Enhancement of the Biologic Effects of Interleukin-3 In Vivo by Anti–Interleukin-3 Antibodies

By A. Tomlinson Jones and H.J. Ziltener

Interleukin-3 (IL-3) has been shown to be a promising agent in the stimulation of bone marrow regeneration following myeloablative therapy. The biologic half-life of this agent is very short (5 to 15 minutes), which limits the effectiveness of low-dose therapy. Here we show that the biologic effects of low-dose IL-3 in mice may be enhanced by concurrent use of polyclonal anti–IL-3 antibodies. The biologic effects of IL-3 in vivo were enhanced dramatically by the combination of the cytokine and polyclonal rabbit anti–IL-3 antibodies, which recognized a peptide comprising the first 29 amino acids of the IL-3 molecule. Enhancing effects were not apparent in vitro, where weak neutralizing properties were observed for these antibodies. The mechanism of this enhancement by the antibody appears to be via a ninefold reduction in the total-body clearance of the cytokine in vivo. The apparent volumes of distribution for IL-3 and for the IL-3/antibody complex were surprisingly similar and exceeded the expected intravascular volume. The prolonged biologic half-life of IL-3 was reproducibly associated with a threefold to fivefold increase in splenic mast-cell precursors over levels observed in mice treated with IL-3 alone; increases in the numbers of mature mucosal-type mast cells in the spleen, but not in the jejunum or lung; increases in IL-3–dependent colony-forming unit–cell in the spleen; and an apparent redistribution of mast cells away from the bone marrow. These experiments demonstrate that antibodies to a cytokine can enhance the biologic activity of that cytokine in vivo.

© 1993 by The American Society of Hematology.

MATERIALS AND METHODS

Production of antibodies. Synthetic peptides corresponding to the first 29 amino acids of murine IL-3 (1-29 mIL-3) and to the full-length molecule (1-140 mIL-3) were synthesized by solid-phase methods and coupled to keyhole limpet hemocyanin (KLH), as described elsewhere. Four Dutch-Belted rabbits were each injected in six subcutaneous sites with 300 μg of peptide (total) emulsified in complete Freund’s adjuvant. After 4 weeks, the rabbits were bled and boosted at two-weekly intervals with decreasing amounts of antigen (200 to 50 μg) emulsified in incomplete Freund’s adjuvant. At monthly intervals, rabbit sera were screened for production of high-titer anti–IL-3 or antipeptide antibodies by enzyme-linked immunosorbent assay (ELISA) as previously described. Antibodies were affinity-purified over activated thiol-sepharose (Pharmacia, Uppsala, Sweden) to which 1-29 (IL3) peptide had been coupled. Antigen-specific antibodies were eluted using 0.1 mol/L glycine, pH 2.5, and dialyzed against 0.9% (wt/vol) saline solution. Purified antibodies were concentrated to 0.5 mg/mL and assayed by ELISA for binding to denatured and recombinant antigen. Binding of antibodies to denatured antigen was detected using a conventional ELISA employing 10 μg/mL of full-length chemically synthesized IL-3 adsorbed to ELISA plates (Becton-Dickinson, Oxnard, CA). Binding of antibodies to recombinant IL-3 was

From The Biomedical Research Centre and the Department of Pathology, University of British Columbia, Vancouver, Canada.

Submitted July 13, 1992; accepted April 23, 1993.

Supported by the B.C. Health Care Foundation and The Biomedical Research Centre. A.T.J. is the recipient of a Medical Research Council of Canada Fellowship.

Address reprint requests to H.J. Ziltener, PhD, Biomedical Research Centre, University of British Columbia, 2222 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1993 by The American Society of Hematology.
detected using a sandwich ELISA method adapted to assay antibody binding.\textsuperscript{18} ELISA plates were coated with 10 µg/mL polyclonal sheep antibody raised against IL-3. Recombinant IL-3 at a concentration of 50 U/mL was added to the plates, which were incubated for 2 hours at room temperature. Serial dilutions of the rabbit anti-1-29 (IL-3) antibodies were transferred to the plate. Antibody binding to captured IL-3 was detected using a peroxidase-coupled anti-rabbit antibody as previously described.\textsuperscript{14}

