



**Isolation and Expression of Human Cytokine Synthesis Inhibitory Factor  
cDNA Clones: Homology to Epstein-Barr Virus Open Reading Frame  
BCRF1**

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## Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: Homology to Epstein–Barr virus open reading frame BCRFI

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**ABSTRACT** We have demonstrated the existence of human cytokine synthesis inhibitory factor (CSIF) [interleukin 10 (IL-10)]. cDNA clones encoding human IL-10 (hIL-10) were isolated from a tetanus toxin-specific human T-cell clone. Like mouse IL-10, hIL-10 exhibits strong DNA and amino acid sequence homology to an open reading frame in the Epstein–Barr virus, BCRFI. hIL-10 and the BCRFI product inhibit cytokine synthesis by activated human peripheral blood mononuclear cells and by a mouse Th1 clone. Both hIL-10 and mouse IL-10 sustain the viability of a mouse mast cell line in culture, but BCRFI lacks comparable activity in this assay, suggesting that BCRFI may have conserved only a subset of hIL-10 activities.

Immune responses are specific both for the antigen against which they are mounted and for the class of response that is induced. Humoral (antibody mediated) and delayed-type hypersensitivity (DTH) responses can be mutually exclusive (1, 2). An organism's ability to deal effectively with the antigen depends on which class of immune response is generated. For example, the strong DTH response elicited in C57BL/6 mice by the parasite *Leishmania major* results in cure of the infection, while BALB/c mice mount a mostly humoral immune response and ultimately die (3, 4). These two types of response to *Leishmania* are also seen in the human disease (5–7). Other human diseases can show similar characteristics: for example, tuberculoid leprosy is accompanied by a strong DTH response that ultimately kills and clears the bacilli, while in lepromatous leprosy, with weak cell-mediated immunity, the organisms multiply and the disease persists (8, 9).

A possible explanation for the two types of immune response is suggested by studies of mouse helper T-cell clones that differ in their effector functions and cytokine secretion patterns. Th1 helper T-cell clones secrete interleukin 2 (IL-2) and interferon  $\gamma$  (IFN- $\gamma$ ) and preferentially induce macrophage activation and DTH (10, 11), while Th2 clones produce IL-4 and IL-5 (12, 13) and provide superior help for humoral responses (14–17). If the functions of Th1 and Th2 clones reflect the different classes of immune response, then these cells may be mutually inhibitory (18–20). Cytokine synthesis inhibitory factor (CSIF; IL-10), a product of murine Th2 clones, inhibits cytokine secretion by Th1 cells (21). cDNA clones encoding murine IL-10 (mIL-10) (22) have been reported.

Although many human T-cell clones do not display the strict Th1/Th2 patterns observed for murine clones, human diseases do exhibit the corresponding differences in immune response class. So far, no human counterpart of IL-10 has

been described. In this report, we describe cDNA clones encoding human IL-10 (hIL-10) and show that the recombinant cytokine has CSIF activity on human and mouse cells.¶ Like mIL-10 (22), hIL-10 shows extensive homology to BCRFI, an open reading frame in the Epstein–Barr virus (EBV) genome (23). While both hIL-10 and the product of BCRFI (24) exhibit CSIF activity on mouse and human cells, the viral cytokine has little of the mast-cell stimulatory activity of IL-10 (41). These results suggest that BCRFI may have conserved only a subset of IL-10 activities.

### MATERIALS AND METHODS

**Cells and Cell Lines.** Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy donors by centrifugation on a density gradient of Ficoll/Hypaque (Sigma). BL-2 and BL-30 are EBV<sup>-</sup> B-cell lymphoma lines (25). BL-2/B95 and BL-30/B95 are EBV-transformed B-cell lines obtained by *in vitro* infection of BL-2 and BL-30, respectively, with the EBV strain B95-8. BL-30/P3 is a subline of BL-30 infected with the nonimmortalizing EBV strain p3HR-1, which lacks the EBV nuclear antigen 2 (EBNA-2) coding sequence (25). These cell lines were a kind gift of G. Lenoir (International Agency for Research on Cancer, Lyon, France). The EBV-transformed lymphoblastoid cell line RPMI8866 was provided by J. Banchereau (Schering-Plough).

