RECEPTORS FOR T CELL-REPLACING FACTOR/INTERLEUKIN 5

Specificity, Quantitation, and Its Implication

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It is well accepted that stimulation with T cells or with T cell-derived soluble factors is necessary in the process of B cell maturation into Ig-secreting cells (1). T cell-derived lymphokines (B cell stimulatory factors, BSFs)[1] were shown to be involved in the regulation of B cell proliferation and differentiation (2–6). The T cell-replacing factor (TRF) is one of BSFs that was defined as a B cell differentiation factor (7–10) and was considered to be an important molecule involved in T-B cell interaction for B cell triggering (11). Naturally produced TRF by a murine TRF-producing T cell hybridoma (B151K12) triggers activated B cells or neoplastic B cell line such as chronic B cell leukemia (BCL_1) to induce differentiation into Ig-secreting cells (8–10), to promote their DNA synthesis (B cell growth factor II [BCGFII] activity) (12), and to induce increased expression for IL-2 receptors (13–15). Murine TRF has been purified to homogeneity and an mAb to TRF has been developed (16).

Recently, cDNA encoding for murine and human TRF was isolated (17–19). The studies with the use of rTRF, i.e., products of recombinant DNA technology, revealed that rTRF exerts all activities demonstrated by using B151-TRF and induces cytotoxic T cell generation in immature thymocytes in conjunction with IL-2 (killer-helper factor [KHF] activity) (20). Moreover, purified rTRF is capable of inducing growth and terminal differentiation of eosinophil precursors (eosinophil differentiation factor [EDF] activity) (19, 21, 22), indicating that TRF, BCGFII, KHF, and EDF activity are mediated by a single molecule. Based on these diverse activities of TRF on different target cells, we proposed that TRF or BCGFII will be called IL-5 (17).

Because IL-5 has an important role in the growth and differentiation of B cells as well as of other hematopoietic cells such as T cells and eosinophils, the nature

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[1] *Abbreviations used in this paper:* BCGF, B cell growth factor; BCL_1, B cell leukemia; BSF, B cell stimulatory factor; DSS, disuccinimidyl suberate; EDF, eosinophil differentiation factor; FPLC, fast protein liquid chromatography; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage CSF; KHF, killer-helper factor; TRF, T cell-replacing factor.

of receptor for IL-5 on the cell surface has become a matter of great interest. It is also of importance to compare the expression of IL-5 receptors with those for BSF-1/IL-4 and BSF-2/IL-6, because a single class of receptor sites for BSF-1/IL-4 and BSF-2/IL-6 was reported (23–26).

Direct measurement of IL-5 binding to cell surface receptor has been made possible by the availability of large quantities of highly purified IL-5. For the initial characterization of the membrane IL-5 receptors, we chose the murine chronic B cell leukemic cell, BCL_1-B20 (in vitro) as a model cell line. This cell line is able to differentiate into IgM-secreting cells in response to IL-5 and constitutes a well-defined model system for IL-5 action (11).

Using radiolabeled murine IL-5 we show that BCL_1-B20 cells bind IL-5 specifically with high and low affinity. We further demonstrate that cell-bound IL-5 is associated with a membrane protein of 46,500 mol wt. The cell types that showed significant levels of high-affinity IL-5 binding are those responding to it. The characterization of IL-5-R, both at the functional and structural levels, should give us new insight in understanding the diverse biological activities of IL-5 on different target cells.

Materials and Methods

Cell Lines. A lymphokine-responsive clone B20 of BCL_1 was isolated by limiting dilution culture of an in vitro BCL_1 line (purchased from American Type Culture Collection, Rockville, MD). This clone was selected for a high level of IgM secretion in response to IL-5. BCL_1-B20 cells were maintained in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FCS (Flow Laboratories, McLean, VA), 5×10^{-5} M 2-ME, penicillin (100 U/ml), and streptomycin (100 μg/ml). Immunological characteristics of BCL_1-B20 will be described elsewhere (Yamaguchi, N., T. Takahashi, N. Harada, and K. Takatsu. Submitted for publication). In vivo BCL_1 tumor line, provided by Dr. E. S. Vitetta (University of Texas Health Science Center at Dallas, TX) was maintained by in vivo passage in BALB/c mice, and was used for IL-5 assay according to the method described (10). HeLa cells transfected with a pKCR plasmid containing the cDNA sequence encoding for mouse IL-5 were established (27) and were kindly provided by Dr. T. Honjo, Kyoto University, Kyoto, Japan. They were maintained in DME supplemented with 10% FCS, 2-ME, penicillin, and streptomycin. Myeloma cells lines (MOPC104E and X5563), P388D1 (macrophage tumor line), P815 (mastocytoma cell line), and BW5147 (thymoma) cell lines were kindly provided by Dr. T. Hamaoka, Biomedical Research Center, Osaka University Medical School, Osaka, Japan. BAL.17 and L10A cell lines were kindly provided by Dr. J. Kim (National Institutes of Health, Bethesda, MD). Stromal cell-dependent early B cell lines were established according to the methods previously described by Ogawa et al. (28) and were then maintained in the presence of IL-5 to let them grow IL-5 dependently. We finally established two cell lines, J-87 and T-88, which were used in the present study.

Monoclonal Antibodies. Anti-IL-5 antibody producing rat-mouse B cell hybridoma (TB13) (16) was transplanted intraperitoneally into BALB/c *nu/nu* mice. The monoclonal TB13 anti-IL-5 antibody (IgG_1 class) was purified as described (16) from the ascites of TB13-bearing *nu/nu* mice. An aliquot of the purified TB13 antibody was coupled to formyl-cellulofine beads (Seikagaku-Kogyo, Tokyo, Japan) and was used for an immunoaffinity column. Ascites of rat B cell hybridoma 11B11 producing anti-murine BSF-1/IL-4 mAb (IgG_1 class) (29) were kindly provided by Drs. J. Ohara and W. E. Paul (National Institutes of Health). Purified mouse mAb 10B1 (30) against human atrial natriuretic peptide was also used.