Production of recombinant mIL-3. The murine nonproducing myeloma X63Ag8-653 (X63) transfected with the expression vector pBValPHA modified to express MGnGNeo-mIL-3 was obtained from F. Melchers (Basel Institute of Immunology, Basel, Switzerland). The resultant stable transformant, X63-omIL-3, constitutively secretes murine IL-3 in high quantities.\textsuperscript{19} Conditioned media from this cell line was concentrated 10-fold using an ultrafiltration concentrator (Amicon, Danvers, MA) with a membrane molecular weight cut off of 5 Kd. The concentrated media was then applied to cyanogen bromide-activated Sepharose (Pharmacia) to which purified rabbit polyclonal antibodies raised against the 1-29 (IL-3) peptide had been coupled. The columns were washed with 10 times their bed volume of phosphate-buffered saline (PBS). IL-3 was then eluted with 0.1 mol/L glycine, pH 2.5, neutralized with saturated Trizma base solution, and dialyzed three times against 0.9% (wt/vol) saline solution. All affinity-purified recombinant IL-3 preparations were filter sterilized and assayed as described below. This method of affinity purification resulted in 90% depletion of IL-3 bioactivity from the conditioned media and approximately 90% of activity was recovered in the eluate. IL-3 preparations were diluted to 10,000 U/mL with 0.9% (wt/vol) saline, stored at −20°C, and thawed before use. Recombinant IL-3 was used for all in vivo studies and was filter-sterilized immediately before use.

Bioassay of mIL-3. Recombinant IL-3 preparations were assayed for biologic activity using the IL-3–responsive cell line, R6-XE4, as described previously.\textsuperscript{20} Experiments were performed in triplicate. Results were compared with a standard curve produced by serial dilution of a known amount of recombinant IL-3. One biologic unit of IL-3 was defined as that amount producing 50% of maximal [\textsuperscript{3}H]thymidine incorporation over a 6-hour period.

Bioassay of recombinant IL-3 in the presence of antipeptide antibodies. Rabbit anti-1-29 (IL-3) antibodies were serially diluted fourfold from a stock of 10 µg/mL and titrated in triplicate against a constant amount of recombinant mIL-3 (1 U/mL) over 10 wells of a Terasaki tray and assayed for proliferative activity as described above. Experiments were performed in triplicate and compared with the proliferative effect of an equivalent concentration of recombinant IL-3 alone.

Gel-filtration profile of antibodies. A 2.5×100-cm column of Sephadex G100 (Pharmacia) was equilibrated with PBS. Fifty micrograms of rabbit anti-1-29 (IL-3) antibodies were preincubated for 30 minutes with 2,000 biologic units of recombinant IL-3 in 200 µL of PBS and then applied to the column and eluted in 1-mL fractions. Fractions were assayed for IL-3 bioactivity using the bioassay described, and for antibody activity using the ELISA method described. This method was used as a screening test for high-affinity antibodies.

To determine the exact molecular weight of high-affinity antibody-antigen complexes and the number and types of complexes formed, similar analyses were performed using a fast protein liquid chromatography (FPLC) apparatus equipped with a Superose 6 column (Pharmacia).

In vivo pharmacology. Balb/c mice were housed in the animal facility of the University of British Columbia under specific pathogen-free conditions with access to sterilized food and water ad libitum. Mice were used at 8 to 12 weeks of age. Groups of mice received intravenous injections into the tail vein of (1) 1,000 biologic units of recombinant IL-3, (2) 10 µg rabbit anti-1-29 (IL-3) antibodies, (3) IL-3 and rabbit anti-1-29 (IL-3) antibodies, or (4) IL-3 and 10 µg rabbit control IgG. All injections were made in a total volume of 120 µL 0.9% (wt/vol) saline. At intervals, groups of four mice were killed and bled by cardiac puncture to obtain serum for estimation of IL-3 bioactivity and antibody titer, as described above. Experiments were repeated on three separate occasions. Clearance data were also obtained following injection of 10,000 biologic units of IL-3, to determine the effect of higher doses on clearance. Clearance data were described statistically using the MULTIFIT program supplied by D.W.A. Bourne (Oklahoma University College of Pharmacy, Oklahoma City, OK).\textsuperscript{21}

In vivo biology. DBA2 mice were allocated to groups of five mice and received intravenously (1) 1,000 biologic units of mIL-3, (2) 10 µg of rabbit anti-1-29 (IL-3) antibodies, (3) both 1,000 biologic units of IL-3 and 10 µg of rabbit anti-1-29 (IL-3) antibodies, or (4) 0.9% (wt/vol) saline injections were removed at 24-hour intervals for 5 days. On the sixth day, the animals were killed and serum, bone marrow, lung, jejunum, and spleen removed for further analysis. In some experiments, mice were injected with 1,000 U of IL-3 and 10 µg rabbit control IgG to act as an additional control.