NP44 and PD67 are DerP1-specific CD4<sup>+</sup> human T-cell clones established from PBMCs of allergic donors (H. Yssel and J.E.deV., unpublished data). GN12 is a *Borrelia burgdorferi*-specific T-cell clone isolated from PBMCs of a patient suffering from Lyme disease (H. Yssel, G. Peltz, and J.E.deV., unpublished data). B21 (M.-G.R. and H. Spits, unpublished data) is a tetanus toxin-specific CD4<sup>+</sup> human T-cell clone obtained from PBMCs of a patient with severe combined immunodeficiency that had been successfully reconstituted by transplantation of fetal liver and thymus (26). This T-cell clone secretes IL-2, IL-4, IL-5, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IFN- $\gamma$  (R.deW.-M., unpublished data). A cDNA library was pre-

Abbreviations: DTH, delayed-type hypersensitivity; IL, interleukin; mIL-10, murine IL-10; hIL-10, human IL-10; EBV, Epstein–Barr virus; PBMC, peripheral blood mononuclear cell; CSIF, cytokine synthesis inhibitory factor; BCRFI, EBV BamHI C fragment, rightward reading frame I; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; IFN- $\gamma$ , interferon  $\gamma$ ; GM-CSF, granulocyte-macrophage colony-stimulating factor; ORF, open reading frame; PCR, polymerase chain reaction; MHC, major histocompatibility complex.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M57627).

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pared (27) in the pcDSR $\alpha$  vector (28) from poly(A)<sup>+</sup> RNA derived from B21.

**Isolation of hIL-10 cDNA Clones.** hIL-10 cDNA clones were isolated by colony hybridization with a mixture of oligonucleotides encoding the entire mIL-10 open reading frame (ORF) (22) as a probe. Hybridization was at 55°C in 5 $\times$  SET (1 $\times$  SET = 0.15 M NaCl/30 mM Tris-HCl/1mM EDTA, pH 7.5)/1% SDS/10 $\times$  Denhardt's solution (1 $\times$  Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/denatured salmon sperm DNA (0.1 mg/ml) (overnight). The filters were then washed three times at room temperature with 2-0.1 $\times$  SET/0.5% SDS, and at 50°C in 2 $\times$  SET/0.5% SDS. DNA sequence analysis utilized M13mp18/-19 or Bluescript KS+ (Stratagene) vectors and the Sequenase 2.0 kit (United States Biochemical). Transfection of COS-7 cells and *in vitro* labeling with [<sup>35</sup>S]methionine were described (22).

**Assays for hIL-10.** The human CSIF assay and RNA blot analysis for IFN- $\gamma$  were performed as described (24). The ability of hIL-10 to inhibit cytokine synthesis by a murine Th1 clone was determined in a mouse CSIF assay (21). The assay for mast cell-stimulating activity was performed as described (29).

**DNA and RNA Blot Analyses.** Human genomic DNA (40  $\mu$ g) from PBMCs of healthy donors (30) was digested with different restriction enzymes, fractionated by gel electrophoresis, blotted, and probed with the 760-base-pair (bp) *Bgl* II (position 159)/*Hind*III (position 919) fragment of the hIL-10 cDNA clone as described (22). RNA blot analysis (22) utilized the same hIL-10 probe.

**Polymerase Chain Reaction (PCR).** PCR analysis of reverse-transcribed RNA was carried out (22) with two hIL-10 primers: primer 1, nucleotides 323-349 (sense strand); primer 2, nucleotides 648-674 (antisense strand). Primer 2 hybridizes to a region with no homology to BCRFI; these PCR primers do not generate an amplified fragment from plasmid templates containing the BCRFI gene. Control reactions from which reverse transcriptase was omitted were done for each RNA sample. Amplified fragments were detected by ethidium bromide staining in 1.5% agarose gels and then by hybridization to an oligonucleotide probe (nucleotides 429-489).