Recombinant IL-5. rIL-5 was prepared by methods previously described (17, 31). In brief, murine IL-5 cDNA (pSP6K-mTRF23) was cleaved with Sal I to linearize plasmid DNA, and mRNAs were synthesized using SP6 RNA polymerase. The synthesized RNAs were injected in *Xenopus* oocytes and were incubated for 2 d at 20°C. Oocytes supernatants were collected

after the incubation, and were applied to an anti-TRF mAb (TB13)-coupled cellulofine beads. The samples were eluted with 50 mM acetic acid after extensive washing by the each of the following solutions: 1 M NaCl, 0.5% NP-40, PBS, pH 7.2, and distilled water. Then, acetic acid was evaporated by a Speed Vac Concentrator (Savant Instruments, Inc., Hicksville, NY). The samples thus obtained were further purified by gel permeation column chromatography with the use of Superose 12 connected with fast-protein liquid chromatography (FPLC; Pharmacia Fine Chemicals, Uppsala, Sweden). Contamination of proteins other than IL-5 was analyzed by SDS-PAGE with the use of silver staining and was <0.5%. We also used immunoaffinity-purifed IL-5 obtained from cultured supernatant of HeLa cells which had been transfected with mouse IL-5 cDNA. Specific activity of purified rIL-5 was calculated from protein concentration determined by fluorescamine analysis (32) and biological activity was determined by TRF assay as described below.

Radiolabeling of IL-5. We used both biosynthetically labeled [^{35}S]IL-5 and externally labeled ^{125}I-IL-5 for binding assay. To prepare biosynthetically labeled IL-5, 0.1 mCi [^{35}S]methionine (1,000 Ci/mmol, Amersham Japan, Tokyo) in 10 μl was added to the 100-μl culture in modified Barth's medium that contained 10 oocytes. After the culture, ^{35}S-labeled IL-5 was immunoaffinity-purified by the methods previously described (30). Purified IL-5 was labeled using diiodo-Bolton-Hunter reagent (33) (4,000 Ci/mmol; New England Nuclear, Boston, MA) according to the manufacturer's instructions. Briefly, 4 μg of immunoaffinity-purified IL-5 dissolved in 10 μl of 0.1 M borate buffer, pH 8.5, was added to a vial containing 500 μCi Bolton-Hunter reagent. Reaction was allowed to proceed for 30 min on ice and was stopped by addition of 500 μl of 0.2 M glycine in 0.1 M borate buffer (pH 8.5). 500 μl of 0.25% gelatin in RPMI 1640 medium (pH 7.2) was added as a carrier, and labeled IL-5 was separated free from Bolton-Hunter reagent by using a gel filtration column (PD-10, Pharmacia Fine Chemicals). Fractions containing protein-bound radioactivity were pooled. ^{125}I-labeled IL-5 was diluted with RPMI 1640, 25 mM Hepes, pH 7.2, 1 mg/ml BSA, 100 μg/ml streptomycin, and 100 U/ml penicillin (binding medium). Purity and molecular weight of radiolabeled IL-5 were examined by SDS-PAGE followed by autoradiography. SDS-PAGE analysis was carried out by the procedures described by Laemmli (34), using 12.5% polyacrylamide gels.

Biological Assay for IL-5. Biological activities of rIL-5 were assessed with the use of BCL_1 (in vivo line) by polyclonal IgM plaque-forming cell (PFC) assay as described (10), and were expressed as units/milliliter. A unit of IL-5 was determined by the amounts of IL-5 required for half-maximal responses in the IgM PFC assay.

Binding Assay. Binding assay was performed according to the protocol described by Robb et al. (35) with some modifications. Cells for assay were washed twice and incubated for at least 2 h at 37°C in 50 ml RPMI 1640, 25 mM Hepes, pH 7.2, and washed three times with the same medium to remove any endogenous IL-5. After washing, cells were immediately used for binding experiments. To determine the level of binding, serial dilutions of radiolabeled IL-5 were incubated at 37°C with 1–10 × 10^6 cells in a total volume of 200 μl binding medium using 1.5-ml micro test tubes (Assist Trading Co., Ltd., Tokyo, Japan). The tubes were stirred every 10 min to avoid the aggregation of the cells. At the end of incubation, the reaction mixture was layered onto a 200-μl mixture of 84% silicone oil (SH-550; Nakarai Chemical Ltd., Tokyo, Japan) and 16% paraffin oil (No. 7162; Merck & Co., Inc., Rahway, NJ) in a 400-μl polypropylene tube (Assist Trading Co., Ltd.) and were centrifuged at 12,000 rpm for 90 s to remove the small amounts of unbound IL-5. The tips of tubes were cut-off, placed in vials, and radioactivity was counted. When [^{35}S]IL-5 was used as a ligand, tips were placed in scintillation vials and the pellets were solubilized by addition of 100 μl of 1% SDS followed by addition of 3 ml Sintisol Ex (Dojindo Laboratories, Kumamoto, Japan).

To assess equilibrium radiolabeled IL-5 binding to the BCL_1-B20 cells, cells were incubated at 37°C for 10 min with various concentrations of ^{125}I-IL-5. The aliquots were removed and centrifuged through a layer of oil mixture. The cell-bound and free radioactivity were monitored as described above. The specific binding was defined as the difference between total binding and nonspecific binding in the presence of 50–100-fold molar excess of unlabeled IL-5. The dissociation constant and an average number of binding sites per cell were calculated by Scatchard analysis of the saturation binding data (36). The lower limit of detection for ligands was 10 binding sites per cell.