Quantification of spleen colony formation in vitro. Spleens were bisected and single-cell suspensions prepared from half of each spleen (the remainder being fixed for histology). Red blood cells were removed by hypotonic lysis using Tris·HCl-NH\textsubscript{4}Cl. Cells were counted and resuspended at a concentration of either 10\textsuperscript{4} or 10\textsuperscript{5} cells/mL in a 0.3% solution of blue bactor agar (Difco Laboratories, Detroit, MI) in RPMI medium containing 10% fetal calf serum (FCS) and β-mercaptoethanol. Ten-fold concentrated WEHI-3B-conditioned media was added at 2% (vol/vol) as a source of IL-3. Agar cultures were incubated for 7 days at 37°C in 5% CO\textsubscript{2} and scored blind for colony formation.

Determination of mast-cell precursor frequency in vivo. The frequency of mast-cell precursor in bone marrow and spleen was determined as described previously.\textsuperscript{22} Single-cell suspensions of spleen cells prepared as above and of bone marrow cells eluted from mouse femurs were plated at five different concentrations into wells of 96-well tissue culture–treated trays (Costar, Cambridge, MA) in 150 µL of RPMI medium containing 10% FCS, β-mercaptoethanol, and 2% (vol/vol) 10× WEHI-3B-conditioned media as a source of IL-3. After 15 days, trays were scored blind by two scorers for mast-cell growth. The frequency of mast-cell precursors was calculated from the dilution-dilution data using a Poisson probability distribution, lines being fitted to the data points by maximal likelihood estimation techniques. Calculations were performed using the dlimt program\textsuperscript{23} to determine the frequency of mast-cell precursors and the 95% confidence limits.

Histology. One centimeter of jejunum, one lung, one popliteal lymph node, and half of the spleen from each animal were fixed in methanol, formalin, and acetic acid (85%, 10%, 5% vol/vol) for 24 hours at 4°C. Sections were stained with either alcan blue-saffrin for mast-cell determination\textsuperscript{24} or hematoxylin and eosin (H&E) for assessment of histopathologic changes. Mast-cell numbers and megakaryocytes were enumerated in 40 randomly chosen high-power (×40) fields from each group. Averaged results for each mouse were then analyzed by analysis of variance (ANOVA) to determine differences between the groups.

Statistics. Statistical analysis of results was performed by ANOVA using the STATVIEW program (Microsoftword, Seattle, WA). This program calculates significance at the 95% level by both the Fisher probability of least significant difference (PLSD) and Scheffe F test.
RESULTS

Rabbit anti-1-29 (IL-3) recognizes synthetic and native IL-3 in solid phase with high titer. Antibodies raised against synthetic peptides recognize linear epitopes within their target molecule and frequently do not recognize the full-length molecule in its non-denatured conformation. Conventional ELISA may detect binding of antibodies to denatured antigen bound to the solid phase and may overestimate binding to the antigen in its native state. To determine the reactivity of our antibodies with recombinant IL-3 in a non-denatured state, we used a sandwich ELISA, which employs a sheep anti-IL-3 antibody to capture recombinant IL-3 in solution. Binding of affinity-purified rabbit anti-1-29 (IL-3) antibodies to both recombinant and denatured IL-3 could be detected to antibody concentrations of less than 0.5 ng/mL.

