Species Specificity of Human Interleukin-3 Demonstrated by Cloning and Expression of the Homologous Rhesus Monkey (Macaca mulatta) Gene

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To enable preclinical studies on homologous interleukin-3 (IL-3) in primate species, we isolated the gene encoding Rhesus monkey IL-3 (RhIL-3). The nucleotide sequence of the RhIL-3 gene displayed 92.9% homology with that of the human IL-3 (hIL-3) gene. The isolated RhIL-3 gene encodes a 143-amino acid (aa) precursor polypeptide, nine C-terminal residues shorter than the human protein. Protein homology was found to be 89.5% for the signal peptide (19 aa) and 80.5% for the mature protein (124 aa). Comparison of the human and RhIL-3 coding sequences showed that the majority of substitutions had occurred at amino acid replacement sites indicating a rapid evolution of the IL-3 protein. After expression of a genomic fragment in COS cells, RhIL-3 cDNA was constructed, which enabled large-scale production of the RhIL-3 polypeptide. RhIL-3 produced by Bacillus licheniformis and purified to homogeneity appeared to be approximately 100-fold more effective in stimulating Rhesus monkey hematopoietic progenitors than hIL-3, whereas RhIL-3 and hIL-3 showed comparable stimulatory activity on normal as well as malignant human hematopoietic cells. Thus, the rapid evolution of hIL-3 has resulted in a unidirectional species specificity, which most likely restricts the in vivo effects of hIL-3 in Macaca species.

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HEMATOPOIESIS IS regulated by a group of soluble polypeptide mediators termed hematopoietic growth factors (HGFs), which are defined by their ability to support proliferation and differentiation of various blood cell progenitors.1,2 Interleukin-3 (IL-3), also referred to as multilineage colony-stimulating factor (multi-CSF), is a HGF with a broad spectrum of biologic activities.3 The reported biologic activities of IL-3 include stimulation of the in vitro proliferation and differentiation of multipotential hematopoietic cells, leading to the production of most of the major blood cell types. Administration of recombinant murine IL-3 to mice showed a dramatic enhancement of hematopoietic activity in these animals.4,5 Analogous or even more profound results were obtained in mice transplanted with retrovirus-infected hematopoietic cells, which supported hematopoiesis as a consequence of endogenous IL-3 synthesis.6 It was shown that human IL-3 (hIL-3) stimulates the in vivo proliferation of normal as well as malignant hematopoietic clonogenic cells.7,8 In addition, the presence of other HGFs or accessory cells are required for terminal differentiation of IL-3-stimulated immature hematopoietic cells.8 In agreement with the limited sequence homology at both DNA and protein level, hIL-3 failed to stimulate proliferation of murine hematopoietic cells.1

The molecular cloning and expression of the gene encoding hIL-37,11 and its subsequent large-scale production has prompted preclinical in vivo studies in monkey species to identify its therapeutic window and side effects.12,13 Contrasting its broad range of action in vitro, recombinant hIL-3 administered to Rhesus monkeys (Macaca mulatta) and cynomolgus monkeys (Macaca fascicularis) exerted only limited, and in part inconsistent, effects on the production of peripheral blood cells. Somewhat larger effects of hIL-3 on peripheral blood cell numbers in these species were noted by sequential administration of other, more pathway-restricted HGFs, such as granulocyte-macrophage CSF (GM-CSF) or erythropoietin.12,13 Based on such evidence it is generally held that IL-3 expands an early cell population that subsequently requires the action of later acting factors such as GM-CSF to complete its development.12 Alternatively, we proposed the hypothesis that the limited effects of human IL-3 in Macaca species should be attributed to its species specificity.

To test this hypothesis we cloned and expressed the gene encoding Rhesus monkey IL-3 (RhIL-3) and studied the stimulatory activity of the expressed IL-3 molecules on human and Rhesus monkey cells.

