent on IL-3 or IL-5 for growth (26). They were carefully washed and incubated in IL-5-free medium for 2 h to remove remaining traces of IL-5. Cytokine samples were serially diluted in 96-well flat-bottomed microtiter plates (Nunc, Roskilde, Denmark) in HL-60 culture medium supplemented with 0.4 mM sodium butyrate or in B13 culture medium. HL-60 or B13 cells were plated at a density of 5×10^3/ml in 200 μl medium. Culture plates were incubated for 3 d at 37°C in a humidified atmosphere. Cell proliferation was determined by [^{3}H]thymidine uptake (0.5 μCi/well, 5 mCi/mmol; Amersham International) during the last 6 h of the culture period. The data are expressed as the mean counts per minute of triplicate cultures.

Chemical Crosslinking of IL-5 Binding Proteins on the HL-60 Clone 15 Surface. 2×10^7 HL-60 clone 15 cells were incubated for 2 h at 4°C in 0.5 ml binding medium with 5 nM ^{125}I-hIL-5 (10 μCi/μg) or 1 nM ^{35}S-mIL-5, alone or in presence of 100-fold excess of unlabeled ligand. Subsequently, the cells were washed twice with ice-cold HBSS to remove unbound IL-5. Crosslinking was carried out with the homobifunctional crosslinker BS^3 (bis [sulfosuccinimidyl] suberate; purchased from Pierce Chemical Co.). The reaction was performed essentially as described by Mita et al. (14). 10^7 cells were resuspended in 1 ml of HBSS, and crosslinker (20 μl at 50 mM in dimethyl sulfoxide) was added to a final concentration of 1 mM. As control, the other half of the cells was resuspended in HBSS with 20 μl dimethyl sulfoxide only. The samples were incubated for 30 min on ice, and the reaction was stopped by addition of 50 μl 1 M glycine during the last 5 min of the incubation. Thereafter, cells were centrifuged, washed once in 1 ml HBSS, and resuspended in 300 μl lysis buffer (PBS con-
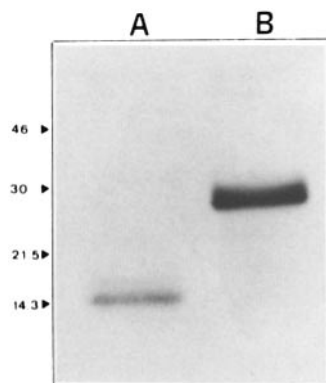
taining 2 mM EDTA, 2 mM EGTA, 2 mM PMSF, 10 μM pepstatin, 10 μM leupeptin, 2 mM o-phenantroline, 200 kallikrein inhibitor U/ml aprotinin, and 1% Triton X-100). Nuclei and other cell debris were removed by centrifugation for 10 min at 4°C in a microcentrifuge. 150 μl of cell extract was analyzed on a 7.5% SDS gel. After electrophoresis, the gel was exposed to X-OMAT AR film (Kodak, Rochester, NY). In case of crosslinking with ^{35}S-mIL-5, the gel was submerged in EN^{3}HANCE (New England Nuclear, Boston, MA) before autoradiography.

