

# Recombinant Human CD40 Ligand Stimulates B Cell Proliferation and Immunoglobulin E Secretion

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## Summary

Signaling through the cell surface molecule, CD40, is known to play an important role in the proliferation and differentiation of B lymphocytes. Using the thymoma cell line EL4, we recently identified and cloned a cDNA encoding a murine ligand for the CD40 molecule (mCD40-L) and showed that it has biological activity *in vitro*. A cDNA encoding a human homologue of the mCD40-L was isolated using crosshybridization techniques from an activated peripheral blood T cell library. The predicted amino acid sequence indicates that this human ligand for CD40 (hCD40-L) is a 261 amino acid type II membrane protein that exhibits 78% amino acid identity with its murine counterpart. Northern blot and FACS<sup>®</sup> analyses suggest that the hCD40-L is restricted in its expression to T lymphocytes, and that it is most abundant on the CD4<sup>+</sup> T cell subpopulation. Cells transfected with hCD40-L caused the proliferation of human tonsil B cells in the absence of costimuli and, in the presence of interleukin 4, induced immunoglobulin E secretion from purified human B cells. A comparison of the efficacy of the hCD40-L and mCD40-L in these assays is presented.

CD40 is a cell surface protein expressed on B lymphocytes, follicular dendritic cells, normal epithelium, and some epithelial carcinomas. The predicted amino acid sequence for CD40 indicates that it is a member of the recently described TNF receptor family of proteins (1–3). This family includes such molecules as the low affinity receptor for nerve growth factor, both forms of TNF receptors, CD27, OX40, and the Hodgkin's lymphoma marker CD30 (3, 4). In addition to structural homology to known cytokine receptors, functional effects in B cells have been shown to be mediated through mAbs directed against CD40. These effects include short- and long-term proliferation (5–7), differentiation (8, 9), induction of intercellular adhesion (10, 11), and tyrosine phosphorylation of a series of intracellular proteins (12). Furthermore, germinal center centrocytes are prevented from undergoing apoptosis when activated through their antigen receptors and by CD40 mAb (13). Taken together, these data strongly suggested that CD40 was the receptor for an unknown ligand.

Recently, we reported the molecular cloning of a ligand for CD40 from the murine thymoma cell line EL4, and showed that this ligand was biologically active on primary B lymphocytes (14). Cells transfected with mCD40-L could induce the proliferation of murine and human B cells in the absence of costimuli. In this paper, we detail the cloning of a human homologue of the ligand for CD40; describe the

relationship of the nucleotide and predicted amino acid sequences of the hCD40-L with those of the mCD40-L; examine the cellular distribution of the hCD40-L by Northern blot analysis and cell surface expression; compare the proliferative activity of both the hCD40-L and mCD40-L on human tonsillar and murine splenic B cells in the presence and absence of costimuli; and evaluate the ability of the two ligands to induce IgE secretion from IL-4-activated B cells.

## Materials and Methods

**Cell Separation.** PBMC were purified from healthy donors by centrifugation over Histopaque<sup>®</sup> (Sigma Chemical Co., St. Louis, MO). Peripheral blood T cells (PB T)<sup>1</sup> were then purified by rosetting with 2-aminoethylisothiuronium bromide (AET)-treated SRBC and further centrifugation over Histopaque<sup>®</sup>. Contaminating monocytes were removed by plastic adherence for 1 h at 37°C. The resulting T cell preparations were always >98% CD3<sup>+</sup>, as determined by flow cytometric analysis (FACScan<sup>®</sup>, Becton Dickinson & Co., Mountain View, CA). Tonsillar tissue was gently teased and the resulting cell suspension centrifuged over Histopaque<sup>®</sup>. T cells were purified as described for PB T cells. Purification of B cells was achieved by removal of cells rosetting with AET-treated SRBC and treatment of the remaining cells with B cell lympho-

<sup>1</sup> Abbreviations used in this paper: AET, aminoethylisothiuronium bromide; FBS, fetal bovine serum; PB T, peripheral blood T cells.

kwik (One Lambda Inc., Los Angeles, CA) for 1 h at 37°C to lyse contaminating non-B cells. The resultant B cell preparations were >96% CD20<sup>+</sup> as determined by flow cytometry. Murine splenic B cells were isolated using anti-T cell antibody and complement, followed by passage over Sephadex G-10 (Pharmacia Fine Chemicals, Piscataway, NJ) to remove adherent cells. B cells were then positively selected by panning on plates coated with 5 µg/ml goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL). Isolated cells were >95% surface IgM<sup>+</sup> as determined by flow cytometry.