MATERIALS AND METHODS

Isolation, characterization, and expression of genomic RhIL-3. Lambda Charon 40 phages containing Rhesus monkey genomic DNA partially digested with EcoRI, kindly provided by Dr Jerry L. Slightom (The Upjohn Company, Kalamazoo, MI), were used to infect the recA- Escherichia coli host ED8767. This library (106 plaques) was hybridized with an hIL-3 cDNA probe devoid of the 3'-noncoding region (nucleotides [nt] 541 through 856) containing repetitive sequences. Hybridization with this probe, isolated from plasmid pLB42 and radiolabeled according to the random primers procedure16 with [α-32P]dCTP, was performed at 65°C in 50 mmol/L Tris, 1 mol/L NaCl, 10X Denhardt’s, 0.5% sodium dodecyl sulfate (SDS), 0.1% PP, in the presence of 100 μg/mL of herring sperm DNA for 16 to 20 hours. Filters were washed at 65°C in 2X SSC, 0.1% SDS, and 0.1% PP, and autoradiographed with Kodak XAR films (Rochester, NY). One positive clone was screened and plaque purified and DNA was isolated as described previously.1 Radiolabeled DNA was digested with various restriction enzymes, separated by agarose gel electrophoresis, blotted onto Gene Screen Plus hybridization transfer membranes (New England Nuclear Research Products, Boston, MA), followed by hybridization with the complete hIL-3 cDNA, the 5' fragment (nt 1 through 253), and the 3' fragment (nt 411 through 910). All fragments were prepared from the pLB4 plasmid1 and radiolabeled16 with [α-32P]dCTP (Amerham, Buckinghamshire, UK).

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A 4.1-kb EcoRI fragment, containing the complete RhIL-3 gene, was digested with AvaI and BamHI, and the resulting fragments were subcloned in pTZ18R (Pharmacia, Uppsala, Sweden) and sequenced in both directions using the KiloBase Sequencing System (Bethesda Research Laboratories, Paisley, Scotland). Oligonucleotides complementary to the established sequences were synthesized and used for subcloning. The sequence data were analyzed using the Microgenie computer program. The complete nt sequence of the 4.1-kb EcoRI fragment has been deposited at the EMBL Data Library and assigned the following accession number: X51890.

The 2.2-kb SmaI-XhoI fragment, comprising all coding sequences, was inserted in the SmaI site of the polylinker downstream of the heterologous SV40 late promoter in the pSVL (Pharmacia) eukaryotic expression vector, resulting in the pSVL/RhIL-3 plasmid. Plasmid DNA was purified and transfected to COS cells using the DEAE-dextran/chloroquine method.

Construction and expression of an RhIL-3 cDNA. To enable expression of RhIL-3 in prokaryotes, a cDNA was constructed from messenger RNA (mRNA) isolated from COS cells transfected with pSVL/RhIL-3. Isolation of cytoplasmic RNA was performed 48 hours after transfection followed by isolation of poly A+ mRNA, using an oligo-dT cellulose column. The first strand reaction of cDNA synthesis was performed on 2 ng of poly A+ mRNA using oligo-dT as primer. The resulting single-stranded cDNA was amplified with the polymerase chain reaction (PCR) technique using oligonucleotides as primers that were designed for RhIL-3 cDNA cloning into a Bacillus expression vector. The first oligonucleotide has the sequence 5'-GGA TCC GAG CTC CCC-3' and is complementary to the 3' end of mRNA synthesized from pSVL constructs. After PCR (25 cycles: 2 minutes 94°C, 2 minutes 55°C, and 3 minutes 72°C) according to the recommendations of the supplier of Taq-polymerase (Cetus, Emeryville, CA), the reaction products were digested with BamHI and XhoI and the resulting fragments were inserted into pGEM-7Zf(-) (Promega, Madison, WI). From several individual transformants the sequence of the cDNA insert was established. The PstI-XhoI restriction sites and the information for the 3' part of the B licheniformis a-amylase signal peptide precisely fused to the mature RhIL-3 sequence. The second oligonucleotide has the sequence 5'-GGA TCC GAG CTC CCC-3' and is complementary to the NdeI-HindIII fragment of pGB/IL-326 (N.L.M. Persoon and R.W. van Leen, 1989, WO 88/04691) directed against the hIL-3 polypeptide. Endotoxin concentrations were below the detection level of the assay (Lumilus Lyse assay) for all hIL-3 and RhIL-3 preparations used.