Results

Induction of IL-5 Binding Sites on Established Eosinophilic Sublines from HL-60. HL-60 promyelocytic leukemia cells can be induced to differentiate to neutrophils, macrophages, and eosinophils. To select for cells expressing IL-5Rs, HL-60 cells were subcloned and cultured under conditions that allowed differentiation along the eosinophilic cell lineage (see Materials and Methods). After differentiation, induced by butyrate, some clones showed significant binding of ^{125}I-hIL-5 to their cell surface (data not shown). One of these, clone 18.16, displayed ∼500 IL-5 binding sites after 8 d of butyrate treatment (Fig. 2). Fischkoff isolated clones of HL-60, previously cultured for prolonged periods under alkaline conditions, that differentiated with a high rate to eosinophils, as judged by morphological and cytochemical criteria (25). The propensity of these cells to differentiate to eosinophils persists for months after they have been removed from the alkaline environment. Two of these cell lines, clone 2 and clone 15, were induced to differentiate with butyrate and were tested for IL-5 binding. As can be seen in Fig. 2, both showed a gradual increase in IL-5 binding that reached maximal levels after 8 d of exposure to butyrate. Later time points showed no further increase (not shown). The number of IL-5 binding sites on clone 2 was comparable with that of clone 18.16. In contrast, clone 15
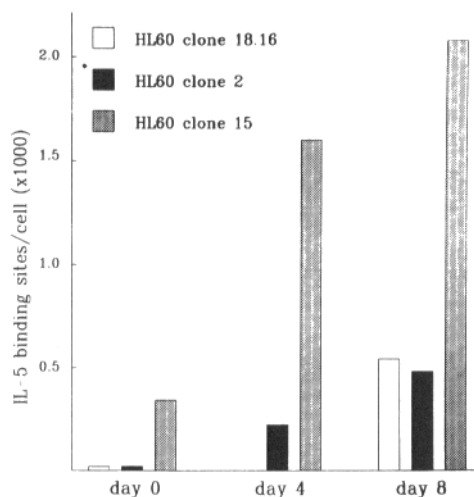
Figure 2. Induction of IL-5 binding sites on HL-60 clones selected for eosinophilic differentiation. Cells were incubated for the indicated times in medium containing 0.4 mM Na-butyrate. 5×10^6 cells were incubated with 0.8 nM ^{125}I-hIL-5 for 1 h at RT in presence or absence of excess cold hIL-5. The number of IL-5 binding sites was calculated after correction for nonspecific binding.

Figure 1. SDS-PAGE analysis of labeled rhIL-5. ^{125}I-IL-5 was run under reducing (lane *A*) or nonreducing (lane *B*) conditions on a 12% acrylamide gel and autoradiographed.

displayed three to four times more IL-5 binding sites at its cell surface. Noteworthy, this clone already showed IL-5 binding before maturation, but treatment with butyrate increased the number of binding sites five- to sixfold. HL-60 clone 15 was chosen for further analysis.

Specificity and Kinetics of Radiolabeled hIL-5 Binding to Butyrate Induced HL-60 Clone 15 Cells. To test the specificity of IL-5 binding to HL-60 cells, assays were performed in the presence of potential competitors for binding. Table 1 summarizes the results of such analysis. Binding of labeled human IL-5 can be successfully blocked with cold hIL-5 and mIL-5, and with anti-hIL-5 mAb 5A5, which is also a potent inhibitor of hIL-5 biological activity. hIL-5 and mIL-5 inhibit binding at similar concentrations. Other cytokines, such as IL-3, GM-CSF, and IL-6, fail to compete for ^{125}I-IL-5 binding. mAb R52.120, which recognizes an epitope of the mIL-5R and

blocks binding of mIL-5 on mouse B cells (16), does not interfere with IL-5 binding on these human cells. To establish optimal parameters for hIL-5 binding to HL60, cells were incubated at different temperatures for different times with radiolabeled hIL-5. Measurement of the time course of ^{125}I-IL-5 binding to these cells at 37°C showed a rapid association of label. At 37°C and at room temperature (RT) (±21°C), maximum levels of specific binding (after subtraction of nonspecific background) were reached after ~10–15 min. Even at 4°C there was no significant change anymore after 15 min. The total amount of cell-associated label at 37°C started to decline after 30 min (Fig. 3). At RT and at 37°C, nonspecific binding kept increasing, while at 4°C, it remained constant after ~1 h. We therefore used incubation at 4°C for further experiments.

Equilibrium Binding of IL-5 to HL-60 Clone 15 Cells. Fig. 4 shows specific binding of ^{125}I-hIL-5 on butyrate-induced HL-60 clone 15 cells as a function of the concentration of labeled IL-5. This experiment demonstrates that the binding is saturable. Scatchard analysis of the data indicates the presence of a single class of binding sites. This particular experiment gave estimates of 1,700 binding sites per cell and a K_d of 22 pM. Table 2 is a summary of several binding experiments performed with labeled hIL-5 and/or mIL-5. From the competition experiment (Table 1), we could expect that mIL-5 would bind to HL60 with an affinity comparable with that of hIL-5. Table 2 shows that this is indeed the case. In separate binding experiments, we measured a K_d value between 10 and 50 pM for both hIL-5 and mIL-5. mIL-5-dependent B cell lines display two classes (high and low affinity) of IL-5Rs (14, 15). However, on HL-60 cells, we found no evidence for the existence of a second receptor with low affinity, even when
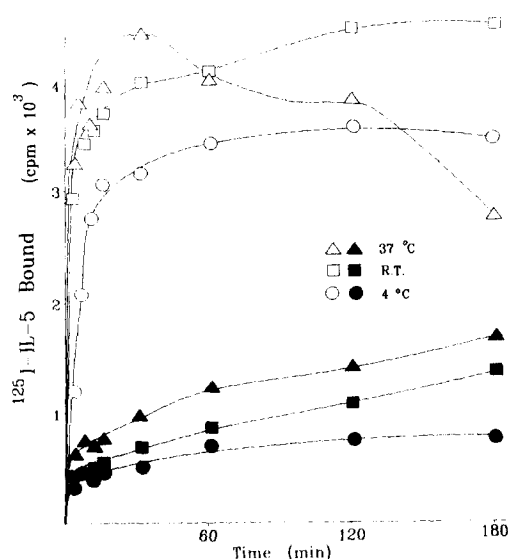
Table 1. *Competition for the Binding of ^{125}I-labeled hIL-5 to HL60 Clone 15 (Induced with 0.4 mM Na-butyrate for 7 d)*