## RESULTS

**Isolation of cDNA Clones Encoding hIL-10.** We screened a number of human T-cell clones for expression of IL-10 mRNA by RNA blot analysis, using a mIL-10 cDNA probe. One human T-cell clone, B21, expressed an  $\approx$ 1.8-kilobase mRNA, which hybridized to mIL-10 (data not shown). Using a set of eight overlapping oligonucleotides spanning the entire coding region of mIL-10 as a probe, we screened a cDNA library of activated B21 mRNA and isolated two human IL-10 cDNA clones: H5C and H15C. The DNA sequence of H15C contains an ORF encoding a polypeptide of 178 amino acids, with an N-terminal hydrophobic leader sequence 18 amino acids long (Fig. 1; the nucleotide and predicted amino acid sequences of hIL-10 are available from the authors upon request). The protein contains one potential N-linked glycosylation site, four cysteine residues, and seven methionines.

Overall DNA sequence homology between hIL-10 and mIL-10 of >80% is observed throughout the entire sequence, except for a region between nucleotide positions 1162 and 1477 where a repetitive element of the human *Alu* family (31) is inserted. This element is flanked by a duplicated stretch of 14 nucleotides whose sequence is present in the mouse cDNA and is not part of the repetitive element (data not shown).

The hIL-10 sequence was compared to sequences in available data bases. No significant homology to known cytokines, other than mIL-10, was detected. However, as ob-

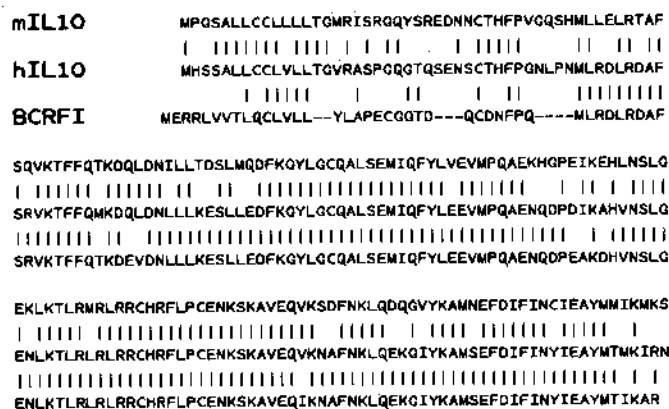


Fig. 1. Comparison of the predicted amino acid sequences of mIL-10, hIL-10, and BCRFI. Amino acid sequence identities are indicated by vertical lines.

served for mIL-10 (22), substantial homology was found between hIL-10 and the EBV ORF BCRFI (23). Fig. 1 compares the predicted amino acid sequences of hIL-10, mIL-10, and BCRFI. The amino acid sequence of hIL-10 is 73% similar to the sequence of mIL-10. This homology is observed throughout the entire protein, including the leader sequence. The amino acid sequence homology of hIL-10 and BCRFI is 78% for the entire protein-coding region. However, if the leader sequence is excluded from the analysis the homology is 84%, and in the region distal to Met<sup>22</sup> 94% homology is observed, with only 10 amino acid differences distinguishing the two sequences. The amino acid sequence homology between the predicted mature hIL-10 and BCRFI proteins (84%) is greater than their nucleic acid sequence homology (71%). In contrast, the nucleotide sequence homology between mIL-10 and hIL-10 is 81%, but amino acid sequence homology is only 73%.