**Reagents.** Murine and human recombinant IL-4 were purified from yeast supernatant as previously described (15). The 57-kD gene product of the extracellular domain of human CD40 fused to the Fc region of human IgG1 (CD40.Fc) was expressed, purified, and labeled with biotin-X-nonhydroxy succinimide (NHS; Calbiochem Corp., La Jolla, CA) as previously described (16). CV1/EBNA cells transfected with plasmids containing the hCD40-L or mCD40-L (see below) as described (14, 17), were cultured for 3 d on 10-cm petri dishes, removed and fixed with 1% paraformaldehyde for 10 min at 4°C, and washed extensively in culture medium before their use in biological assays.

**Culture Conditions.** For the preparation of RNA, PB T cells were activated for 20 h with immobilized CD3 mAb. Tonsil T cells were activated for 20 h with 10 ng/ml PMA and 1 µg/ml Con A. For proliferation assays, purified B cells (10<sup>5</sup>/well) were cultured in triplicate with a titration of CV1/EBNA cells expressing human or murine CD40 ligand in flat-bottomed 96-well microtiter plates in 200 µl RPMI in a humidified atmosphere of 10% CO<sub>2</sub>. For the proliferation of murine B cells, medium was supplemented with 5% heat-inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 50 µM 2-ME. For human B cells, medium was supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Wells were pulsed with 2 µCi [<sup>3</sup>H]thymidine (25 Ci/mmol) for the final 8 h of culture, cells were harvested, and incorporated cpm was determined by tritium-sensitive avalanche gas ionization detection on a beta counter (Matrix 96 Direct; Packard Instrument Co., Inc., Meriden, CT). Cultures for the determination of murine IgE secretion were performed as described for murine B cell proliferation assays but in supplemented RPMI 1640 containing 20% FBS. Culture conditions for the determination of human IgE were basically as described for human B cell proliferation assays but were conducted in round-bottomed 96-well microtiter plates. IgE levels in culture supernatants were determined by ELISA and were sensitive to 100 pg/ml and 4 ng/ml of human and murine IgE, respectively.

**Flow Cytometric Analysis.** Purified human PB T cells (10<sup>6</sup>/ml) were cultured with 10 µg/ml immobilized OKT3 (CD3, American Type Culture Collection) mAb in the presence or absence of 10 ng/ml PMA for the times indicated. Cells were harvested and preincubated with 100 µg/ml human IgG1 in PBS plus 0.02% NaN<sub>3</sub> for 30 min at 4°C, to prevent nonspecific binding of staining reagents. Surface expression of CD40-L was determined by staining with 5 µg/ml biotinylated CD40.Fc for 30 min at 4°C. Cells were washed twice with PBS plus 0.02% NaN<sub>3</sub> and incubated for 30 min longer at 4°C with streptavidin-PE diluted 1:5 in PBS plus 0.02% NaN<sub>3</sub> and then analyzed using a FACScan® (Becton Dickinson & Co.). A minimum of 5,000 cells were analyzed for each sample.

**cDNA Library Construction.** Pooled human PB T were isolated from normal donors and cultured in 10 ng/ml IL-2 for 6 d followed by stimulation with 10 ng/ml PMA and 1 µg/ml Con A for 8 h. Polyadenylated RNA was used as a template to construct a random

primed cDNA library using a cDNA synthesis kit (Amersham Corp., Arlington Heights, IL). cDNA was adapted using EcoRI linkers and cloned into a λgt11 vector (Stratagene Inc., La Jolla, CA). The library was screened with a <sup>32</sup>P-labeled mCD40-L riboprobe which was generated using SP6 polymerase (Promega Corp., Madison, WI) according to the supplier's protocol. This probe corresponds to the complete coding region of the mCD40-L gene, and was hybridized to the λgt11 library at 55°C in Stark's solution followed by washing in 2 × SSC 0.1% SDS at 63°C.