Exp.	Inhibitor	Dose	^{125}I-hIL-5 bound
		$\mu g/ml$	cpm
1	None		2,530
	rhIL-5	0.17	473
		1.70	82
		17.00	29
	rmIL-5	0.17	357
		1.70	67
		17.00	65
	rhIL-3	0.13	2,461
		1.30	2,519
		13.00	2,554
	rhGM-CSF	0.17	2,207
		1.70	2,321
		17.00	2,233
	rhIL-6	0.17	2,396
		1.70	2,344
		17.00	2,690
2	None		1,522
	rhIL-5	6.66	137
	5A5	1	196
		10	140
		100	150
	R52.120	0.8	1,396
		8.4	1,470
		84.0	1,440

2.5 × 10⁶ cells and 3 × 10⁻¹⁰ M of ^{125}I IL-5 were incubated for 1 h at room temperature in 250 μl binding medium with indicated amounts of cytokines or antibodies. The fraction of label bound to the cells was determined as described in Materials and Methods.

Figure 3. Kinetics of binding of ^{125}I-hIL-5 to HL60 clone 15 cells at different temperatures. Cells were induced for 7 d with butyrate, as described in Materials and Methods. 2.5 × 10⁶ induced cells were incubated with 2 nM ^{125}I-hIL-5 at indicated temperatures in presence (*filled symbols*) or absence (*open symbols*) of 100-fold cold IL-5.
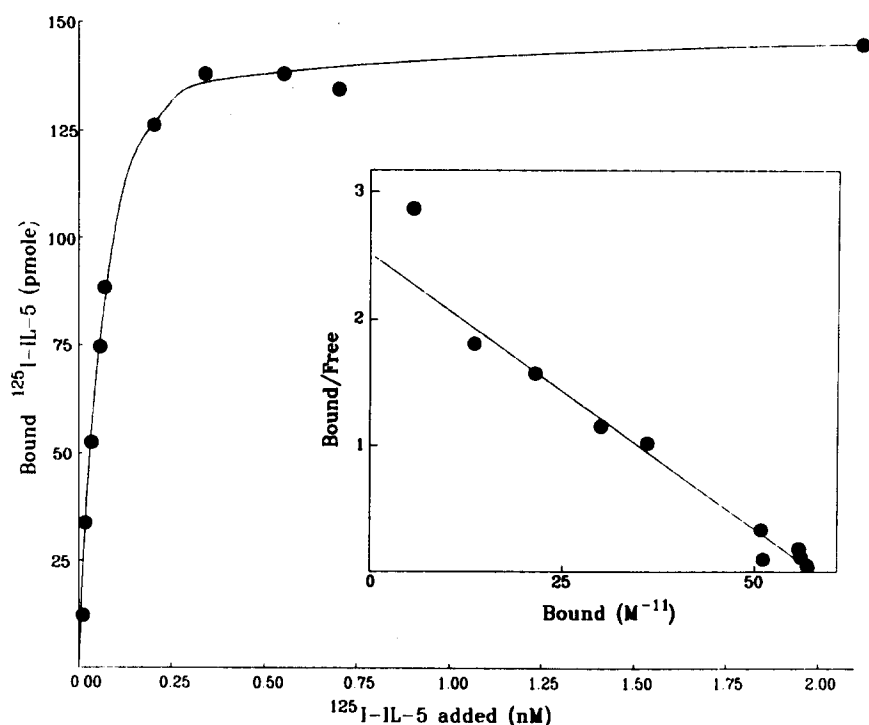
Figure 4. Saturation binding analysis of human ^{125}I-IL-5 binding to butyrate-induced HL-60 clone 15 cells and (inset) Scatchard transformation of the data. Cells (5 × 10^6) were incubated for 2 h at 4°C in 250 μl binding medium containing increasing amounts of labeled IL-5. The values were corrected for nonspecific binding in presence of 100-fold excess of unlabeled IL-5.