**Expression and Biological Activity of Recombinant hIL-10.** COS-7 cells were transfected with mIL-10, BCRFI, and hIL-10 cDNA clones and cultured *in vitro* with [<sup>35</sup>S]methionine in the presence or absence of tunicamycin B2 (22). Aliquots of supernatants were subjected to SDS/PAGE under reducing conditions. <sup>35</sup>S-labeled supernatants from mock-transfected cells were used as a negative control. Fig. 2 shows that supernatants from COS-7 cells transfected with the H5C or H15C cDNA clones contain a polypeptide of  $\approx$ 18 kDa, not present in mock-transfection supernatants, whose mobility is unaffected by culture with tunicamycin B2. This suggests that, in contrast to mIL-10 (22), hIL-10 has little or no N-linked carbohydrate, as also found for BCRFI (24). A rat antiserum against BCRFI/hIL-10 (24) immunoprecipitates this single 18-kDa protein from supernatants of COS-7 cells

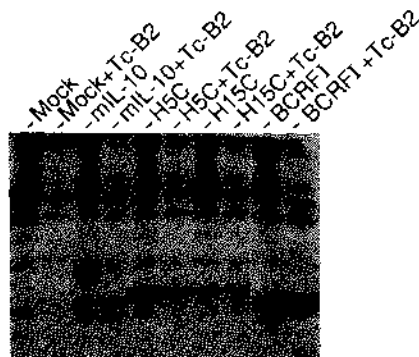


Fig. 2. *In vitro* labeling of IL-10 expressed in COS-7 cells transfected with mIL-10, BCRFI, or the two hIL-10 cDNA clones (H5C and H15C). Labeling with [<sup>35</sup>S]methionine was performed in the absence or presence of Tc-B2 as noted.

transfected with the H5C or H15C cDNA clones (data not shown). Despite extensive sequence similarity, recombinant hIL-10 (18 kDa) can be distinguished from recombinant BCRFI (17 kDa) by the apparent molecular mass in SDS/PAGE (Fig. 2).

The ability of hIL-10 to inhibit cytokine synthesis by human PBMCs was tested in a human CSIF assay (24) with supernatants from transfected COS-7 cells used as a source of recombinant hIL-10. The results (Fig. 3) show that hIL-10 inhibits synthesis of IFN- $\gamma$  and GMCSF by human PBMCs (Fig. 3A). hIL-10 inhibits cytokine secretion by both phytohemagglutinin (PHA) and anti-CD3-stimulated peripheral blood lymphocytes (Fig. 3B). mIL-10 is not active on human cells and BCRFI has similar activity to hIL-10 (24). Similar results were observed at the RNA level (Fig. 3C). hIL-10 reduces the level of IFN- $\gamma$  mRNA that can be detected in stimulated PBMCs. A  $\beta$ -actin probe served as a control for the amount of RNA loaded on the gel (Fig. 3C).

Like mIL-10 (21), recombinant hIL-10 inhibits IFN- $\gamma$  secretion by an activated murine Th1 clone (Fig. 4A). In addition, Thompson-Snipes *et al.* (41) have found that mIL-10 supports the viability of mast cell lines in culture and synergizes with IL-3 and IL-4 in stimulating mast cell proliferation. Fig. 4B shows that hIL-10 also supports the viability in culture of the murine mast cell line MC/9. However, recombinant BCRFI, which inhibits IFN- $\gamma$  synthesis by mouse and human cells (24), exhibits little or no activity on MC/9 cells under the conditions tested. Similarly, BCRFI lacks significant mast cell growth cofactor activity in the presence of IL-4 or IL-3 (Y. Liu and K.W.M., unpublished data).

**Detection of the hIL-10 Gene.** We analyzed restriction enzyme digests of human genomic DNA by hybridization to an hIL-10 cDNA probe. Identical band patterns were observed in *Taq* I and *Bgl* II digests of DNA from four donors tested (data not shown; this result is available upon request).