**Transient Expression of hCD40-L in Mammalian Cells.** The coding region of the hCD40-L was cloned into the mammalian expression vector pDC302 (17); DEAE transfections were performed in COS cells as described (18). Alternatively, pDC302 expression vector carrying the hCD40-L gene was cotransfected with the plasmid pSV3neo (19) using DEAE dextran into CV-1 EBNA cells (18).

**Northern Blot Analysis.** Northern blots were prepared essentially as described (20). Hybridization was performed in Stark's solution at 63°C followed by several washings in 0.2 × SSC, 1% SDS at 55°C. Blots were exposed for 1–3 d at –70°C.

**Radiolabeling and Immunoprecipitations.** COS cells expressing the hCD40-L protein or PB T cells cultured with immobilized CD3 as described for 3 h were radiolabeled with 100 µCi/ml <sup>35</sup>S Translabel (ICN Biomedicals, Inc., Irvine, CA) for 2–3 h. Cells were lysed in PBS containing 1% Triton X-100 and clarified at 10,000 g for 30 min. Lysates were incubated with purified CD40.Fc protein (1 µg/ml) for 30 min followed by the addition of protein A-Sepharose. After extensive washing, pellets were resuspended in sample buffer in the presence or absence of β-ME and analyzed by PAGE.

## Results

**Characterization of the hCD40-L cDNA Clone.** A <sup>32</sup>P-labeled riboprobe corresponding to the coding region of the mCD40-L was used to screen a stimulated human PB T library under reduced stringency (see Materials and Methods). This resulted in the isolation of a 1,803-bp cDNA that contained the complete coding region of the hCD40-L. This clone contained a single open reading frame that could encode a polypeptide of 261 amino acids, a 45-bp 5' noncoding region, and 975 bp of 3' noncoding sequences which included a poly(A) tail. The predicted amino acid sequence of the hCD40-L and an alignment with the CD40-L is shown in Fig. 1. Like the mCD40-L, the hCD40-L is a type II membrane protein with a short 22-amino acid NH<sub>2</sub>-terminal cytoplasmic domain followed by a 24-amino acid hydrophobic signal anchor region. The hCD40-L has a 215-amino acid COOH-terminal extracellular domain compared to 214 amino acids in the mCD40-L and five cysteine residues compared to four in the murine ligand. There is an N-linked glycosylation site in the extracellular domain that is conserved between the human and murine ligands, and an additional, but likely not utilized, glycosylation site in the cytoplasmic domain of hCD40-L. The two sequences exhibit 78% amino acid identity overall. More specifically, there is 81% amino acid identity between the cytoplasmic domains, 96% between the transmembrane regions, and 75% between the extracellular domains.

**Expression and Immunoprecipitation of Recombinant hCD40-L.** Oligonucleotide primers designed to include incompatible restriction sites were used in a PCR reaction to amplify