ligand concentration was increased to 30 nM (not shown). Treatment with butyrate increases the number of IL-5Rs but does not alter significantly their affinity. The total number of IL-5 binding sites varied considerably from one induction to another. In the best case, we found ~2,000 receptors per cell. The reason for this variability is unclear. Perhaps subtle differences in culture conditions during butyrate treatment

Table 2. *Characterization of IL-5 Rs on HL-60 Clone 15 Cells*

			IL-5 Rs	
Exp.	Induction	^{125}I-IL-5	K_d(M^{-11})	Binding sites per cell
1	None	Human	1.1	60
	Butyrate (7 d)	Human	3.2	300
2	Butyrate (7 d)	Human	4.6	450
	Butyrate (7 d)	Mouse	1.2	210
3	Butyrate (7 d)	Human	2.2	1,700
	Butyrate (7 d)	Mouse	5.0	1,150
4	Butyrate (7 d)	Mouse	4.7	630

Summary of binding studies on HL-60 clone 15. Cells (5 × 10^6) were incubated at 4°C for 1.5–2 h in 250 μl binding medium. 1 to 4 are separate experiements. For each assay, between 6 and 12 different doses of ^{125}I-IL-5 were used to calculate dissociation constant and number of binding sites.

such as cell density or pH of the medium influence the efficiency of the induction.

Biological Activity of IL-5 on HL-60 Clone 15 Cells. We investigated if the IL-5Rs that we could detect on these cells were functional, i.e., if they could transmit a biological signal. Clone 15 cells are not dependent on IL-5 for proliferation. However, we found that IL-5 increased their uptake of [^{3}H]thymidine by a factor of two (Table 3). hIL-5 and mIL-5 were equally efficient in stimulating this response. The concentration of IL-5 that, on average, induced 50% of maximal stimulation is in the range of 100 pg/ml or 3 × 10^{-12} M, i.e., ~1/10 of the K_d. GM-CSF also has a proliferative effect on these cells, which is consistent with published data (35). While hIL-5 and mIL-5 have comparable specific activities when tested on HL60, we found, in agreement with others (12, 36), that the specific activity of hIL-5 on murine B cells is >100-fold lower than that of mIL-5.

Affinity Crosslinking of Radiolabeled IL-5 to HL-60 Cells. Normal HL-60 and clone 15 cells, cultured in presence or absence of butyrate, were incubated with ^{125}I-hIL-5. Bound IL-5 was crosslinked with BS^3, as described in Materials and Methods, and the reaction products were analyzed by SDS-PAGE. The results are shown in Fig. 5 *A*. Two major IL-5-crosslinked complexes were identified in clone 15 cell extracts. In lysates of cells induced to differentiate for 7 d with butyrate, the same bands could be observed but their intensity was markedly increased. The crosslinking products were specific for IL-5 binding since excess of unlabeled IL-5 or absence of crosslinking reagent abolished the presence of the bands on the gel. No bands could be detected in wild-type HL-60, suggesting that their expression is correlated with the selection for eosinophilic phenotype. The molecular mass of the ra-

Table 3. *IL-5 Induced Proliferation of HL-60 Clone 15 and B13 Cells*

Agent added	Dose (ng/ml)					
	10²	10	1	10⁻¹	10⁻²	10⁻³
HL60 clone 15						
hIL-5	44,118 ± 1,885	45,027 ± 1,394	43,603 ± 3,513	<u>36,058 ± 1,305</u>	24,499 ± 1,286	21,133 ± 949
mIL-5	42,852 ± 999	45,141 ± 631	42,556 ± 2,288	<u>39,553 ± 440</u>	25,794 ± 639	21,957 ± 665
hGM-CSF	35,659 ± 210	42,295 ± 1,442	38,837 ± 433	<u>35,140 ± 826</u>	23,634 ± 1,122	22,385 ± 1,292
None	21,759 ± 1,024					
B13						
hIL-5	22,154 ± 2,318	<u>8,553 ± 958</u>	705 ± 129	55 ± 18	95 ± 44	75 ± 35
mIL-5	26,839 ± 717	25,713 ± 497	23,816 ± 419	<u>9,937 ± 372</u>	796 ± 30	146 ± 35
None	95 ± 36					