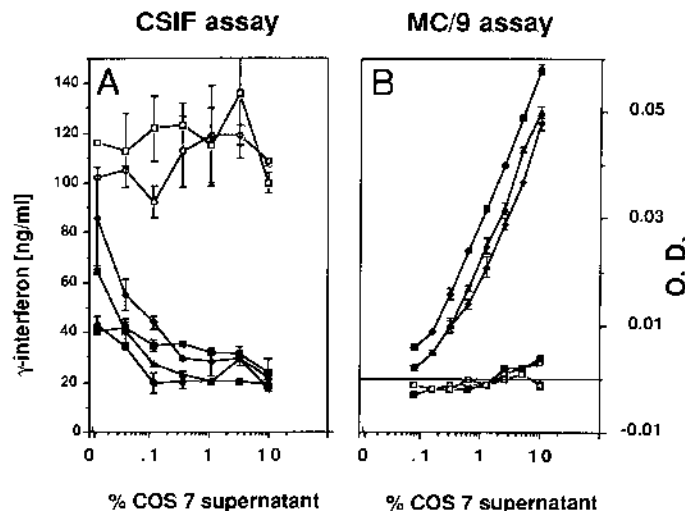


FIG. 4. (A) mIL-10, hIL-10, and BCRFI inhibit IFN- $\gamma$  secretion by the murine Th1 clone HDK1. HDK1 cells ( $5 \times 10^4$  cells per well) were stimulated with TNP-KLH ( $1 \mu\text{g}/\text{ml}$ ) and irradiated spleen cells ( $5 \times 10^5$  cells per well) in the presence of transfection supernatants from COS-7 cells. Mouse IFN- $\gamma$  in cultures was measured by ELISA after 24 hr. The mean values and range of duplicate samples are shown. (B) mIL-10 and hIL-10 sustain the viability of a murine mast cell line. The viability of MC/9 cells was assessed by MTT assay (29). Results are expressed as the mean OD and range of duplicate cultures containing dilutions of mIL-10 (●), BCRFI (◆), H5C (▲), or H15C (◆). Negative controls were supernatants from mock-transfected cells (○) or H17C (□). COS-7 supernatants in A and B were from the same transfection.

**Expression of hIL-10 mRNA.** RNA from different cell lines under varied conditions of activation was tested by RNA blot analysis (Fig. 5). Two T-cell clones, B21 and NP44, express IL-10 mRNA. In NP44, only anti-CD3 plus phorbol 12-myristate 13-acetate (PMA), but not PMA and  $\text{Ca}^{2+}$  iono-