Rabbit anti-1-29 antibodies show neutralizing properties in bioassay. Previous work has shown that the first 16 N-terminal amino acids of IL-3 are not essential for receptor binding and that biologic activity is observed for peptides derived from the IL-3 molecule, which lack the first 16 amino acids. We have demonstrated by fluorescence-activated cell sorter (FACS) analysis that rabbit anti-1-29 (IL-3) antibodies recognize IL-3 when this molecule is bound to its receptor. We therefore hypothesized that rabbit anti-1-29 (IL-3) antibodies will bind at sites that do not compromise the biologic activity of IL-3. To test this hypothesis, rabbit anti-1-29 (IL-3) antibodies were assayed for neutralizing or enhancing properties in vitro on the IL-3-responsive cell line, R6-XE4. Rabbit anti-1-29 (IL-3) antibodies produced modest neutralization of the bioactivity of recombinant mIL-3 over the range of concentrations tested (Fig 1). Inhibition was only apparent when concentrations of greater than 50 ng/mL were used in combination with 1 U of IL-3 (∼2,000-fold excess of antibody combining sites over IL-3). The ratio of concentrations used in subsequent experiments (10 ng antibody to 1 U IL-3) was associated with negligible inhibition of biologic activity.

Rabbit anti-1-29 (IL-3) antibodies augment the apparent molecular weight of IL-3 as assessed by gel-filtration chromatography. To determine whether rabbit anti-1-29 (IL-3) antibodies will form tight complexes with IL-3 in solution, rabbit anti-1-29 (IL-3) antibodies were incubated together with recombinant IL-3 and then passed over a gel-filtration column. Biologic activity of IL-3 and antibody titer were determined for each of the fractions eluted and compared with the elution profile of IL-3 alone. IL-3 alone eluted in fractions 10 to 16, with the peak of biologic activity occurring in fraction 12 (Fig 2A). This corresponds to a molecular weight of 20 to 50 Kd. When the mixture of IL-3 and rabbit anti-1-29 (IL-3) antibodies were applied to the column, IL-3 bioactivity and antibody titer coeluted in the void volume (Fig 2B) corresponding to a molecular weight in excess of 100 Kd. It was evident that despite the neutralizing properties of rabbit anti-1-29 (IL-3) antibodies, IL-3 bioactivity was still readily detectable in the eluted complex.

To determine the exact size of the complex, 10 μg rabbit anti-1-29 (IL-3) antibodies were preincubated with 1,000 U IL-3 and passed over a Superose 6 gel-filtration column. A representative elution profile is shown in Fig 3. The IL-3 bioactivity in the complex eluted in fractions 14 to 17, with a peak of activity in fraction 15. This corresponded to a molecular weight of 200 to 250 Kd. However, the antibody titer eluted in a broader peak and was greatest in fractions 14 to 18, suggesting that the majority of the antibody was uncomplexed. This finding is not unexpected, since antibody was present in a 1,000-fold excess. If a lower antibody to IL-3 ratio is used, the predominant complexes have the same molecular weight as above; however, a proportion of the IL-3 dissociates and may be detected in fractions 18 and 19 (corresponding to a molecular weight of 25 to 50 Kd; data not shown). The shape of the elution profile in these experiments suggested that a single species of immune complex predominated (Fig 3). There was no evidence for complexes of greater molecular weight, indicating that the major component of the complex was a single IL-3 molecule together with one antibody molecule; however, the resolution of the method is not sufficient to exclude the presence of