AML-193 assay and in vitro cultures of bone marrow cells. Recently an IL-3-dependent human AML cell line (AML-193) was established. AML-193 cells, grown in the presence of recombinant hIL-3 (10 µg/L), were collected, washed, and resuspended in modified Dulbecco’s medium supplemented with 4.8 mg/L human transferrin (Boehringer, Marburg, BRD) and 5 mg/L insulin (GIBCO, Middlesex, England). After culturing 2 x 10^6 cells/200 µL/well for 4 days in microtiter plates, cells were radiolabeled with [2-3H]thymidine (0.08 µCi/well) for 1 day, and incorporation was measured and expressed as counts per minute (cpm). The amount of IL-3 per milliliter required to give half-maximal response is designated as 1 unit. Similar unit calculations were performed on human as well as Rhesus monkey bone marrow, if appropriate.

Rhesus monkey bone marrow cells were obtained and subjected to discontinuous density gradient centrifugation, E-rosette sedimentation, and cryopreservation until further use as previously described. After thawing and removal of dimethyl sulfoxide by step-wise dilution, T-lymphocyte-depleted bone marrow progenitor cell concentrates were labeled with the MoAb MO1 (Coulter Immunology, Hialeah, FL), and the MO1 positive cells were removed by immunomagnetic bead separation to deplete the suspension of accessory cells influencing colony growth. The resulting MO1 depleted suspension was further labeled with anti–HLA-DR, an antigen known to be densely present on stem cells and progenitor cells. HLA-DR positive cells were conjugated to protein-A, which was covalently bound to immunomagnetic beads and then removed from the suspension by a magnet. The cells were eluted from the beads by excess immunoglobulin in normal bovine serum. The resulting T-lymphocyte-depleted, MO1/DR- stem cell concentrates were used for IL-3–stimulated cultures at a concentration of 10^4 cells/mL. Because the IL-3 response is known to be impaired in the absence of accessory cells, 10^4 MO1 positive cells, prepared by the same immunomagnetic bead separation method, were added to the cultures. The methylcellulose cultures and scoring of hematopoietic colonies (day 14) were performed according to methods previously described. Although multiple types of colonies emerged, typing of colony morphology was considered to be irrelevant for the purpose of this report. Human marrow was obtained as described, cryopreserved and depleted of T lymphocytes and accessory cells by complement-mediated lysis using MoAbs OKT-3 and Vim2 and selection on the basis of CD34 expression according to established procedures. The cultures were supplemented with recombinant human erythropoietin as described and scored according to established criteria for erythroid, granulocytic, and mixed colonies. Standard deviations were calculated on the assumption that crude colony counts are Poisson distributed.

RESULTS

Characterization of the RhIL-3 gene and comparison with the human homologous gene. The gene encoding RhIL-3 was isolated by screening a Lambda Charon 40 library containing Rhesus monkey genomic DNA with hIL-3 cDNA. Characterization of a 4.1-kb EcoRI fragment containing the hIL-3 homologous sequences by various molecular biologic methods, including restriction enzyme analysis, Southern blot analysis, and nucleotide sequence analysis, showed that the overall gene structures of RhIL-3 and hIL-3 were virtually identical (Fig 1). RhIL-3 shares 92.9% nucleotide sequence homology with the complete (3,600 bp) hIL-3 sequence, and an identical percentage was found for the
coding regions with the intron/exon boundaries specified by a single consensus splice donor and acceptor sequence fully conserved. The 5' flanking region upstream of the transcription initiation site, which spans the putative promoter, contains structural elements common to many eukaryotic promoters including a "TATAA"-related sequence, a "CAT"-related sequence, a G + C rich sequence, and a well-conserved "decanucleotide" that is present in the 5' untranslated region of many HGFs.27 The 3' noncoding region upstream of the polyadenylation site contained 8 "ATTAA" repeat units from which 5 were conserved in the hIL-3 gene.2 Such repeat units were shown to mediate selective mRNA degradation of lymphokines28 or to preclude mRNA translation.29