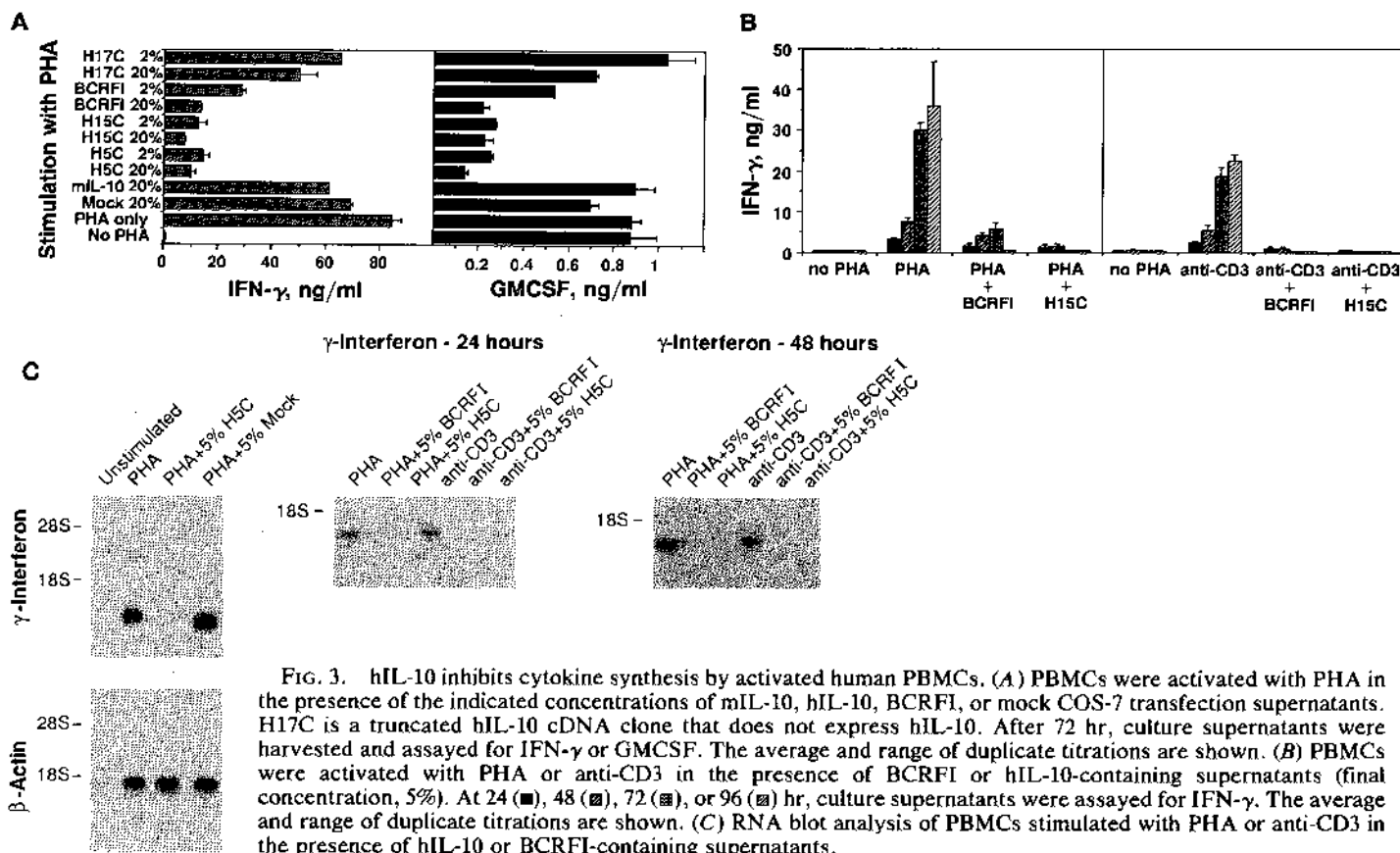


FIG. 3. hIL-10 inhibits cytokine synthesis by activated human PBMCs. (A) PBMCs were activated with PHA in the presence of the indicated concentrations of mIL-10, hIL-10, BCRFI, or mock COS-7 transfection supernatants. H17C is a truncated hIL-10 cDNA clone that does not express hIL-10. After 72 hr, culture supernatants were harvested and assayed for IFN- $\gamma$  or GMCSF. The average and range of duplicate titrations are shown. (B) PBMCs were activated with PHA or anti-CD3 in the presence of BCRFI or hIL-10-containing supernatants (final concentration, 5%). At 24 (■), 48 (▣), 72 (▤), or 96 (▥) hr, culture supernatants were assayed for IFN- $\gamma$ . The average and range of duplicate titrations are shown. (C) RNA blot analysis of PBMCs stimulated with PHA or anti-CD3 in the presence of hIL-10 or BCRFI-containing supernatants.

