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Immunology

A soluble, high-affinity, interleukin-4-binding protein is present in the biological fluids of mice

(interleukin 4 receptor/immunoregulation)

RAFAEL FERNANDEZ-BOTRAN AND ELLEN S. VITETTA*

Department of Microbiology and Cancer Immunobiology Center, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75235

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ABSTRACT **Cytokines such as interleukin 4 (IL-4) play a key role in the regulation of immune responses, but little is known about how their multiple activities are regulated *in vivo*. In this report, we demonstrate that an IL-4-binding protein (IL-4BP) is constitutively present in the biological fluids of mice (serum, ascites fluid, and urine). Binding of ^{125}I-labeled IL-4 to the IL-4BP is specific and saturable and can be inhibited by an excess of unlabeled IL-4 but not IL-2. The IL-4BP binds IL-4 with an affinity similar to that reported for the cellular IL-4 receptor ($K_d \approx 7 \times 10^{-11}$ M) and has a molecular mass of 30–40 kDa and pI values of 3.6–4.8. IL-4BP-containing biological fluids or purified IL-4BP competitively inhibit the binding of ^{125}I-labeled IL-4 to mouse T or B cells and inhibit the biological activity of IL-4 but not IL-2. The serum levels of IL-4BP in severe combined immunodeficiency (SCID) mice are lower than those of normal mice. The above findings suggest that IL-4BP plays an important immunoregulatory role *in vivo*.**

Interleukin 4 (IL-4) is a pleiotropic lymphokine that plays a central role in the generation of IgG1 and IgE responses in mice (reviewed in refs. 1 and 2). IL-4 is also a growth factor for thymocytes, activated T cells, and mast cells and synergizes with other hemopoietic growth factors to induce the growth and differentiation of hemopoietic precursor cells. It is also an activation factor for macrophages (reviewed in refs. 1 and 2). Although it was initially thought that IL-4 is produced exclusively by activated T_{H2} cells (3), there is now evidence that IL-4 can also be secreted by mast cells (4) and certain B-cell lines (5). High-affinity receptors for IL-4 (IL-4Rs) have been identified on a wide variety of both hemopoietic and nonhemopoietic cells (6–9). Recently, the cloning and expression of the murine IL-4R have been reported (10, 11), including the description of a cDNA clone encoding a truncated soluble form of the IL-4R that appears to be secreted (10).

The mechanisms that regulate the multiple functions of IL-4 *in vivo* are not yet understood, and it is unclear whether IL-4 acts solely as a mediator between interacting cells or at sites distant from its production. In this report, we present evidence for the existence of a soluble, high-affinity, IL-4-binding protein (IL-4BP) in mouse serum, ascites fluid, and urine that may play an important role in regulating the effects of IL-4 *in vivo*. IL-4BP-containing fluids or purified IL-4BP preparations are able to compete with mouse T or B cells for the binding and biological activities of IL-4. The relationship of the IL-4BP found in biological fluids to the soluble form of the murine IL-4R reported by Mosley *et al.* (10) is discussed.

MATERIALS AND METHODS

Cells. The interleukin 2 (IL-2)/IL-4-responsive T-cell line HT-2 (12) was maintained in the presence of rIL-2 at 10 units/ml.

Antibodies, Lymphokines, and Reagents. Monoclonal antibodies or paraproteins were obtained by injection of hybridoma or myeloma cells into the peritoneal cavity of pristane-primed BALB/c *nu/nu* mice. Recombinant murine IL-4 (rIL-4) was a generous gift from K. Grabstein (Immunex, Seattle); recombinant human IL-2 was purchased from Am-Gen Biologicals. ^{125}I-labeled IL-4 (^{125}I-IL-4) was prepared by iodination of rIL-4 by following the procedure described by Lowenthal *et al.* (9) to a specific activity of 2–4 × 10^6 cpm/pmol. The concentration of the labeled IL-4 preparation was determined on the basis of its biological activity in the HT-2 proliferation assay (13). The crosslinking agent 3,3′-dithiobis(propionic acid *N*-hydroxysuccinimide ester) (DSP) was obtained from Sigma.