**Fig 1.** Effect of rabbit anti-1-29 (IL-3) antibody on the IL-3 proliferative response of R6-XE4 cells; 500 R6-XE4 cells were incubated for 24 hours with 1 U/mL recombinant IL-3 in the presence or absence of serial 1:4 dilutions of 10 μg/mL rabbit anti-1-29 (IL-3). Proliferative response is expressed as mean counts per minute of 3H-thymidine uptake for triplicate wells during a 6-hour pulse. IL-3 alone (●); IL-3 plus antibody (■).
Anti-IL-3 antibodies extend the biologic half-life of IL-3 in vivo. In a series of experiments, mice were injected with either IL-3, rabbit anti-1-29 (IL-3) antibodies, or a combination of both. At specific time intervals following injection, the mice were killed and serum obtained for estimation of IL-3 bioactivity and antibody titer. A representative experiment is shown in Fig 4A. No IL-3 bioactivity was detected at any time point in mice treated with the antibody alone. The data for the clearance of IL-3 alone and for the IL-3/anti-body complex were best described by a two-compartment model and were fitted to this model by the MULTIFORTE program using the Gauss-Newton method and weighting the data by the reciprocal of the plasma concentration squared. For this data set, this gave an excellent fit to the data with a weighted sum of the squares (WSS) value of 0.05356 and an $R^2$ value of 1.00 for IL-3 alone and a WSS value of 0.03885 and an $R^2$ value of 1.00 for IL-3 and antibody. In this experiment, the $\alpha$ half-life for IL-3 alone was calculated to be 2.1 minutes and the $\beta$ half-life was calculated to be 10.0 minutes. The $\alpha$ half-life for IL-3 together with antibody was 5.7 minutes and the $\beta$ half-life was 72.5 minutes. No IL-3 bioactivity could be detected in the group treated with IL-3 alone after 1 hour. The total-body clearance of the IL-3-antibody complex was 5.1 mL/h, compared with 45.5 mL/h for IL-3 alone. The calculated volume of distribution was slightly smaller for the complex at 8.9 mL compared with 10.9 mL for IL-3 alone, but was still in excess of the volume of the intravascular compartment (1 to 2 mL), suggesting that the complex is not restricted to this compartment. Antibody titer remained unchanged throughout the duration of the experiment (data not shown). This is compatible with the expected half-life for heterologous antibody, which is 4 to 5 days.

Clearances were performed in the presence of rabbit control IgG. Rabbit control IgG did not produce significant enhancement of the half-life of IL-3. The $\alpha$ half-life of IL-3 in these experiments was 2.1 minutes and the $\beta$ half-life was 15 minutes.

To determine the effect of a higher dosage on IL-3 clearance, mice were injected with 10,000 U IL-3. The clearance of this high-dose IL-3 was prolonged compared with low-dose IL-3. The initial $\alpha$ half-life was 3.6 minutes and the $\beta$ half-life was 30.8 minutes. The calculated volume of distribution was 13.6 mL and the total-body clearance was 18.09 mL/h.

Anti-IL-3 antibodies increase CFU-c in the spleen. To determine whether prolongation of the biologic half-life of IL-3 would result in augmentation of the biologic effects of IL-3 treatment, mice were allocated to one of four treatment regimens. To maximize any effect attributable to prolongation of IL-3 half-life, the dosing interval chosen for each group was 24 hours. The biologic effects of these regimens were assessed using a number of IL-3-responsive assays. The frequency of IL-3-responsive CFU-c in the spleen of treated mice was determined by an in vitro colony assay (Fig 5). The frequency of IL-3-responsive colonies obtained from spleen cells from mice injected with IL-3 alone or from mice injected with rabbit anti-1-29 (IL-3) antibody alone was not significantly different from that of control animals. The combination of this antibody and IL-3 resulted in a threefold to sevenfold increase in the number of IL-3-dependent colonies in repeat experiments. Colonies contained cells exhibiting both macrophage and neutrophil morphology, consistent with the known biologic effects of IL-3. The data indicate that the combination of rabbit anti-1-29 (IL-3) antibody with IL-3 results in an enhancement of the biologic effects of IL-3. Control IgG was ineffective in augmenting the response to IL-3 and the number of colonies scored was not significantly different from controls in experiments where the combination of rabbit anti-1-29 (IL-3)
antibody and IL-3 produced threefold more colonies than IL-3 alone (data not shown).

Anti-IL-3 antibodies increase mast-cell precursor frequencies in the spleen. The frequency of mast-cell colony precursors detected when spleen cells are maintained in liquid culture for 15 days has been shown to be a sensitive and specific assay of the in vivo response to treatment with IL-3. The observed frequency of mast-cell precursors among spleen cells from mice treated with both antibody and IL-3 was threefold to fivefold greater than that seen in control animals or in animals treated with IL-3 or rabbit anti-1-29 antibody alone (Fig 6A). The frequencies of mast-cell precursors in animals treated with a combination of control rabbit IgG and IL-3, rabbit anti-1-29 (IL-3) antibody alone, or IL-3 alone were not significantly different from values of animals treated with control saline (Fig 6A and B).

The frequency of mast-cell precursors in the bone marrow was slightly reduced in mice treated with both rabbit anti-1-29 (IL-3) antibody and IL-3, compared with IL-3- or saline-treated controls (Fig 7).