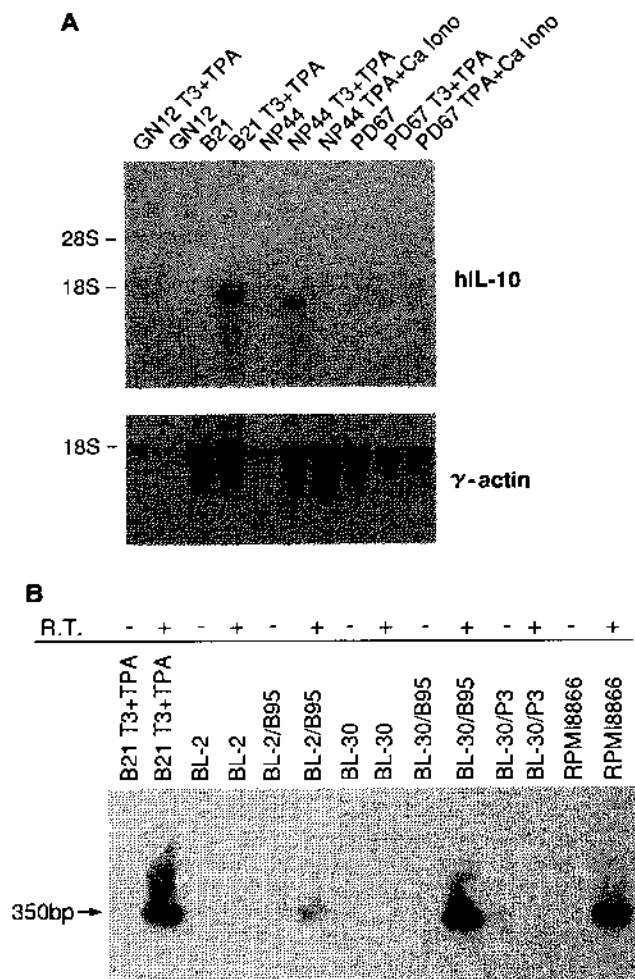


FIG. 5. Expression of hIL-10 mRNA in various cell lines. Expression of hIL-10 mRNA was detected either by RNA blot analysis (A) or by amplification of reverse-transcribed (R.T.) RNA by PCR (B). Positions of the 18S and 28S ribosomal RNA bands are noted, as is the size (350 bp) of the PCR-amplified hIL-10 fragment. TPA, PMA; Ca Iono,  $Ca^{2+}$  ionophore.

phore induces detectable transcription of the hIL-10 gene (Fig. 5A). PCR analysis allowed detection of low levels of hIL-10 mRNA in all T-cell RNA samples (data not shown).

Mouse B-cell lines (22, 29) and normal B cells (29) produce IL-10. We therefore examined hIL-10 mRNA expression by PCR analysis of human B-cell lines (Fig. 5B). Two EBV-lymphoma lines, BL-2 and BL-30, do not transcribe detectable levels of IL-10 mRNA, nor does BL-30/P3, infected with EBNA-2-deficient EBV. However, hIL-10 mRNA can be detected in the EBV-transformed B-cell lines BL-2/B95, BL-30/B95, and RPMI8866. RNA blot analysis was not sufficiently sensitive to detect hIL-10 mRNA in these two cell lines (data not shown). We also detected expression of IL-10 mRNA in FACS-purified peripheral blood T and B lymphocytes, after activation with anti-CD3 and PMA, or *Staphylococcus aureus* Cowan I, respectively (data not shown).

## DISCUSSION

We have established the existence of and cloned cDNAs encoding a human cytokine, hIL-10. hIL-10 cDNA clones were isolated from a cDNA library of an activated human T-cell clone by using oligonucleotides encoding mIL-10. Their identity as hIL-10 cDNA clones was established by several criteria. The nucleotide sequence of hIL-10 is >80% homologous to that of the mouse cytokine throughout the

entire cDNA sequence, except where the human cDNA has an insertion of a human *Alu* repetitive sequence element. The amino acid homology between h- and mIL-10 is 73% (Fig. 1). Moreover, hIL-10 cDNA probes hybridize to restriction fragments in human genomic DNA with similar patterns and intensities in different individuals. This observation, along with the high degree of homology of hIL-10 to its murine counterpart and the fact that mIL-10 is the product of a gene in the mouse genome (22), all argue for a genomic origin of hIL-10. Finally, hIL-10 has similar activities to mIL-10. It inhibits cytokine synthesis by activated T cells (Figs. 3 and 4A), is a growth-stimulatory cofactor for mouse thymocytes and T cells (32, 33) and mast cells (Fig. 4B) (41) and induces class II major histocompatibility complex (MHC) expression on and sustains the viability in culture of small, dense, resting mouse B cells (34).