^{125}I-IL-4 Binding Assays. Binding of ^{125}I-IL-4 to intact cells (in the presence and absence of IL-4BP) was determined as previously described (14). Binding to soluble proteins in serum, ascites fluid, or urine was determined by using gel filtration through Sephadex G-50 minicolumns (Pharmacia) to separate free from bound ligand. Briefly, 5–50 μl of sample per tube was incubated for 60 min at 4°C with different concentrations of ^{125}I-IL-4 (5–200 pM) in a final volume of 120 μl of RPMI 1640 medium containing 10% fetal calf serum. The mixtures were then applied to 1-ml Sephadex G-50 columns previously washed with 20 mM sodium phosphate-buffered saline, pH 7.2 (PBS), containing 10% fetal calf serum and centrifuged at 600 × *g* for 90 sec. Bound ^{125}I-IL-4 was excluded, whereas free ^{125}I-IL-4 was retained in the column. Specific binding was calculated by subtracting the cpm bound in the presence of a 100-fold molar excess of unlabeled IL-4 (nonspecific binding) from the cpm bound in its absence (total binding).

Crosslinking of ^{125}I-IL-4 to IL-4BP. Aliquots (50 μl) of ascites fluid were incubated with ^{125}I-IL-4 (200 pM) in a final volume of 75 μl for 60 min at 4°C. Then 75 μl of a 1 mM DSP solution in PBS was added and the mixtures were incubated for 30 min at 4°C. Samples were analyzed by SDS/PAGE and autoradiography.

IL-4 Bioassay. The effects of the IL-4BP on the biologic activity of IL-4 and IL-2 were determined by measuring the rIL-4- or rIL-2-mediated proliferation of HT-2 cells (13) in the presence and absence of purified IL-4BP.

Purification of IL-4BP. Saturated ammonium sulfate (pH 7.4) was added to mouse ascites fluid to a final concentration of 50% saturation. The precipitate was dissolved in PBS and subjected to gel filtration on a Sephacryl S-200 column equilibrated with PBS. The fractions containing the IL-

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Abbreviations: IL-4, interleukin 4; IL-4BP, IL-4-binding protein; IL-4R, high-affinity receptors for IL-4; rIL-4, recombinant murine IL-4; IL-2, interleukin 2; DSP, 3,3′-dithiobis(propionic acid *N*-hydroxysuccinimide ester); SCID, severe combined immunodeficiency.

*To whom reprint requests should be addressed at: Department of Microbiology, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, TX 75235.

4-binding activity were pooled and passed through a Blue Sepharose column to remove contaminating albumin. The effluent was concentrated by precipitation with ammonium sulfate (50% saturation) and subjected to a second gel filtration step on a new Sephacryl S-200 column. Fractions containing the activity were again precipitated, dissolved, dialyzed against 25 mM imidazole/HCl (pH 7.4), and subjected to chromatofocusing in the 3–7 pH range. Fractions eluted in the presence of Polybuffer 7–4 (Pharmacia) (pI > 4.0) or 0.1 M HCl (pI < 4.0) were immediately neutralized and assayed for IL-4-binding activity. Active fractions were pooled, brought to 2 M in ammonium sulfate, applied to a phenyl-Sepharose column, and eluted with 50 mM sodium phosphate (pH 7.0). The active fractions were concentrated and subjected to SDS/PAGE under nonreducing conditions. After elution in the presence of 6 M urea/0.1 M Tris·HCl (pH 8.0), passage through Extractigel D columns (Pierce), and dialysis against PBS, the IL-4-binding activity was determined to be associated with a single band in the 30- to 40-kDa range. The molecular mass of the IL-4BP has been confirmed by a "ligand blot" assay, involving the transfer of the IL-4BP to a nitrocellulose membrane (after SDS/PAGE) and incubation with ^{125}I-IL-4 followed by autoradiography.

RESULTS

Inhibition of Binding of ^{125}I-IL-4 to Cells by Mouse Ascites Fluid. Initially, we observed that all samples of murine ascites fluid had the ability to inhibit the binding of ^{125}I-IL-4 to its receptors on T or B lymphocytes by as much as 90% (Fig. 1). Moreover, such samples also showed dose-dependent inhibition of IL-4 activity, as measured in the HT-2 proliferation assay (results not shown). Removal of the immunoglobulin from the ascites fluid by affinity chromatography did not eliminate the inhibitory activity, indicating that inhibition was mediated by a molecule other than immunoglobulin.