The intron/exon structure of the RhIL-3 gene, deduced from the nucleotide sequence of the hIL-3 cDNA and confirmed by sequence analysis of the isolated RhIL-3 cDNA construct, shows that the RhIL-3 gene, like the murine, rat, and human counterparts,26,30,31 is divided into five small exons, encoding in this case a 143-amino acid (aa) polypeptide. This RhIL-3 polypeptide is most likely processed at the amino terminus by the removal of 19 residues to yield a mature protein comprising 124 aa (molecular weight [mol wt] 13,974), which is nine residues shorter than the human protein (mol wt 15,074). The hIL-3 and RhIL-3 protein homology was found to be 89.5% for the signal peptide and 80.5% for the mature protein, as shown in Fig 2. The two cysteine residues at positions 16 and 84 present in the human and RhIL-3 are conserved in the Rhesus monkey (Fig 2) and may play an essential role in protein folding. The potential N-linked glycosylation site present at position 70 of hIL-3 is conserved in RhIL-3, but that at position 15 is deleted due to substitution of the asparagine residue in hIL-3 sequences between humans and Rhesus monkeys showed resulting in a change of the aa sequence.

Expression of the RhIL-3 coding sequences and large-scale production of the encoded RhIL-3 polypeptide. COS cells were transfected with plasmid pSVL/RhIL-3 and the presence of functional RhIL-3 in the COS supernatant was demonstrated by Western blot analysis using polyclonal as well as MoAbs directed against the hIL-3 polypeptide, and by the capacity of the supernatant to stimulate hematopoietic cells. The number of IL-3 units present in the supernatant was assessed on human AML-193 cells as well as on Rhesus monkey bone marrow progenitor cells and appeared to be approximately 10^2 U/mL in both assays. Purification and assessment of the specific activity of RhIL-3 expressed in COS cells were not done because of the low expression level. (The reciprocal experiment, the capacity of hIL-3 expressed in COS cells to stimulate Rhesus monkey bone marrow cells, yielded no significant response, which actually was the basis for the study presented here. Due to the low expression level in COS cells, ie, <10^2 U/mL, a detailed analysis of the difference in specific activity could not be pursued.) To enable expression of RhIL-3 in prokaryotes, a cDNA was constructed from transfected COS cell mRNA followed by cloning the cDNA into an appropriate expression vector. The resulting vector pGB/RhIL-326 was used for expression of RhIL-3 in B licheniformis. Transformants of B licheniformis strain T9 produced high amounts of RhIL-3 (>5 mg/L), of which more than 95% was secreted into the culture medium. The secreted RhIL-3 was purified to homogeneity by a series of chromatographic steps. SDS-PAGE demonstrated the presence of a single Coomassie brilliant blue-stained band with a purity of >99%.

Comparative studies between human and RhIL-3. The human and RhIL-3 polypeptides used for the comparative studies were produced by B licheniformis and subsequently purified to homogeneity. From the effect of human and RhIL-3 on the AML-193 cells, depicted in Fig 3A, it is apparent that RhIL-3 is capable of stimulating these human cells, although it is a consistent finding that the specific activity of RhIL-3 is threefold less than the specific activity of hIL-3. Along with the stimulation of human malignant hematopoietic cells, RhIL-3 also promotes colony formation of purified normal human bone marrow cells (Table 1). Furthermore, similar dose-effect relationships were observed using either hIL-3 or RhIL-3, and differences in colony morphology and/or differentiation lineages were not found.
absence of erythropoietin) appeared to be at least 100-fold more effective than by its human homologue (Fig 3B). Except for the erythroid lineage in the presence of erythropoietin, there was no spontaneous proliferation of progenitors observed without IL-3 addition in the human as well as the Rhesus monkey bone marrow cultures, indicating that accessory cells capable of producing endogenous HGFs did not contribute to the observed difference. Thus, the results depicted in Fig 3 and Table 1 demonstrate a unidirectional species specificity.