Proliferation of butyrate-induced HL-60 clone 15 cells and of B13 cells was determined as described in Materials and Methods. The data represent mean cpm ± SD. Values that correspond roughly to 50% of maximal growth stimulation are underlined.

diolabeled complexes was 145 and 75 kD. After substraction of one IL-5 monomer (the samples were loaded in presence of reducing agent), this corresponds to molecules of 130 and 60 kD. Essentially similar results were obtained using ^{35}S-mIL-5 labeled to high specific activity in the oocyte system (Fig. 5 B). Two complexes of ~175 and ~95 kD could be observed under nonreducing conditions from butyrate-induced clone 15 cells. Under reducing conditions, an upper band of 150 kD and two lower bands of 82 and 75 kD could be identified. The double band probably reflects heterogeneity of the biosynthetically ^{35}S-labeled IL-5 due to differential glycosylation (see uncrosslinked IL-5 molecules). The formation of these radiolabeled complexes could be competed with excess cold mIL-5 or hIL-5, indicating that both IL-5 species bind to the same molecules.

Effect of Various Cytokines on IL-5R Expression. Butyrate-induced clone 15 cells expressed maximally ~2,000 IL-5Rs per cell. We tried to increase this number by growing the cells in the presence of different cytokines. Clone 15 cells were incubated for 3 d in butyrate. Subsequently, various agents were added during the last 4 d of the induction period. From the different cultures, the same number of cells were incubated with ^{125}I-IL-5, and specifically bound IL-5 was determined. As can be seen in Fig. 6, none of the cytokines tested could further increase IL-5R expression. In contrast, some factors like TNF and GM-CSF, as well as supernatant from Con A–stimulated lymphocytes, decreased IL-5 binding. None of the factors had a negative effect on the viability or the growth rate of these cells (not shown). IgE can activate eosinophils, as monitored by the secretion of eosinophil peroxidase and
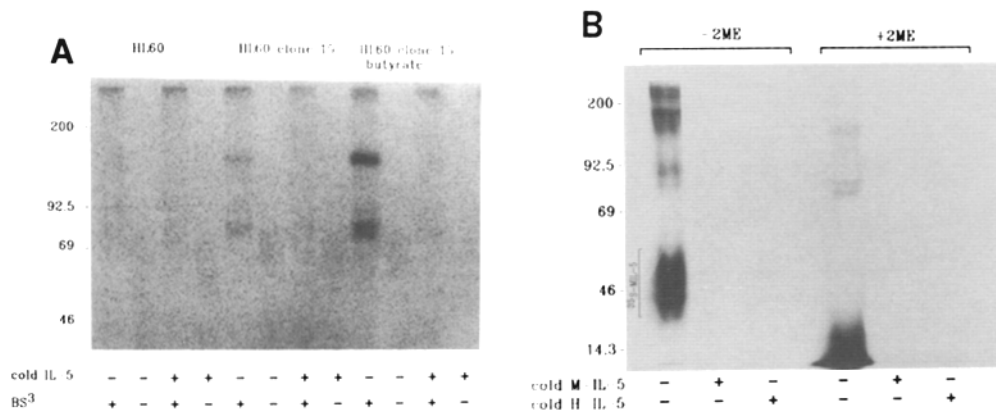