TGCCACCTTCTCTGCCAGAAGATACCATTTCACCTTTAACACAGCATGATCGAAACATAC 60  
 hCD40.L M I E T Y 5  
 mCD40.L  
 AACCAACTTCTCCGGCATCTGCGGCCACTGGACTGCCCATCAGCATGAAAATTTTATG 120  
 hCD40.L N Q T S P R S A A T G L P I S M K I F M 25  
 mCD40.L S P V A  
 TATTACTACTGTTTTTCTTATCACCCAGATGATGGGTGAGCACTTTTGTGTGTAT 180  
 hCD40.L Y L L T V F L I T Q M I G S A L F A V Y 45  
 mCD40.L V  
 CTTCATAGAAGTTGGACAAGATAGAAGATGAAAGGAATCTTCATGAAGATTTGTATT 240  
 hCD40.L L H R R L D K I E D E R N L H E D F V F 65  
 mCD40.L V E V  
 ATGAAAACGATACAGAGATGCAACACAGGAGAAAGATCCTTACTGAACTGTGAG 300  
 hCD40.L M K T I Q R C N T G E R S L S L L N C E 85  
 mCD40.L I K L K K G  
 GAGATTAAGCCAGTTTGAAGGCTTTGTGAAGGATATAATGTTAAACAAGAGGAGAGC 360  
 hCD40.L E I K S Q F E G F V K D I M L N K E E T 105  
 mCD40.L M R R D L T  
 AAGAAAGAAAACAGCTTTGAAATGCAAAAAGGTGATCAGAATCCTCAAATGCGGCACAT 420  
 hCD40.L K K E N S F E M Q K G D Q N P Q I A A H 125  
 mCD40.L R E D  
 GTCATAAGTGAAGCCAGCAGTAAACAACATCTGTGTACAGTGGGCTGAAAAGGATAC 480  
 hCD40.L V I S E A S S K T T S V L Q W A E K G Y 145  
 mCD40.L V N N A A K  
 TACACCTGAGCAACACTTGGTAACCCCTGGAAAATGGGAAACAGCTGACCGTTAAAAGA 540  
 hCD40.L Y T M S N N L V T L E N G K Q L T V K R 165  
 mCD40.L K S M  
 CAAGGACTCTATTATATCTATGCCCCAAGTCACCTTCTGTTCATCGGGAAGCTTCGAGT 600  
 hCD40.L Q G L Y Y I Y A Q V T F C S N R E A S S 185  
 mCD40.L E V T P  
 CAAGCTCCATTTATAGCCAGCCTCTGCCTAAAGTCCCGCGTAGATTTCGAGAGAATCTTA 660  
 hCD40.L Q A P F I A S L C L K S P G R F E R I L 205  
 mCD40.L R V G W P S I G S  
 CTCAGAGCTGCAAAATACCCACAGTTCGCGCAACCTTGGCGCAACAATCCATTCACCTG 720  
 hCD40.L L R A A N T H S S A K P C G Q Q S I H L 225  
 mCD40.L K S Q L E V  
 GGAGGATTTTGAATGCAACCAGGTGCTTCGGTGTGTGCAATGTGACTGATCCAAGC 780  
 hCD40.L G G V F E L Q P G A S V F V N V V D P S 245  
 mCD40.L A E A  
 CAAGTGAGCCATGGCACTGGCTTCACGTCTTTGGCTTACTCAAACCTGAAACAGTGTCA 840  
 hCD40.L Q V S H G T G F T S F G L L K L \* 261  
 mCD40.L I R V S  
 CCTTGCAGGCTGTGGTGGAGCTGACGCTGGGAGTCTTCATAATACAGCACAGCGTTAAG 900  
 CCCACCCCTGTTAACTGCCTATTTATAACCTAGGATCCTCCTTATGGAGAATTTTA 960  
 TTATACACTCCAAGGCATGTAGAATCTGATAAATGTAATTACAGGTACATGAAACCAA 1020  
 ACGGGCCCTGCTCCATAAGAGCTTATATATCTGAAGCAGCAACCCCACTGATGCAGACAT 1080  
 CCAGAGAGTCTATGAAAAGCAAGGCCATTATGCACAGGTTGAATTTCTGAGTAAACAGC 1140  
 AGATAACTTGCCAAAGTTCAGTTTTGTTCTTTGGTGCAGTGTCTTTCCATGGATAATGC 1200  
 ATTTGATTTATCAGTGAAGATGCAGAAGGAAATGGGAGCCTCAGCTCACATTGAGTTA 1260  
 TGGTTGACTCTGGTCTCTATGGCCTTGTGGAGGGGCCAGGCTCTAGAAGTCTAACA 1320  
 CAGTGGAGAACCGAAACCCCGCCCGCCCGCCCGCCCGCCCGCCCGCCCGCCCGCCCGCC 1380  
 CTTTCAATCTCTCTCTCCATCTCTCTCTTTCAGTCTCTCTCTCAACCTCTTTCTTC 1440  
 CAATCTCTTTCTCAATCTCTCTGTTTCCCTTTGTCAGTCTCTTCCCTCCCGCAGTCTC 1500  
 TCTTCTCAATCCCTTTCTAACACACACACACACACACACACACACACACACACACACA 1560  
 CACACACACACAGAGTCAAGCCGTTGCTAGTCAAGTCTCTCTTCTTCCACCTGTCCTAT 1620  
 CTCTACCACTATAGATGAGGGTGAAGGAGTGGGAGTGCAGCCCTGAGCCTGCCACTCCT 1680  
 CATTACGAAATGACTGTATTTAAAGGAAATCTATGTATCTACCTGCACTGTCATTGTT 1740  
 TCCAGAGTGAACCTGTAATTATCTGTTATTTATTTTGAATGATAAGCCTCTTAAC 1800  
 ATT 1803