Binding of ^{125}I-IL-4 by a Factor in Murine Biological Fluids. To determine the nature of the inhibitory molecule, we developed a soluble-phase ^{125}I-IL-4-binding assay based on size separation of free from bound ligand on Sephadex G-50 minicolumns. Using this assay, we detected specific binding of IL-4 in all samples of mouse ascites fluid (13/13), mouse serum (15/15), and mouse urine (4/4) tested (Table 1). No specific binding of ^{125}I-IL-2 was observed in these samples. Binding of ^{125}I-IL-4 to the IL-4-BP in biological fluids was specific and could be inhibited by a 100-fold excess of unlabeled IL-4 (Table 1) or by an anti-IL-4 antibody, 11B11 (15) (data not shown), but not by IL-2 (Table 1). The activity of the IL-4BP in mouse serum is species specific: normal serum from rabbit, chicken, and human showed no detectable binding, and rat serum showed only trace binding of murine ^{125}I-IL-4 (Table 1). Binding of ^{125}I-IL-4 to the IL-4BP is saturable and concentration dependent. Scatchard analysis (16) of the binding data indicates that the IL-4BP has a single class of binding sites with a dissociation constant ($K_d \approx 7 \times 10^{-11}$ M) similar to that reported for cellular IL-4Rs (6–9). Assuming a 1:1 molar ratio of IL-4BP to IL-4, 1 ml of ascites fluid has $0.5–1.0 \times 10^{12}$ IL-4-binding sites (≈50 ng/ml, see below) (Fig. 2).

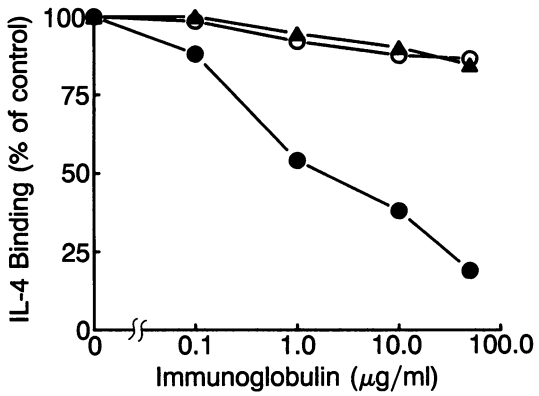

FIG. 1. Inhibition of the binding of ^{125}I-IL-4 to murine HT-2 cells by mouse ascites fluid or purified myeloma proteins. HT-2 cells were incubated with a fixed concentration of ^{125}I-IL-4 (5×10^{-11} M) in the presence of purified 1B711 IgG1 (10 μg per tube; ○), purified MOPC-21 myeloma IgG1 (▲), or 1B711 ascites fluid (●). 1B711 ascites fluid was added on the basis of its immunoglobulin concentration (μg/ml). Binding of ^{125}I-IL-4 to intact cells was determined as previously described (14).

Crosslinking of ^{125}I-IL-4 to the IL-4BP in Ascites Fluid. To determine the molecular mass of the IL-4BP, samples of ascites fluid were incubated with a saturating amount of ^{125}I-IL-4 for 60 min at 4°C in the presence or absence of a 100-fold molar excess of unlabeled IL-4, crosslinked with DSP, and subjected to SDS/PAGE and autoradiography. Such experiments showed a single band of approximately 45 kDa, indicating that the IL-4BP has a mass of ≈30 kDa (after subtraction of the 15 kDa corresponding to IL-4) (Fig. 3*A*). Addition of an excess of unlabeled IL-4 completely inhibited the detection of this band (Fig. 3*A*).

Purification of the IL-4BP. Recently, Mosley *et al.* (10) have reported the cloning of a cDNA encoding a soluble form of the murine IL-4R. The soluble rIL-4R binds IL-4 with an affinity comparable to that of the membrane form of the IL-4R and blocks the activity of IL-4 in a T-cell proliferation assay. Because of these similarities, it was hypothesized that the IL-4BP might be the natural secreted form of the IL-4R. Since the monoclonal anti-IL-4R antibody was not made available for us to test this directly, we have purified the IL-4BP from murine ascites fluid and characterized some of its physicochemical properties. A partially purified IL-4BP preparation was obtained (Fig. 3*B*, lane 3) with an average

Table 1. Binding of ^{125}I-IL-4 by a soluble factor in mouse ascites fluid, serum, and urine

Sample	Competitor	^{125}I-IL-4 binding, cpm per tube
Ascites fluid*		
1B711	—	2946
1B711	IL-4	240
1B711	IL-2	3022
1B711 (anti-Ig adsorbed)	—	2408
MOPC-104E	—	3268
MARK-1	—	3064
BCL1.X63	—	3133
29.13	—	2466
Serum		
Mouse	—	2155
Mouse	IL-4	0
Rat	—	231
Chicken	—	0
Human	—	0
Urine		
Mouse	—	2050

Binding of ^{125}I-IL-4 to soluble protein(s) in serum, ascites fluid, or urine was determined by using gel filtration through Sephadex G-50 minicolumns. Unlabeled IL-4 or IL-2 was used at a 100-fold molar excess over that of ^{125}I-IL-4. This table depicts a representative experiment of three performed. The SEM was lower than 10% for all points.