hIL-10 is expressed by some human T-cell clones after induction with anti-CD3 and PMA, and by several EBV-infected B-cell lines (Fig. 5). Of particular interest was the observation that the original BL-30 line and its defective EBV-infected counterpart BL-30/P3 did not express detectable hIL-10 mRNA, but the EBV-infected BL-30/B95 cells did (Fig. 5B). PCR analysis of RNA from BL-30/B95 and BL-2/B95 reveals that these cell lines do not transcribe detectable levels of BCRFI mRNA (D.-H. Hsu and K.W.M., unpublished data). In addition, the hIL-10 PCR primers do not generate an amplified fragment from templates containing the BCRFI gene (data not shown). Thus, hIL-10 is apparently one of a number of cellular genes whose transcription is activated in latently infected EBV B-cell lines (25). In contrast, BCRFI is expressed during the late phase of the lytic virus cycle (35) when cells are producing virus.

As found for mIL-10, the mature protein coding region of hIL-10 is homologous to the EBV ORF BCRFI (Fig. 1). Like mIL-10 and hIL-10, the BCRFI protein has CSIF activity (24) (Figs. 3 and 4A) and also sustains the viability of resting mouse B cells in culture (34). However, despite its substantial similarity to IL-10, BCRFI does not significantly support the viability in culture of a murine mast cell line (Fig. 4B). Similarly, in two other assays in which both mIL-10 and hIL-10 have comparable activity—costimulation of growth of mouse thymocytes and T cells (33) and induction of class II MHC antigens on resting splenic mouse B lymphocytes (34)—the activity of BCRFI is greatly diminished or absent. Taken together, these results suggest that the viral cytokine may have conserved only a subset of the activities of IL-10. Because IFN- $\gamma$  inhibits the generation and outgrowth of EBV-transformed B cells *in vitro* (36–38), it is not surprising that EBV has conserved the CSIF activity of hIL-10. Moreover, these findings argue for a degree of heterogeneity in IL-10 receptors, receptor number, or differences in the threshold of activation. Since mIL-10, hIL-10, and BCRFI all suppress cytokine synthesis, but the viral cytokine has substantially reduced activity in the mouse mast cell, thymocyte, and class II MHC induction assays, the receptor/signal transduction mechanisms operating in the latter systems apparently respond much more poorly to BCRFI.

Although in some disease situations human T-cell clones can be isolated that express cytokine secretion profiles resembling the murine Th1 or Th2 patterns, many human CD4<sup>+</sup> T-cell clones obtained from healthy donors produce IL-2, IL-4, IL-5, and IFN- $\gamma$  (39, 40) and thus do not fit the Th1/Th2 pattern. Preliminary observations indicate that hIL-10 inhibits production of both IL-4 and IFN- $\gamma$  in this latter type of human T-cell clones (R.deW.-M. and J.E.deV., unpublished data). Thus, while mIL-10 does not inhibit cytokine synthesis by Th2 clones (21), hIL-10 can inhibit synthesis of a "Th2" cytokine expressed by a human T-cell clone with a non-Th2 phenotype. We also note the previous suggestion that the action of IL-10 may be indirect (21), and

the observation (D.F.F., A. Zlotnik, P.V., T.R.M., M. Howard, K.W.M., and A. O'Garra, unpublished data) that IL-10 inhibits the ability of macrophages to stimulate cytokine synthesis by murine Th1 but not Th2 clones. These observations suggest that some types of T cell can be stimulated by IL-10-treated macrophages while others cannot. Therefore, we suggest that sensitivity to inhibition of cytokine synthesis by IL-10 may reflect a property of the differentiated T cell, rather than regulation of expression of individual cytokines.

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