Lymphokines. Human rIL-1α was a gift from Ohtsuka Pharmaceutical Co. Ltd. (Tokushima, Japan); human rIL-2 was from Takeda Central Research Institutes (Toyonaka, Japan); and murine IFN-γ was from Shionogi Research Institutes (Osaka, Japan). Murine rIL-3, murine recombinant granulocyte colony-stimulating factor (G-CSF), and granulocyte/monocyte colony-stimulating factor (GM-CSF) were generously provided by Drs. K. Arai and A. Miyajima (DNAX Research Institute, Palo Alto, CA).

Chemical Crosslinking of IL-5 Binding Proteins on the BCL_1-B20 Cell Surface. Crosslinkers disuccinimidyl suberate (DSS), and ethylene glycol bis succinimidyl succinate (EGS) were purchased from Pierce Chemicals Co. (Rockville, IL) and used in the present study. Radiolabeled IL-5 was crosslinked to IL-5 binding proteins according to the following protocol described by Sabe et al. (37). In brief, BCL_1-B20 cells (10^7 cells) in 200 μl of binding medium alone or binding medium containing 100-fold excess unlabeled IL-5 were incubated with radiolabled IL-5 for 10 min at 37°C. Subsequently, cells were centrifuged and washed once with 1 ml HBSS and were resuspended in 500 μl HBSS. 10 μl of crosslinker (50 mM) in DMSO were added to give a final concentration of 1 mM, and the mixture was incubated for 30 min on ice followed by the addition of 50 μl of 0.5 M glycine in HBSS to quench the reaction for 5 min on ice. The cells were then washed twice with HBSS and finally resuspended in 300 μl PBS containing 1% Triton X-100, 2 mM EGTA, 2 mM EDTA, 2 mM PMSF, 10 μM pepstatin, 10 μM leupeptin, 2 mM *O*-phenanthroline, and 200 KIU/ml aprotinin. The detergent extraction mixture was incubated for 5 min on ice and then was centrifuged for 10 min at 4°C in a microfuge to remove nuclei and other cell debris. For SDS-PAGE analysis, 100 μl of cell extract was mixed with 2 × SDS sample buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol) supplemented with or without 10% 2-ME, were boiled for 5 min, and were subjected to SDS-PAGE analysis in 7.5% SDS polyacrylamide gels using stacking gel procedure of Laemmli (34). After electrophoresis, the gel was stained with Coomassie Brilliant Blue G-250, and exposed to AIF new RX 50 film (Fuji Photo Film Co. Ltd., Tokyo, Japan) at −80°C.

Results

Purity of Radiolabeled Il-5. Fig. 1 shows SDS-PAGE analysis on a slab gel of the [^{35}S]methionine internally labeled IL-5 preparation and the ^{125}I-Bolton-Hunter reagent-labeled IL-5 preparation used in the binding studies. The radiolabeled IL-5 showed a broad band with a molecular weight of 45,000–50,000 under nonreducing conditions and migrated to a molecular weight of 23,000–26,000, indicating that IL-5 consists of a homodimer (31). When the radiolabeled IL-5 was treated with *N*-glycanase, the sample migrated to an apparent M_r of 13,000, as shown by SDS-PAGE under reducing conditions (data not shown), indicating that heterogeneous glycosylation may result in relatively heterogeneous IL-5 preparations.

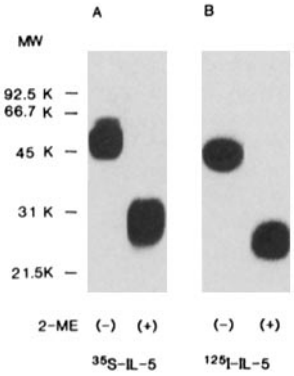

FIGURE 1. Autoradiograph of radiolabeled IL-5 analyzed by SDS-PAGE. An aliquot of [^{35}S]methionine-labeled IL-5 (10^5 cpm) (*A*) or ^{125}I-labeled IL-5 (10^5 cpm) (*B*) was boiled with the sample buffer described by Laemmli (34) in the presence or absence of 2-ME for 5 min, and was applied on polyacrylamide gel with 12.5% acrylamide gels. SDS-PAGE was performed at a constant current of 25 mA according to the methods of Laemmli (34) with a slight modification (16). Marker proteins used were soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,700), and phosphorylase B (92,500).

Comparison between biological activity and determination of protein concentration of purified rIL-5 revealed that a unit of biological activity of purified IL-5 corresponds to ~42 pg of protein. The specific activity of the [^{35}S]IL-5 and ^{125}I-IL-5 was estimated to be 6,000 cpm/42 pg or 6.5 × 10^{15} cpm/mmol (mol wt 46,000) and 484 cpm/42 pg or 5.3 × 10^{14} cpm/mmol, respectively. The specific biological activity of ^{125}I-IL-5 was ~70% of biological activity of the material before iodination. However, comparison of the binding of radiolabeled ^{125}I-IL-5 with that of unlabeled IL-5 measured by inhibition revealed little decrease in binding activity for BCL_1-B20 cells (see below) Although biosynthetic labeling with [^{35}S]methionine yielded the radiolabeled IL-5 with high specific activity, it was impractical. In contrary, radioiodination of IL-5 using Bolton-Huntor reagent was practically easy to handle. We chose to use mainly ^{125}I-IL-5 for further studies. However, when we would confirm the existence of high-affinity IL-5 binding sites, we used [^{35}S]IL-5 preferentially.