Anti-IL-3 antibodies increase the numbers of mature mast cells in the spleen, but not in other tissues. Spleen sections taken from mice treated with both IL-3 and rabbit anti-1-29 (IL-3) antibody showed significant increases in
the numbers of mature mast cells observed compared with other groups (Table 1) \( (P < .05) \). Many of the mast cells observed in the IL-3-treated and rabbit anti–1-29 (IL-3) antibody–treated sections were small, immature mast cells with few alcian-blue–positive granules. These observations are compatible with a relatively recent increase in proliferation of precursor cells. The numbers of megakaryocytes in the sections examined were not significantly different between the groups. Mast-cell numbers were not increased in either lung, jejunum, liver, or lymph nodes (data not shown). No abnormal histopathologic changes were seen on any sections stained with H&E.

**IL-3 bioactivity is not detectable in serum of treated mice.**

**24 hours after injection.** IL-3 bioactivity was not detected in serum samples obtained from each group at the conclusion of the experiment. The data indicate that no treatment regimen resulted in accumulation of IL-3.

**DISCUSSION**

The data presented here demonstrate the ability of anti–IL-3 antibodies to enhance the biologic activity of IL-3 in vivo. The extent of the enhancement observed varied with the antibody preparation used, as would be expected for a polyclonal antibody, and correlated best with the ability to

![Graph A](image1)

![Graph B](image2)

**Fig 6.** Frequency of mast-cell precursors in spleens of mice undergoing different treatments. (A) Balb/c mice were treated for 5 days with either saline, rabbit anti–1-29 (IL-3) antibody alone, IL-3 alone, or IL-3 plus rabbit anti–1-29 (IL-3) antibody. At day 6, mice were killed and spleen cells disaggregated and serially diluted into 96-well tissue culture plates containing 150 μL/well RPMI, 10% FCS, and 2% WEHI-3B–conditioned media. After 15 days of incubation at 37°C in 5% CO_2_, plates were scored blind for mast-cell colony formation. The frequency of negative wells in each tray was used to derive a precursor frequency. Precursor frequencies ± 95% confidence limits are shown for pooled spleens from five mice for each treatment group. (B) Similar analysis is shown for mice treated with saline, IL-3 alone, IL-3 plus rabbit control IgG, and IL-3 plus rabbit anti–1-29 (IL-3).

**Fig 7.** Frequency of mast-cell precursors in bone marrows of mice undergoing different treatments. (A) Balb/c mice were treated for 5 days with either saline, rabbit anti–1-29 (IL-3) antibody alone, IL-3 alone, or IL-3 plus rabbit anti–1-29 (IL-3) antibody. At day 6, mice were killed and bone marrow cells serially diluted into 96-well tissue culture plates containing 150 μL RPMI, 10% FCS, and 2% WEHI-3B–conditioned media. After 15 days of incubation at 37°C in 5% CO_2_, plates were scored blind for mast-cell colony formation. The frequency of negative wells in each tray was used to derive a precursor frequency. Precursor frequencies ± 95% confidence limits are shown for pooled spleens from five mice for each treatment group.

![Graph C](image3)

**Table 1.** Mast-Cell and Megakaryocyte Frequencies in the Spleen

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mast Cells per High-Power Field (&gt;40)</th>
<th>Megakaryocytes per High-Power Field (&gt;40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0.27 ± 0.09</td>
<td>1.4 ± 0.33</td>
</tr>
<tr>
<td>Rabbit anti–1-29 (IL-3) antibody</td>
<td>0.32 ± 0.16</td>
<td>1.2 ± 0.37</td>
</tr>
<tr>
<td>IL-3</td>
<td>0.40 ± 0.13</td>
<td>0.9 ± 0.21</td>
</tr>
<tr>
<td>IL-3 + rabbit anti–1-29 (IL-3) antibody</td>
<td>1.92 ± 0.36*</td>
<td>1.5 ± 0.07</td>
</tr>
</tbody>
</table>

Balb/C mice were treated for 5 days with saline, rabbit anti–1-29 (IL-3) antibody alone, IL-3 alone, or IL-3 plus rabbit anti–1-29 (IL-3) antibody. At day 6, mice were killed and spleens fixed with methanol:formalin:acetic acid (85:10:5 vol/vol) overnight at 4°C. Sections were stained with either alcian blue and saffranin-O or H&E for determination of mast-cell and megakaryocyte numbers and histopathologic changes.