**DISCUSSION**

In the present study we report the isolation, identification, and expression of the RhIL-3 gene. The encoded RhIL-3 protein, which appeared to be nine residues shorter than the human protein, displayed a protein homology of 80.5% for the mature protein. The two cysteine residues are conserved, whereas only one potential N-linked glycosylation site is conserved. Proposed regulatory elements were similar to those identified in IL-3 genes of other species, indicating a common regulating mechanism of IL-3 expression with an essential role for the structural elements.

Despite approximately 80% homology of the aa sequences of hIL-3 and RhIL-3, the specific activity of RhIL-3 appeared to be about 100-fold higher than hIL-3 as tested on highly purified Rhesus monkey bone marrow cells. This contrasted the biologic activity of RhIL-3 on human cells, which was comparable with hIL-3. Experiments in which we compared biologic activities, as tested on human bone marrow as well as AML-193 cells, different preparations of recombinant hIL-3 expressed in COS cells, in a mouse cell line, in the yeast Kluyveromyces lactis, and in E coli (L.C.J. Dorssers, G. Wagemaker, Y.J. Vos, R.W. van Leen, 1987, WO 88/04691) all yielded dose-effect relationships similar to those shown for hIL-3 produced by B licheniformis. Purified glycosylated K lactis-derived hIL-3 (G. Wagemaker et al, manuscript in preparation) and homogenous nonglycosylated hIL-3 expressed either by E coli (L.C.J. Dorssers et al, manuscript in preparation) or B licheniformis yielded identical specific activities as assessed on AML-193 cells or on human bone marrow. Apparently, the type and extent of glycosylation does not alter the specific activity of hIL-3, which is in agreement with reports on mouse IL-3. In addition, we demonstrate here that glycosylated COS cell-derived RhIL-3 and nonglycosylated RhIL-3 produced by B licheniformis were equally effective on human AML-193 cells and Rhesus monkey bone marrow cells. Furthermore, we stress that the specific activity of B licheniformis-derived RhIL-3 assessed on Rhesus monkey bone marrow was identical to that found for B licheniformis-derived hIL-3 on human bone marrow. Based on these data, we have no reason to assume that the diminished capacity of hIL-3 to support colony formation of Rhesus monkey bone marrow progenitor cells may be attributed to differential posttranslational...
processing, because there is no evidence that differential processing influences the biologic activity of mouse, Rhesus monkey, and hIL-3. Because endotoxin was not detected in the *B. licheniformis*-produced homogeneous IL-3 preparations, the observed species specificity could not be the result of indirect IL-3 effects such as endotoxin-mediated growth factor production by bone marrow cells.

The nature of the species specificity of hIL-3 is as yet a matter of speculation. However, the unusual high number of substitutions at aa replacement sites indicates that IL-3 is subject to a high evolution rate. Indeed, we will shortly publish results obtained from a pairwise comparison of IL-3 coding sequences of various primate and rodent species demonstrating that, in general, IL-3 evolves at a rate that approaches that of substitutions in pseudogenes that are generally considered to accumulate substitutions at the neutral mutation rate, because these genes are not believed to be subject to functional constraint. Thus, the species specificity of hIL-3 as encountered for Rhesus monkey cells can be explained from its rapid evolution. Furthermore, the differential stimulation of Rhesus monkey bone marrow progenitors by human and RhIL-3 provides an interesting model to elucidate the functional domains by subjecting hIL-3/ RhIL-3 polypeptide hybrids, constructed by in vitro mutagenesis, to structure-function relationship analysis.

The species specificity of hIL-3 also implies that preclinical in vivo studies in Cynomolgus and Rhesus monkeys might very well be insufficiently representative for the effects of hIL-3 in human patients. In fact, the effects of hIL-3 in these *Macaca* species were restricted to a small increase in white blood cells, predominantly caused by basophilia, whereas the first clinical trial in human patients showed rises in neutrophilic and eosinophilic granulocytes as well as in thrombocytes. We will shortly publish results which demonstrate that in vivo administration of homologous IL-3 to Rhesus monkeys results in a strong dose-dependent stimulation of the production of all bone marrow-derived blood cells, in line with the stimulation of multipotent bone marrow cells observed in vitro.

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