Time-Course of Association and of Dissociation of Radiolabeled IL-5. It was observed in our preliminary experiments that BCL_1-B20 responded to IL-5 resulting in IgM-secreting cells and bound the highest amount of radiolabeled IL-5. Therefore, the optimal parameters for cell binding of radiolabeled IL-5 were initially determined using cloned BCL_1-B20 cells. Measurement of the time-course of association of ^{125}I-IL-5 with these cells at 37°C revealed a rapid uptake of radiolabel with maximum levels within 10 min (Fig. 2 *A*); all data were corrected for nonspecific binding. When the sodium azide (0.02%) that is known to inhibit membrane fluidity was added during binding assay at 37°C, maximum level of binding was observed, but only after 60 min of culture. By contrast, a less than maximum level of binding was observed at 4°C even after 3 h of incubation. We observed essentially identical binding

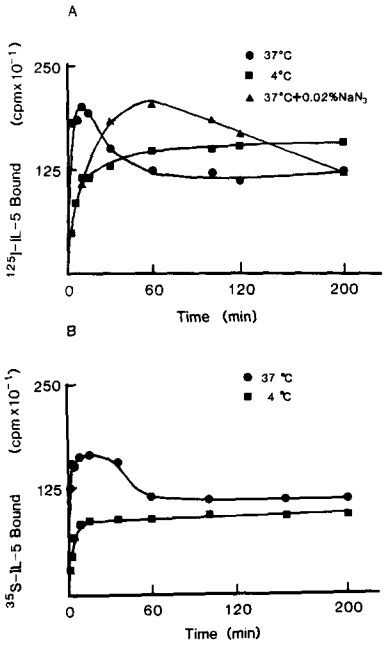

FIGURE 2. Kinetics of radiolabeled IL-5 binding to BCL_1-B20 cells. 2 × 10^6 BCL_1-B20 cells in 200 μl binding medium with 5 × 10^4 cpm ^{125}I-IL-5 (*A*) or with 3 × 10^4 cpm [^{35}S]IL-5 (*B*) were incubated for the indicated time at 37°C in the presence or absence of 0.02% sodium azide or at 4°C in the absence of sodium azide. Data represent the mean specific binding of duplicate samples, which was defined as the difference between the total binding and nonspecific binding that was obtained in the presence of 100-fold excess unlabeled IL-5.

profile of [^{35}S]IL-5 to the target with that of ^{125}I-IL-5 (Fig. 2 *B*). Although we don't show the data, only low levels of internalization of radiolabeled IL-5 was observed at 37°C within 10 min. Therefore, the standard experimental condition; 10 min of incubation at 37°C, was used throughout the study.

Radiolabeled IL-5 Binding: Number of Receptors, and Affinity. Incubation of increasing concentrations of ^{125}I-IL-5 with BCL$_1$-B20 cells demonstrated that the binding consisted of predominantly a saturable component (Fig. 3). Nonsaturable binding was estimated by including 100-fold molar excess unlabeled IL-5 during the incubation. The level of binding that remained was linearly dependent upon the concentration of free ^{125}I-IL-5 and generally constituted 10% of the total bound fraction in the absence of unlabeled IL-5.

Scatchard plot analysis of the binding data revealed that there were two classes of binding sites on BCL$_1$-B20 cells (Fig. 3, *inset*). The negative inverse of the regression coefficient gave a dissociation constant of 66 pM (high affinity) and 12 nM (low affinity) and an average number of high- and low-affinity receptor sites were 400 and 7,500 binding sites per cell, respectively. The [^{35}S]IL-5 gave similar estimates of the number of binding sites and affinity constants (data not shown). We also estimated the numbers of binding sites and affinity to radiolabeled IL-5 to BCL$_1$-B20 cells at 37°C for 60 min in the presence of 0.02% NaN_3. The results revealed that high-affinity binding sites (K_d = 55 pM) are 250 per cell and low-affinity binding sites (K_d = 9.4 nM) are 6,200 per cell.

Characterization of IL-5-R on BCL$_1$-B20, in terms of number of binding sites, could be modulated by stimulating the BCL$_1$-B20 cells in vitro with LPS for 24 h. As shown in Fig. 4, an average number of high-affinity (K_d = 92 pM) and low-affinity (K_d = 5.2 nM) binding sites were 1,200 and 8,400 binding sites per cell, respectively.

Specificity of Binding of Radiolabeled IL-5 on BCL$_1$-B20 Cells. The specificity of radiolabeled IL-5 binding for BCL$_1$-B20 cells was examined by introducing a variety of growth factors and hormones as potential competitors for the binding. As shown in Table I (Exp. 1) and Fig. 5, unlabeled IL-5 inhibited the binding of ^{125}I-IL-5 in a dose-dependent manner. As much as 90% of the total radioactivity of ^{125}I-IL-5 bound to BCL$_1$-B20 without competitor was inhibited by 100-fold excess unlabeled IL-5. None of the recombinant lymphokines tested had any measurable effect on the binding of ^{125}I-IL-5 to BCL$_1$-B20 cells. Of special note are the findings that

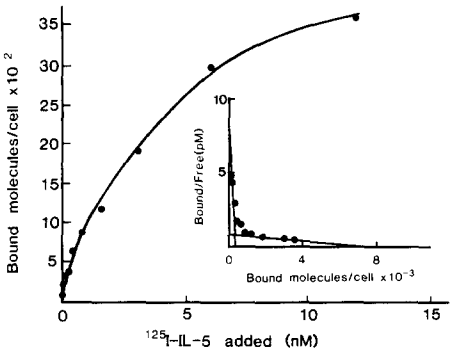

FIGURE 3. Scatchard plot analysis of equilibrium binding analysis of ^{125}I-IL-5 to BCL$_1$-B20 cells. BCL$_1$-B20 cells were incubated at 37°C with various amounts of ^{125}I-IL-5 for 10 min. Specific equilibrium binding was determined after subtraction of nonspecific binding and the data were reexpressed as a Scatchard plot (*inset*).