* \( P < .05 \) values are considered to differ significantly from other values.
augment the molecular weight of IL-3 as demonstrated in gel-elution experiments. In contrast, the antibody used did not enhance the response to IL-3 in vitro; rather, weak neutralizing properties were observed. The enhancing effect observed in vivo is therefore not mediated via an antibody-induced conformational change, resulting in an increase in the receptor affinity of the target molecule, as has been suggested for enhancing antibodies to growth hormone,27 or via cross-linking of receptors, as has been demonstrated using antibodies to epidermal growth factor and insulin,28,29 as both these mechanisms would result in enhanced activity in vitro. Rather, we present direct evidence that the enhancement of biologic activity observed in vivo reflects prolongation of the in vivo half-life of the cytokine by combination of the cytokine with antibody. The total-body clearance of the IL-3 bioactivity is reproducibly reduced approximately sevenfold to 10-fold in independent experiments.

The biologic half-life of 10 to 15 minutes for IL-3 alone, reported here, is in keeping with some,13-16,30 but not all studies of IL-3 half-life, and values of 30 to 60 minutes have been reported.14,30 The short half-life we observed appears to be related to the small amount of IL-3 injected (<5 to 10 ng). However, in combination with antibody, this amount of IL-3 is still able to exert marked biologic effects. Higher concentrations of IL-3 exhibit more prolonged half-lives and in our studies the β half-life of 10,000 U IL-3 was 30.8 minutes. This value is consistent with the studies of Metcalf et al14 and of Crapper et al30 in which longer half-lives were reported following injection of 100-fold to 1,000-fold greater quantities of IL-3 than we used in these low-dose clearances. In the experiments of Crapper et al30 and Garland et al,13 injection of low-dose IL-3 produced similar clearance profiles as observed in our experiments, with no IL-3 bioactivity detectable after 1 hour. A possible explanation for this discrepancy in the half-lives of IL-3 at different doses is that higher serum concentrations of IL-3 may saturate normal clearance mechanisms, resulting in a prolongation of the elimination half-life. Alternatively, continuing distribution of IL-3 to tissue during the β-phase may have resulted in a shorter half-life for low-dose IL-3 than can be accounted for by elimination alone. Thus, the enhancing effects of the antibody might be attributable to effects on both distribution and elimination. This is suggested by the slightly greater volume of distribution observed for high-dose IL-3. The half-life of radioisotopically labeled IL-3 is reported to be 40 to 50 minutes15; however, this may not represent biologically active protein.

We speculate that the mechanism of the enhancement observed in our experiments is a result of the reduction in renal clearance of IL-3. The antibody used is able to augment the molecular weight of the biologically active cytokine, as demonstrated by elution from gel-filtration columns. The size of the eluted complex, as indicated by the elution profile of IL-3 bioactivity, is on the order of 200 to 250 Kd. This augmented molecular weight would result in diminished glomerular filtration and hence reduced renal clearance of IL-3. Both α and β half-lives are extended and the volume of distribution of the antibody-IL-3 complex is slightly smaller than that observed for IL-3 alone. The data suggest that the complex is not confined to the intravascular space and may be able to penetrate tissue, possibly following disassociation of the complex, or may be preferentially trapped within tissue due to Fc-receptor binding.

The increase in mast-cell precursors in the spleen observed is of a similar magnitude to that observed in mice bearing the IL-3-producing tumour WEHI-3B (fivefold)26-31 or following T-lymphocyte activation in graft-versus-host reactions (fourfold to fivefold). Observations in these pathologic conditions associated with chronic low levels of IL-3 in the serum indicate that increases in the frequency of mast-cell and hematopoietic precursor levels of this magnitude are associated with dramatic increases in hematopoietic tissue, eg, red pulp in spleen.4

The marked increase in primitive IL-3-responsive precursor cells in the spleen is associated with a similar increase in the numbers of mature mast cells in the spleen. It is unlikely that this results from stimulation of early progenitors by IL-3 as this would be followed by a delayed increase in mature cells, which would not become apparent during the time frame of these experiments. IL-3 will stimulate proliferation of mature mast cells and of immature megakaryocytes and the increases in the numbers of mast cells observed in the spleen may reflect this effect. No increases in mature mast cells were observed in lung or jejunum, and higher concentrations of IL-3 or longer time courses may be required to produce effects in these tissues. Alternatively, IL-3 may not be distributed as effectively to these tissues due to a degree of trapping within the vasculature. The failure to observe biologic effects in mice treated with IL-3 alone probably reflects the rapid renal elimination of this small dose of IL-3 when administered as a single daily bolus injection.