*1B711 is a mouse hybridoma secreting anti-trinitrophenyl antibodies (IgG1); MOPC-104E is a mouse IgM myeloma; MARK-1 is a mouse hybridoma secreting anti-rat κ chain antibodies (IgG1); BCL1.X63 is a murine hybridoma secreting both IgM and IgG1; and 29.13 is a mouse IgG3 myeloma.

increase in specific activity of 1500-fold and a recovery of 30%. Chromatofocusing experiments indicated considerable charge heterogeneity, with pI values in the 3.6–4.8 range. Assays for IL-4-binding activity carried out on material eluted from SDS/PAGE in 6 M urea and renatured by dialysis against PBS indicated that IL-4-binding activity was associated with a single 30- to 40-kDa protein. These results have also been confirmed by a "ligand blot" assay, carried out on a nitrocellulose filter blot of the SDS gel and incubation with ^{125}I-IL-4 (results not shown).

Inhibition of IL-4 Activity by Purified IL-4BP. As shown in Fig. 4*A*, purified IL-4BP inhibited the binding of ^{125}I-IL-4 to HT-2 cells. The degree of inhibition was inversely related to the concentration of IL-4, suggesting that IL-4BP acts as a competitive inhibitor of IL-4. In addition, the IL-4BP specifically blocked the biological activity of IL-4, as demonstrated by the dose-dependent inhibition of IL-4-, but not IL-2-, mediated proliferation of HT-2 cells (Fig. 4*B*).

IL-4BP Levels in Sera of Mice with Normal or Altered Immune Systems. We have measured the IL-4-BP in the sera of mice with "normal" immune systems (BALB/c) or altered immune systems [immunodeficient—i.e., severe combined immunodeficiency (SCID), *nu/nu*, or anti-CD4 treated; immune—i.e., following immunization; and autoimmune—i.e., NZB]. As shown in Table 2, with the exception of the SCID mice, which had low levels of the IL-4BP in their sera, changes in the serum levels of the other groups were modest. Nevertheless, in three experiments performed, there were decreased levels of IL-4BP in the sera of mice receiving anti-CD4 antibodies (17) and in nude mice. Conversely, animals with activated immune systems (NZB or immunized mice) had marginally increased IL-4BP in their sera. IL-4-binding activity was also detected, albeit at levels lower than those found in sera, in culture supernatants from mitogen-activated T and B cells.

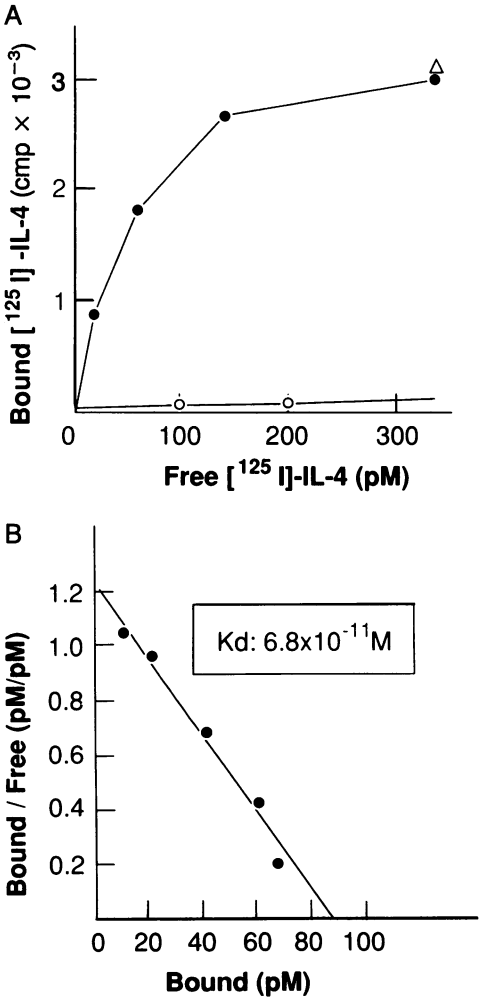

FIG. 2. Binding of ^{125}I-IL-4 to an IL-4BP present in murine ascites fluid. (*A*) A constant amount of murine ascites fluid (1B711, 5 μl per tube) was incubated with increasing concentrations of ^{125}I-IL-4 in the absence (●) and presence of a 100-fold molar excess of unlabeled IL-4 (○) or IL-2 (△). Binding of ^{125}I-IL-4 was determined by gel filtration. (*B*) Scatchard analysis of binding data shown in *A*.