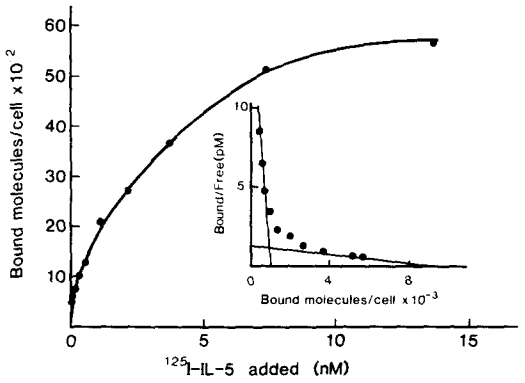

FIGURE 4. Increased expression of IL-5 binding sites by stimulating BCL_1-B20 cells with LPS. The BCL_1-B20 cells (10^6/ml) were cultured with 50 μg/ml LPS for 24 h. After the culture cells were harvested, washed, and subjected to the binding assay according to the procedures as described in Fig. 3. Specific equilibrium binding was determined after subtraction of nonspecific binding and the data were reexpressed as a Scatchard plot (*inset*).

IL-3, GM-CSF, and IFN-γ, which share amino acid sequence homology with IL-5 in short segment, did not compete for ^{125}I-IL-5 binding. The binding of [^{35}S]IL-5 to BCL_1-B20 cells was equally inhibited by ^{125}I-IL-5 with unlabeled IL-5 (Fig. 5), indicating that radiolabeled ^{125}I-IL-5 has essentially similar binding capacity with that of unlabeled IL-5.

The binding of ^{125}I-IL-5 to its receptor was also inhibited by the TB13 anti-IL-5 (TRF) mAb, which is also a potent inhibitor of the biologic function of IL-5. Unlabeled naturally produced IL-5 from B151K12 cells (purified by using immunoaffinity column and gel permeation) could inhibit ^{125}I-IL-5 binding to BCL_1-B20 cells. Antibody to murine BSF-1/IL-4 or to human natriuretic atrial peptide failed to inhibit binding of IL-5 to its receptor (Table I, Exp. 2).

Comparison of IL-5 Biological Activity and Radiolabeled IL-5 Binding. In evaluating the biological relevance of the binding demonstrated for radiolabeled IL-5, it was

TABLE I

Competition for the Binding of ^{125}I-IL-5 by Unlabeled IL-5 and other Lymphokines to BCL_1-B20

Exp.	Inhibitor	^{125}I-labeled IL-5 bound
		cpm
1	None	2,879
	IL-5 (4.0×10^{-8} M)	256
	IL-1α (1.4×10^{-7} M)	2,802
	IL-2 (1.5×10^{-6} M)	2,752
	IL-3 (6.3×10^{-7} M)	2,998
	IFN-γ (8.3×10^{-7} M)	2,890
	GM-CSF (5.3×10^{-8} M)	2,588
2	None	3,372
	IL-5 (2.0×10^{-8} M)*	416
	Anti-IL-5 (50 μg/ml)	605
	Anti-IL-4 (50 μg/ml)	3,238
	Anti-hANP (50 μg/ml)	3,428

5×10^6 BCL_1-B20 cells and 4×10^{-10} M of ^{125}I-IL-5 were incubated in 200 μl binding medium for 10 min at 37°C with indicated amounts of lymphokines or antibodies. Data are mean of duplicate samples.

* Immunoaffinity-purified naturally produced IL-5 by B151K12 cells.

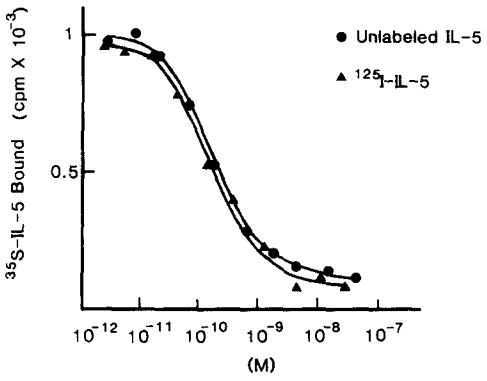

FIGURE 5. Comparison for binding inhibitory effect of radiolabeled IL-5 with unlabeled IL-5. The standard binding assay was conducted using BCL_1-B20 cells (2×10^6) and [^{35}S]IL-5 (12 pM). Various concentrations of either unlabeled IL-5 or ^{125}I-IL-5 were added as inhibitors. Since counts of ^{125}I-IL-5 (γ-ray) overlapped with ^{35}S (β-ray), we monitored the overlapping of ^{125}I counts onto ^{35}S counts, subtracted them from total ^{35}S counts, and recalculated the [^{35}S]IL-5 real counts that bound on BCL_1-B20.

of particular interest to compare the concentrations of IL-5 that result in significant binding with those responsible for in vitro differentiation. Serial twofold dilutions of [^{35}S]IL-5 were incubated with the BCL_1-B20 cells as in a standard TRF assay. After 3 d of culture, the cells were used for PFC assay to measure their response to the exposure to IL-5. When the data from this experiment were compared with the binding curve for [^{35}S]IL-5 to BCL_1-B20 cells, the concentration of IL-5 that promoted half-maximal IgM PFC response was 2.5 pM (2.7 U/ml) and the concentration of [^{35}S]IL-5 giving half-maximal binding was 15 pM (16.3 U/ml) (Fig. 6). Moreover, the maximum biological response occurred at a concentraion of IL-5 that corresponded to 40–50% saturation of the high-affinity binding sites. The similarity between the biological dose-response curve and the [^{35}S]IL-5 binding curve suggests that the biological response is proportional to the high-affinity IL-5 binding site occupancy.