The frequency of mast-cell precursors was markedly increased in spleen following treatment with the antibody-cytokine complex; however, the frequency of mast-cell precursors in the bone marrow was consistently diminished. This observation is in agreement with the findings of Moore,12 but in contrast to those of Metcalf.22 It appears from our observations that low-dose IL-3 treatment causes a release of mast-cell precursors from the bone marrow, which redistribute to the spleen. A possible mechanism for this effect is via downregulation of the c-kit receptor.33

The numbers of IL-3-responsive colonies formed in soft agar by spleen cells derived from mice treated with the rabbit anti-1-29 (IL-3) antibody/IL-3 complex were significantly higher than in controls and significantly higher than in mice treated with IL-3 alone or IL-3 and control IgG. Colonies contained both neutrophils and macrophages by morphology, consistent with the known biologic effects of IL-3.1 CFUs in the bone marrow were not increased by any of the treatments used (data not shown), and this may require higher concentrations of IL-3 (John Schrader, personal communication, May 1991).

Binding of the Fc portion of the antibody/cytokine complex to Fc receptors might be expected to reduce the biologic half-life of the complex by initiating clearance by the reticuloendothelial system; nevertheless, in these experiments, the biologic half-life of the complex was increased. The efficiency of Fc receptor binding is determined by antibody
class and by the size of the immune complexes formed. The binding efficiency of this antibody/cytokine complex is likely to be low, as sizing experiments using a Superose 6 column suggest that the majority of the complex comprises one IL-3 molecule bound to one or less likely, two antibody molecules. This type of complex would activate complement with low efficiency and bind only to high-affinity Fc receptors. Once bound to receptors, it would be slowly internalized. The use of antipeptide antibodies therefore allows for the formation of small immune complexes, which are more effective as carriers for their target cytokine. Some binding of the complex to Fc receptors in hematopoietic mast cells in response to the large apparent volume of distribution observed for the complex. Binding of IL-3 within tissue would reduce the concentration of IL-3 within the vasculature and would therefore increase the apparent volume of distribution.

Autoantibodies to interleukin-1α, interleukin-6, interferons, and tumor necrosis factor-α have been reported as arising spontaneously in normal individuals. These antibodies have traditionally been thought to inhibit the effects of their target; however, it has also been speculated that these antibodies may function as carrier molecules in the circulation, much as described above, and may actually target their cytokine to high-affinity receptors. The production of these antibodies is augmented during chronic infections and may function physiologically during these periods to augment the effects of the target cytokine. We have demonstrated how an antibody/cytokine complex may serve to enhance the biologic effect of its target cytokine and may lead to sustained delivery of the cytokine to the sites at which it is biologically active. These findings have implications for both the physiologic and pathologic functions of autoantibodies.

The use of antipeptide antibodies in these experiments suggests that the therapeutic effects of autoantibodies may be exploited by generating antibodies that are nonneutralizing, bind at a single exclusive site and form complexes that retain biologic activity, have reduced elimination, and produce little or no activation of the immune system. The specificity of these antibodies might be further enhanced by producing chimeric antibodies that are able to target their cytokine to specific tissues.

ACKNOWLEDGMENT

We thank H. Merkens and M. Rebettay for excellent technical assistance, M. Iagallo for histochemistry, and M. Welham and J. Schradler for critical reading of the manuscript.

REFERENCES

11. Chan WL, Ziltener HJ, Liew FY: Interleukin 3 protects mice from acute herpes simplex virus infection. Immunology 71:358, 1990


Enhancement of the biologic effects of interleukin-3 in vivo by anti-interleukin-3 antibodies

AT Jones and HJ Ziltener