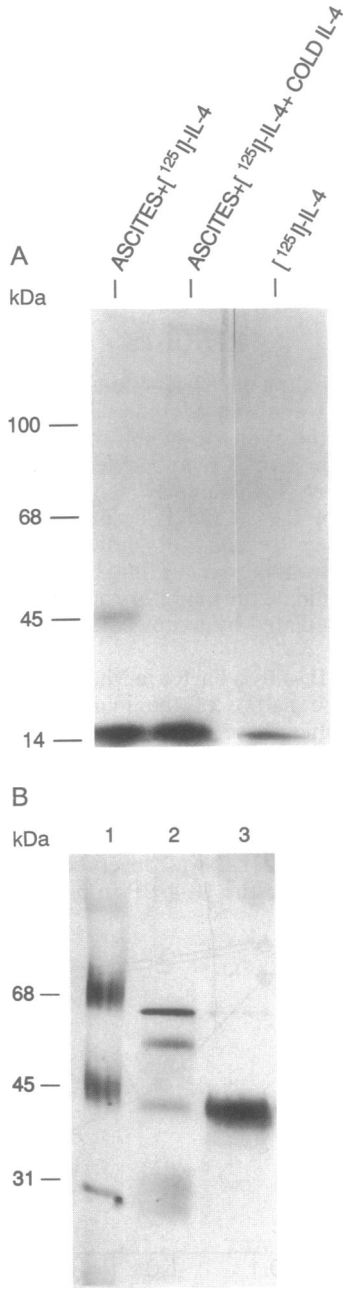

FIG. 3. Biochemical characterization of IL-4BP. (*A*) Crosslinking of ^{125}I-IL-4 to an IL-4BP in mouse ascites fluid. Mouse ascites fluid was incubated with a saturating amount of ^{125}I-IL-4 for 60 min at 4°C and crosslinked with DSP. (*B*) SDS/PAGE of purified IL-4BP (after silver staining). Lane 1, molecular mass standards; lane 2, partially purified IL-4BP after phenyl-Sepharose chromatography step; lane 3, purified IL-4BP after elution from an SDS gel. Between 50% and 60% of the IL-4-binding activity originally loaded onto the gel was recovered in association with this band.

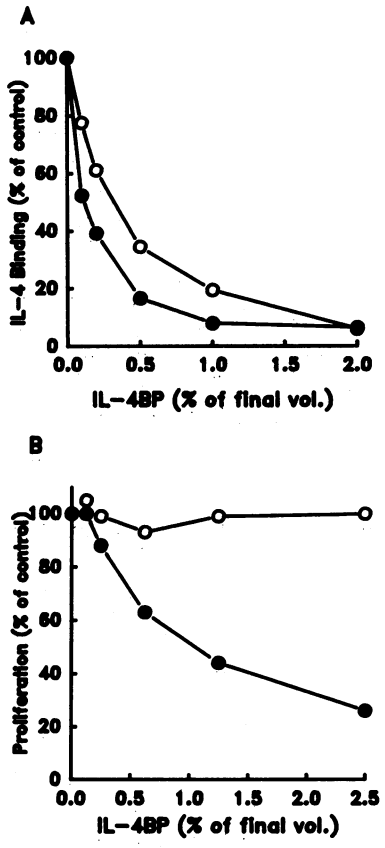

FIG. 4. IL-4BP can compete with HT-2 cells for IL-4 binding. (*A*) Inhibition of ^{125}I-IL-4 binding to HT-2 cells by purified IL-4BP. HT-2 cells were incubated with ^{125}I-IL-4 at 10 pM (●) or 50 pM (○) in the presence of increasing amounts of purified IL-4BP for 60 min at 4°C. Specific binding was assessed as described. (*B*) Inhibition of IL-4-, but not IL-2-, mediated proliferation of HT-2 cells by purified IL-4BP. HT-2 cells were cultured with rIL-4 at 10 pM (○) or rIL-2 at 10 pM (●) in the absence or presence of increasing amounts of purified IL-4BF (see Fig. 3*B*, lane 3). Proliferation was assessed by [^{3}H]thymidine incorporation after 24 hr of culture (13).