Affinity Crosslinking of Radiolabeled IL-5 to BCL_1-B20 Cells. IL-5 bound to receptors on BCL_1-B20 cell surface was crosslinked with DSS or EGS according to the procedures described in the Materials and Methods. Fig. 7 shows an SDS-PAGE analysis of detergent lysates from BCL_1-B20 cells that were incubated with [^{35}S]IL-5 in the presence or absence of unlabeled IL-5, washed, and exposed to bivalent lysine-directed crosslinking agents. Under the conditions used, DSS produced a radiolabeled band at ~92,500 M_r when cell lysates were analyzed under nonreducing conditions (Fig. 7, lane *1*). The crosslinking of IL-5 to the higher molecular weight species was specific,

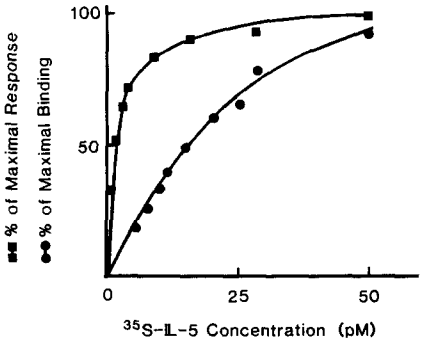

FIGURE 6. Comparison between the percentage of the maximal biological response (IgM-secretion inducing assay, ■) and the maximal level of [^{35}S]IL-5 binding (●) as a function of the concentration of IL-5. Each point represents the mean of triplicate (■) or duplicate (●) determinations.

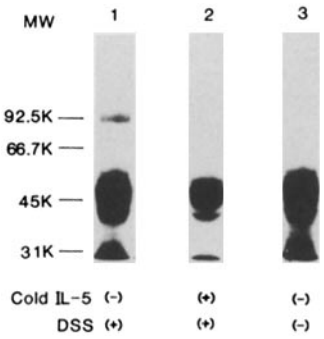

FIGURE 7. Autoradiograph of crosslinking of [^{35}S]IL-5-binding protein. BCL$_1$-B20 cell were incubated with [^{35}S]IL-5 (2×10^5 cpm) for 10 min at 37°C, and were washed. Then, DSS (1 mM) was added and kept at 4°C for another 30 min. After several washings, cells were lysed with the buffer as described in Materials and Methods and the lysate was analyzed with SDS-PAGE with 7.5% acrylamide gels. Autoradiography was made after the electrophoresis.

since unlabeled IL-5 abolished the presence of bands on the gel (Fig. 7, lane *2*). Furthermore, in the absence of crosslinking reagent, no cell-associated radiolabeled species was detected other than IL-5 (Fig. 7, lane *3*). The uncrosslinked IL-5 ran at the bottom of the 7.5% gels, which was 46,000 M_r. Essentially similar results shown in Fig. 7 were obtained using ^{125}I-IL-5 as shown in Fig. 8. EGS as well as DSS produced a radiolabeled band at 92,500 under nonreducing conditions (Fig. 8 *A*). There was a faint band in some but not all samples, migrating with 160,000 and 210,000 M_r (data not shown).

Lysates of cells treated with IL-5 and these crosslinkers were also analyzed under reducing conditions (Fig. 8 *B*). Use of crosslinkers DSS and EGS resulted in the detection of one band in the gel: a major band of 75,000 M_r. The 75,000 M_r band was detected under all conditions examined.

Cellular Distribution of IL-5-R. Binding assay for IL-5 on various murine cell lines was carried out. Table II summarizes the results of a survey of number of cell types, including primary cells and established cell lines, for the presence of IL-5-R. For

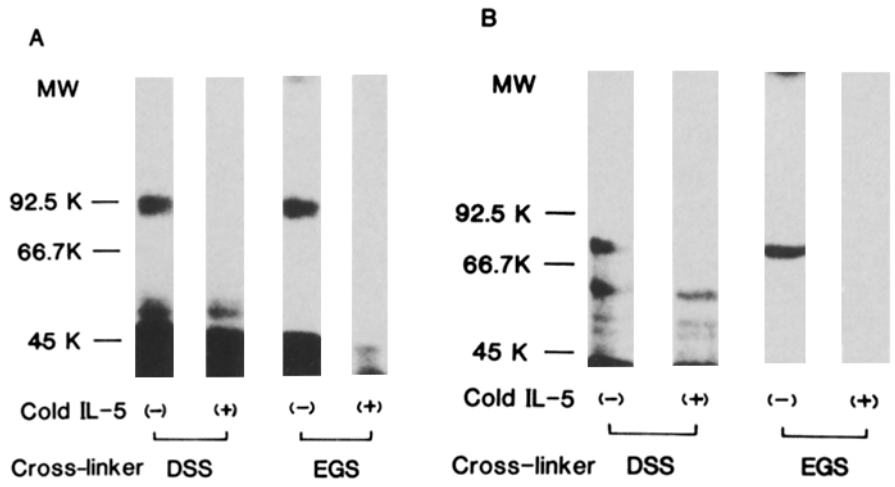

FIGURE 8. Autoradiograph of crosslinking of ^{125}I-IL-5-binding protein. BCL$_1$-B20 cells were incubated with ^{125}I-IL-5 (10^6 cpm), and crosslinked with the use of either DSS (1 mM) or EGS (1 mM) for 30 min. After the washing, cells were lysed with Triton and the lysates were subjected to SDS-PAGE under either nonreducing (*A*) or reducing conditions with 5% 2-ME (*B*).

TABLE II

Cellular Distribution of Murine IL-5 Receptors

Primary cells		High-affinity IL-5 receptors: Binding sites per cell (Mean ± SEM)	High-affinity IL-5 receptors: K_d (Mean ± SEM)
			pM
Bone marrow cells		<10	ND
Splenic B cells		<10	ND
LPS-stimulated B blasts*		40 ± 10	217 ± 50
Thymocytes		<10	ND
Splenic T cells		<10	ND
Con A-stimulated T blasts‡		<10	ND
	In vitro cell lines		
Designation	Cell type		
BCL_1-B20	B-cell leukemia (in vitro line)	243 ± 100	46 ± 13
BCL_1	B-cell leukemia (in vivo line)	120 ± 52	43 ± 15
MOPC104E	Myeloma (IgM producer)	56 ± 7	61 ± 10
X5563	Myeloma (γ_2a producer)	<10	ND
J-87	Stromal cell and IL-5-dependent early B cell line	45 ± 9	41 ± 17
T-88	IL-5-dependent early B cell line	76 ± 11	38 ± 16
BAL.17	B cell lymphoma	<10	ND
L10A	B cell lymphoma	<10	ND
MTH	IL-2-dependent CTLL	<10	ND
B151K12	T cell hybridoma	<10	ND
P815	Mastocytoma	<10	ND
P388D1	Macrophage tumor	<10	ND

Cells were analyzed by usual binding assay. In some cells, at least five different doses of [^{35}S]IL-5 were used for the assay. The averages of duplicate determination were taken for analysis.