Figure 5. Chemical crosslinking of IL-5 binding proteins on HL-60 cells. (*A*) Wild-type HL60 cells and clone 15 cells, cultured with or without butyrate for 7 d, were incubated with ^{125}I-labeled hIL-5. Membrane-bound labeled IL-5 was crosslinked, as described in Materials and Methods, and the reaction products were analyzed by SDS-PAGE in presence of 2-ME. The autoradiography was developed after 4 wk of exposure. The uncrosslinked IL-5 ran off the 7.5% gel. (*B*) Fluorograph of crosslinking of ^{35}S-labeled mIL-5 bound to butyrate-induced clone 15 cells. The crosslinked products were analyzed under reducing and nonreducing conditions. The autoradiography was exposed for 4 d.
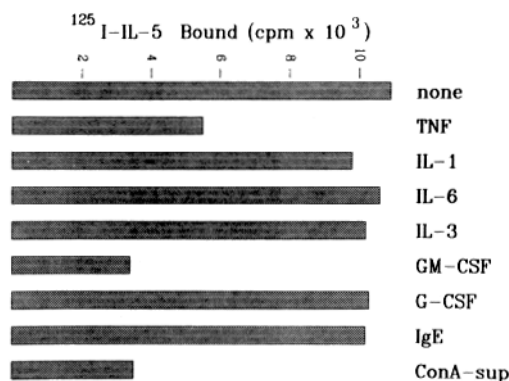
^{125}I–IL–5 Bound (cpm x 10^3)

Figure 6. Effect of various reagents on IL-5R expression. Clone 15 cells were cultured for 3 d in presence of butyrate. The suspension was distributed at 2.5 × 10^5 cells/ml into separate culture bottles in medium with butyrate. Various agents were added at the following concentrations: hTNF-α, 10^3 U/ml; hIL-1β, 1 ng/ml; hIL-6, 6 ng/ml; hIL-3, 0.15 μg/ml; hGM-CSF, 10 ng/ml; hG-CSF, 2 ng/ml; hIgE, 1 μg/ml; Con A-sup, 25%. The cells were further cultured for 4 d. 5 × 10^6 cells were incubated for 1 h at RT in 250 μl binding medium containing 1 nM ^{125}I-hIL-5. The values were corrected for nonspecific binding in presence of 100-fold excess of unlabeled IL-5.

platelet-activating factor, and low affinity receptors for IgE (Fc$_\epsilon$R$_2$, CD23) have been found on eosinophils (37, 38). But, we found no effect of IgE on IL-5R expression of clone 15.

Discussion

So far, the only well-established biological role of IL-5 in the human system is its activity as an eosinophil differentiation factor. Consequently, we searched for suitable eosinophilic cells to screen for hIL-5Rs. The promyelocytic leukemia cell line HL-60 is capable of being selectively induced to differentiate to eosinophils with butyric acid if they have previously been cultured under alkaline conditions (23). Here, we describe the detection and characterization of IL-5Rs on HL-60 sublines that were selected for their propensity towards eosinophil differentiation. To our knowledge, this is the first report on IL-5R expression by human cells.

The production and function of eosinophils is controlled by at least three hemopoietic growth factors, namely IL-3, GM-CSF, and IL-5. IL-3 and GM-CSF bind to high affinity receptors on the surface of human eosinophils. The reciprocally inhibit each others binding, suggesting a common binding protein (39). The present study demonstrates that binding of IL-5 cannot be competed by IL-3, GM-CSF, or IL-6. While IL-3 and GM-CSF show potent stimulatory activity for the proliferation of human eosinophil precursors, IL-5 primarily supports the terminal maturation of eosinophils (40, 41). The kinetics of IL-5R expression on HL-60, as shown in Fig. 2, coincides with the appearance of cells with a mature eosinophil phenotype (23), supporting the hypothesis that IL-5 acts at a later stage during eosinophilopoiesis.

Kinetic studies show that binding of radiolabeled hIL-5 at 37°C is rapid and saturable. Scatchard analysis of equilibrium binding at 4°C provides evidence for a single class of binding sites with a K_d of 1–5 × 10^{-11} M. The crosslinking

experiments provide some structural information on IL-5 binding molecules on HL-60. Crosslinking with ^{125}I-labeled hIL-5 or biosynthetically ^{35}S-labeled mIL-5 resulted in the detection of two complexes of 145–150 and 75–85 kD. The increased mobility of the radiolabeled complexes under reducing conditions is probably due to the loss of one of the IL-5 monomers present in the crosslinked ligand molecule. If this is correct, our data indicate that the receptor is not composed of disulphide-linked subunits. We further show that IL-5 increases [^{3}H]thymidine incorporation by clone 15 cells, which indicates that these IL-5Rs are functional. We can observe important differences in the amount of IL-5 binding sites from one butyrate induction to another (see Table 2). We do not know if this reflects a difference in numbers of receptors per cell or whether the frequency of HL-60 that differentiate to IL-5R-positive cells varies from one experiment to another. However, if we assume that induced clone 15 cells display on average 1,000 binding sites/cell with a K_d of 30 pM, ~10%, or 100 receptor/cell, are occupied at a half-maximal proliferative dose of IL-5 (3 × 10^{-12} M).