DISCUSSION

Most cytokines have multiple activities and act on a variety of cells from hemopoietic and nonhemopoietic origins (reviewed in ref. 2). Because of the pleiotropism and potency of cytokines, their activity *in vivo* must be highly regulated. The presence in serum and other biologic fluids of a soluble protein (IL-4BP) with the ability to bind IL-4 with high affinity and block the binding of IL-4 to cellular IL-4Rs suggests an important immunoregulatory role for this protein *in vivo*. Soluble forms of the low-affinity IL-2 receptor (p55) (18–23) and the Fc portion of IgE (CD23) (24–27) have been reported. More recently, soluble forms of receptors for other cytokines, such as interferon γ, interleukin 6, and tumor necrosis factor α, have also been described (28). Thus, it is possible that soluble receptors in biological fluids may be part of immunoregulatory circuits *in vivo*. For example, soluble CD23 has been implicated in the regulation of IgE synthesis (29). Although the low affinity of the soluble form of the IL-2R is difficult to reconcile with a regulatory function at normal serum concentrations (22, 23), elevated levels of soluble IL-2Rs have been observed in the sera of patients with various forms of leukemias (30–32), rheumatoid arthritis (33), multiple sclerosis (34), and human immunodeficiency virus-induced disease (35). These observations raise the possibility that at these concentrations, soluble IL-2Rs might have an immunoregulatory role.

Table 2. Relative levels of soluble IL-4BP in mouse sera, ascites fluids, and culture supernatants

Sample	Relative binding activity*
Normal mouse serum†	1.0
Ascites fluid (hybridoma)‡	1.8
SCID serum	0.2
nu/nu serum	0.7
NZB serum	1.2
Serum from anti-CD4 (GK1.5)-treated mice	0.5
Immune serum	1.1
Supernatant from Con A-stimulated spleen cells	0.3
Supernatant from lipopolysaccharide-stimulated spleen cells	0.2

*IL-4 binding is expressed as the ratio of the IL-4-binding activity of each sample to that of normal mouse serum (assigned a value of 1.0). The SEM was less than 10% for all samples (n = 3).
†Value represents the average of 6 different sera; 15 sera were tested, and all gave similar values.
‡Value represents the average of 12 different ascites fluids.

Very recently, the cloning and expression of cDNAs encoding the murine IL-4R have been reported by two different groups (10, 11). Interestingly, Mosley *et al.* (10) describe three different types of cDNAs, one of which appears to encode the extracellular domain but not the transmembrane or intracytoplasmic domains. This form of rIL-4R retains its ligand-binding activity but is secreted, rather than membrane bound. It is thus possible that the IL-4BP described in our studies might be the natural secreted form of the IL-4R, although the relatively high levels of the IL-4BP in serum and ascites fluid are difficult to reconcile with the reported low abundance of cDNAs encoding the secreted (5–10% of the total), as compared to the membrane, form of the IL-4R. Although the properties of the IL-4BP investigated so far appear similar to those of the soluble IL-4R (affinity, molecular mass), definitive proof of their identity must await the availability of monoclonal anti-IL-4R antibodies.

There was no definitive correlation between the serum levels of IL-4BP and the immune status of different mice, with the exception of SCID mice. We emphasize, however, that our binding assay detects only "free" IL-4BP and not IL-4BP already bound to IL-4. Hence, undetected *bound* IL-4BP could mask detection of elevations of this protein in the different serum samples. Because the sera from SCID mice had significantly less IL-4BP compared with sera from normal animals and because the IL-4-BP was detected in supernatants from mitogen-activated T and B cells, mature lymphoid and plasma cells might be a major source of this protein.

The high affinity and concentration of the IL-4BP in serum suggest an important immunoregulatory function. This concept is attractive because of the requirement for antigenic and cell lineage specificity in the regulation of the immune response. However, there are no data to indicate how cytokine activity might be restricted to a local site and be rapidly inhibited in an *in vivo* setting. If the IL-4BP can compete with cellular IL-4Rs for free IL-4 (as suggested by our *in vitro* results), then the IL-4BP should be capable of precluding IL-4 from acting on cells at anatomical sites distant from its site of production. Alternatively, IL-4BP might serve as a "transport" molecule, binding IL-4 in the circulation and delivering it to sites where the concentration of cellular IL-4Rs would greatly exceed that of IL-4BP.

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