* Normal resting splenic B cells were stimulated with LPS (50 μg/ml) for 2 d before binding assay.

‡ Normal splenic T cells were stimulated with Con A (4 μg/ml) for 2 d before binding assay.

some cells, binding assay was conducted over a range of five different concentrations of [^{35}S]IL-5 and an average number of high-affinity binding sites per cell, and dissociation constants were calculated by Scatchard analysis. In most cases, however, for a preliminary survey, a single concentration of [^{35}S]IL-5 was used, to determine whether IL-5-R were present and their approximate abundance. Among the analyzed B cell lines, BCL_1-B20 and MOPC104E (mouse myeloma) cells displayed significant numbers of high-affinity binding sites for IL-5. Mouse thymoma cell line, mastocytoma cell line, or macrophage tumor cell line did not express detectable number of IL-5 binding sites (Table II). LPS-stimulated normal B cells expressed detectable numbers of IL-5 binding sites, whereas normal resting B cells, bone marrow cells, and Con A–stimulated T blasts expressed few, if any. Intriguingly, two early B cell lines (J-87 and T-88) whose growth is IL-5 dependent expressed substantial numbers of high-affinity binding sites for IL-5. The data clearly show that none of the cells have more than 10^3 IL-5-R with high affinity.

Discussion

Cloning of cDNA for murine IL-5 (17, 19) and production of mAb against TRF (16) enabled us to yield large quantities of highly purified rIL-5 and has made it possible to examine binding sites for IL-5. We used both biosynthetically labeled [^{35}S]IL-5 and externally labeled ^{125}I-IL-5. These ligands allowed us to analyze the binding properties of IL-5 and to detect the presence of specific receptors for IL-5 on plasma membrane. In our experience, it seems that too much iodination of IL-5 molecule changes its conformation. In fact, TB13 anti-IL-5 antibody does not bind efficiently to heavily iodinated IL-5. Therefore, we rather preferred to use [^{35}S]IL-5 (4,000–8,000 cpm/U) for surveying the characteristics and the distributions of high-affinity binding sites. Under the condition used there was no significant difference in the binding affinity between radioiodinated IL-5 and unlabeled IL-5 to its receptor, as shown in Fig. 5.

The distribution of high-affinity IL-5-R among the various cell types broadly matches the pattern of cellular IL-5 responsiveness (6, 10, 13). This is in favor of the hypothesis that the biological effects of IL-5 are mediated by the plasma membrane receptor(s) identified in these studies.

Kinetic study of cell binding of radiolabeled IL-5 has demonstrated that binding of radiolabeled IL-5 to BCL_1-B20 cells was saturated within 10 min at 37°C (Fig. 2). This level was almost the same in the presence of 0.02% NaN_3 at 37°C. In comparison, at 4°C less than maximum binding was observed even after 3 h of incubation. The binding properties of IL-5 to its receptor are similar to that for IL-2 in terms of its rapid association to receptor sites within 10 min (34), but are different from that for BSF-2/IL-6, in that the level of binding at 0°C is higher than that at 37°C (26). From the binding profile and the Scatchard plot analysis (Figs. 3 and 4), two classes of IL-5 binding sites were detected on BCL_1-B20 cells. This is somehow similar to IL-2-R system (38), and is different from that of BSF-1/IL-4-R (23–25) and BSF-2/IL-6-R system (26), in which a single class of high-affinity binding sites are detected. Although BSF-1/IL-4 and BSF-2/IL-6 have diverse activities and targets (39, 40) like IL-5, regulation of their signal transduction may be different from that of IL-5.

Unlabeled native IL-5 purified from a TRF-producing T cell hybridoma B151K12 as well as rIL-5 gave the similar inhibitory effect on the binding of radiolabeled IL-5 on BCL_1-B20 cells (Table I). The present study clearly demonstrates that IL-5-R only bind IL-5 but not other cytokines, especially murine IL-3, murine GM-CSF, or IFN-γ (Table I). As we have previously described, the primary structure of IL-5 has some structural homology at the NH_2-terminal region with murine IL-3, murine GM-CSF, and murine IFN-γ (17). The results revealed that IL-5 is using different receptors from those for IL-3, IFN-γ, or GM-CSF.

Recent studies on receptors for cytokines such as IL-1, IL-2, BSF-1/IL-4, BSF-2/IL-6 demonstrated that cytokines do not have strict target cell specificity (23, 26, 39, 40). Our present studies using cloned tumor cell lines revealed that BCL_1 cells and MOPC104E cells expressed detectable levels of receptors for IL-5 (Table II). As we described previously (10, 17), IL-5 induces growth and differentiation of BCL_1 cells. Although we do not have concrete evidence for supporting that IL-5 acts on myeloma cells, IL-5 may promote growth of MOPC104E cells. Low numbers

of high-affinity IL-5 binding sites were detected on LPS-stimulated normal B cells, whereas resting B cells appear to express few IL-5 binding sites with high affinity, if any. Interestingly, early B cell lines of which growth are promoted by IL-5 expressed significant, but relatively low numbers of high-affinity binding sites for IL-5.