Binding of radiolabeled human IL-5 can be competed for with cold hIL-5 and mIL-5 at similar concentrations (Table 1), and the number and affinity of binding sites for hIL-5 and mIL-5 are comparable (Table 2). Both mIL-5 and hIL-5 inhibit the formation of ^{35}S-mIL-5-crosslinked complexes (Fig. 5 B), which shows that they interact with the same membrane components. Consistent with these findings, we found that mIL-5 has similar specific biological activity on HL-60 proliferation as hIL-5 (Table 3). This is apparently in contradiction with the results of Clutterbuck et al. (12), who found that mIL-5 is ~20-fold less active on human bone marrow than on homologous bone marrow. Since IL-5 has been shown to potentiate some eosinophil functions, such as antibody-dependent cytotoxicity (42) and the production of superoxide anion (7), it may well be that IL-5, besides its growth-stimulating activity, has other effects on HL-60. Indeed, it has been reported that IL-5 can upregulate mRNA levels for the granule contained proteins eosinophil cationic protein and eosinophil-derived neurotoxin, in an eosinophilic subline of HL60 (43).

As mentioned before, on murine B cells, high and low affinity IL-5 binding sites can be detected. Experimental data suggest that only the high affinity receptor can transmit a biological signal (15). Crosslinking of radiolabeled IL-5 (14, 15, 17), and the characterization of cell surface molecules by anti-IL-5R mAbs (16, 17), revealed that at least two polypeptides (45–60 kD, L chain; and 130–140 kD, H chain) comprise the IL-5R. From their results, Mita et al. (15) concluded that the H chain is associated with the high affinity IL-5R. Apparently, there is a good correlation between the amount of low affinity receptors on a particular cell line and the abundance of the L chain, as detected by affinity crosslinking. Based on these data, two models for the IL-5R have been proposed: (a) H and L chains correspond to high and low affinity binding sites, respectively; and (b) IL-5 binds with low affinity to the L chain, and the high affinity receptor is composed of the H and L chain, a situation comparable with the IL-2R (44). On HL-60, we can detect IL-5 binding sites with a K_d

similar to that of the high affinity IL-5R on murine B cells. Affinity crosslinking indicates that membrane-bound IL-5 is associated with two polypeptides with molecular masses comparable with H and L IL-5R chains on murine B cells. Induction of IL-5 binding on HL-60 correlates with a concomitant increase in intensity of the two crosslinked complexes (Fig. 5 A). Furthermore, butyrate treatment increases the number of binding sites but does not alter significantly their affinity (Table 2). Together, these observations suggest that both polypeptides form part of the high affinity receptor on human eosinophils, which would be compatible with the second model for the mouse receptor.

Assuming that the IL-5R on HL-60 is related to that on mouse B cells, we think of two possible explanations why we only find high affinity receptors. Perhaps the L chain on HL-60, in contrast to its counterpart on mouse B cells, cannot bind IL-5 independently of the H chain. Alternatively, there is an excess of H chain at the cell surface, so that all the binding sites are converted to high affinity receptors. This hypothesis would predict that HL-60 display constitutively a great number of the high molecular weight component and that maturation to eosinophilic phenotype induces the expression of the L chain.

We were not able to increase the number of IL-5Rs with a variety of reagents (Fig. 6), and further subcloning of clone 15 did not yield a line with higher IL-5 binding (not shown). The presence of GM-CSF or TNF during butyrate induction decreases the number of IL-5R on clone 15 cells. It is surprising that GM-CSF has a negative effect on IL-5R expression on this eosinophilic HL-60 subline, since we found that this cytokine significantly enhanced IL-5 binding on wild-type HL60 cultured at high pH with butyrate (data not shown). Moreover, GM-CSF has been shown to increase the frequency of eosinophilic colonies in the parent culture of HL-60 (24). We do not know the reason for this apparent discrepancy. Perhaps, clone 15, which is committed to single lineage differentiation, responds differently to GM-CSF than the multipotential parent cell from which it was derived.

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