The level of expression of IL-5-binding sites was low, compared, for example, with receptor levels of IL-2 on T cells (38). The maximum biologic response occurred at IL-5 concentrations that only yield 40–50% of maximal IL-5 binding. These data are in contrast to the situation with IL-2, which gives coincident binding and biological response curves (38). Our results suggest that IL-5 may exert its biological effects at levels of 10–100 molecules bound per cell. In this sense, the IL-5 and IL-5-R systems seem to have analogy with IL-1 and IL-1-R Systems, as discussed by Dower et al. (41). The reason for that occupancy of low numbers of IL-5 binding sites is sufficient to transduce biological effect is unclear. The point to be considered is that the absolute values for the dissociation constant and number of binding sites per cell are dependent upon preliminary estimates of the purity and specific activity of the radiolabeled IL-5 preparations. While [^{35}S]IL-5 and ^{125}I-IL-5 are reasonably uniform with respect to size on SDS-PAGE, it has some microheterogeniety regarding isoelectric point, which is probably due to different glycosylation. Therefore, it may be necessary in the future to characterize IL-5 binding sites using radiolabeled IL-5 translated in *Escherichia coli*.

The characteristics of IL-5 binding sites with multiple affinities resemble the results obtained with other polypeptide growth factors. The receptor for epidermal growth factor was found to display low- and high-affinity binding sites, which could be interconverted (43). The receptor for IL-2 was also found to show in low and high binding sites (38). In this case, it is well accepted that high-affinity IL-2-R consist of heterodimers, p55 and p75 (43–45). We have no concrete evidence at this moment to support either possibility.

The crosslinking experiments provided some preliminary structural information for IL-5-R protein on the murine BCL$_1$ cell. The most likely candidate for the IL-5-binding protein is the one with a molecule of 46,500 M_r. This protein, when crosslinked to ^{125}I-IL-5, resulted in a 92,500 M_r band on SDS-PAGE under nonreducing conditions (Figs. 7 and 8). The 92,500 M_r protein was detected under all conditions used so far. The protein mixture of 92,500 M_r protein migrated on reducing gels at the 75,000 M_r protein band (Fig. 8 *B*). There are at least three possibilities to account for this observation. If there are ways that can be considered to interconvert high- and low-affinity IL-5 binding sites, as was done for the nerve growth factor receptor (46, 47), the IL-5-binding protein may be covalently linked to the monomer form of IL-5. If we assume that IL-5-R consists of a heterodimer, as was demonstrated for the IL-2-R as well as IL-3 (43–45, 48), the IL-5-binding protein (29,000 M_r) may not be covalently linked to the other (17,500 M_r) as part of a receptor complex. Third, unidentified receptor-associated molecules exist that may not be detected by crosslinking studies using DSS or EGS. Neither possibility is mutually exclusive at this moment.

IL-5 seems to be engaged as a potent factor, as far as we can see, which is capable of inducing growth and differentiation of murine B cells in an antigen-specific manner or polyclonal way. It is not clear, however, how IL-5 manipulates growth and differentiation of B cells in a single cell level. In other words, IL-5 can provide both growth-

and differentiation-inducing signals to B cells at the same time, or each signal is effective on B cells in a certain stage of differentiation or on B cells in different stages of every cell cycle. Moreover, IL-5 has diverse activities on different targets. In this respect, analysis of regulatory mechanisms of expression of IL-5-R is extremely important.

Experimental systems described in this study will give us further insight for investigating the molecular nature of IL-5-R. The availability of an assay system for the presence of IL-5-R makes it possible to screen for mAbs directed against IL-5-R, and to survey for cell lines expressing high levels of IL-5-R. These will be important steps toward isolating and characterizing the receptor protein(s) and the gene(s) that encode for IL-5-R.

Summary

T cell-replacing factor (TRF)/IL-5 is a glycosylated polypeptide that acts as a key factor for B cell growth and differentiation. Since IL-5 action is probably mediated by specific cell surface receptor(s), we have characterized the binding of IL-5 to cells using biosynthetically [^{35}S]methionine-labeled IL-5 and ^{125}I-IL-5 that had been prepared using Bolton-Hunter reagent. The radiolabeled IL-5 binds specifically to BCL_1-B20 (in vitro line) (a murine chronic B cell leukemic cell line previously shown to differentiate into IgM-secreting cells in response to IL-5) within 10 min at 37°C. There are two classes of binding sites with high affinity (K_d = 66 pM) and low affinity (K_d = 12 nM) for IL-5 and an average number of binding sites for high affinity and for low affinity were 400 and 7,500 per cell, respectively. The specificity of binding of radiolabeled IL-5 has been confirmed by demonstrating that only unlabeled IL-5 and anti-IL-5 mAb but not by IL-1, IL-2, IL-3, IFN-γ, and GM-CSF inhibit radiolabeled IL-5 binding to BCL_1-B20 cells. Treatment of surface-bound radiolabeled IL-5 with bivalent crosslinkers identified a membrane polypeptide of M_r 46,500 to which IL-5 is crosslinked.

A variety of cell types have been surveyed for the capacity to bind specifically radiolabeled IL-5 with high affinity. BCL_1 cells MOPC104E (murine myeloma cell line) expressed IL-5-R, whereas BAL. 17 and L10 A (B cell lymphoma) did not. T cell line, mastocytoma cell line, or macrophage tumor cell line did not display detectable levels of IL-5-R. IL-5-R were hardly detectable on normal resting B cells but were expressed on LPS-activated B cells, fitting the function of IL-5 that acts on activated B cells for their differentiation into Ig-secreting cells. Intriguingly, early B cell lines (J-87 and T-88) that grow in the presence of IL-5 expressed significant but low numbers of high-affinity binding sites for IL-5. The biological effects of IL-5 were mediated by high-affinity binding sites. The identification and characterization of IL-5-R should provide new insight into the apparent diverse biological activities of IL-5.

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