**Figure 1.** Nucleotide sequence exclusive of poly(A) of a cDNA encoding the hCD40-L protein (Genbank accession no. X67878). The predicted amino acid sequence of hCD40-L is given and compared with that of mCD40-L. Amino acid differences are indicated. The putative NH<sub>2</sub>-terminal signal/anchor sequence is underlined, and potential N-linked glycosylation sites are shaded. A consensus sequence for addition of poly(A) is located at position 1784 (boxed), and a possible alternative poly(A) site occurs at position 989 (boxed).

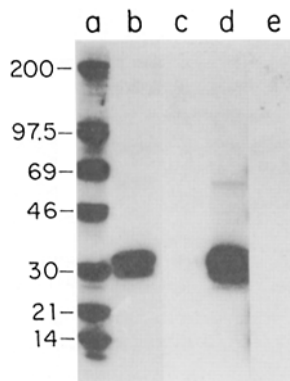
the coding region of the hCD40-L gene. After digestion with the appropriate enzymes, the resulting PCR product was subcloned into a mammalian expression vector. Sequence analysis of the coding region insert confirmed that no nucleotide changes had occurred as a result of the PCR amplification. This plasmid was then used to transfect COS cells. The resulting recombinant hCD40-L was compared with the hCD40-L expressed on stimulated human PB T cells by metabolic labeling and precipitation using a previously described soluble CD40.Fc chimeric molecule (16). The results of this experiment are shown in Fig. 2. Immunoprecipitates electrophoresed under reducing conditions (lane *b*) indicate an apparent *M<sub>r</sub>* of 33–35 kD for the recombinant hCD40-L.

This protein comigrates with the hCD40-L protein derived from CD3 mAb-stimulated PB T cells (lane *d*). Immunoprecipitates electrophoresed under nonreducing conditions are essentially indistinguishable (data not shown), suggesting that the hCD40-L does not dimerize through disulphide bonding. A larger band (~ 69 kD) is present in the samples from stimulated PB T cells (lane *d*) or cells transfected with the hCD40-L (lane *b*), but not in control samples (lanes *c* and *e*). We addressed the possibility that this band might be due to oligomerization of the ligand through noncovalent bond formation by electrophoresing precipitated samples on polyacrylamide gels containing 7 M urea in the presence and absence of reducing agents. When these experiments are performed,

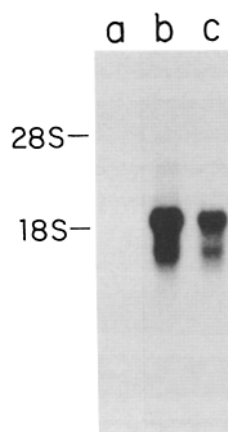
the results are similar to those in Fig. 2 (data not shown). Thus, the exact nature of the higher molecular weight material is still unclear, but it is not likely to be a simple oligomer of the hCD40-L.

**Characterization of hCD40-L Expression by Northern Blot Analysis.** Previously, we reported that mCD40-L mRNA was induced in T cells activated with CD3 mAb (14). We examined purified human PB T and tonsil T cells for their expression of hCD40-L mRNA in response to various stimuli. Total RNA from PB T cells activated with CD3 mAb, or tonsil T cells stimulated with PMA and Con A, was analyzed on Northern blots using a <sup>32</sup>P-labeled antisense RNA probe (Fig. 3). No detectable hCD40-L was present in unstimulated PB T or tonsil T cell total RNA, but was inducible under both stimulation conditions. It is interesting that both PB T and tonsil T cells express what appear to be two hCD40-L-specific mRNAs. The larger band is typically more prominent than the smaller band and appears to be ~2,000 bases in length, comparable to the size of the single mCD40-L mRNA seen in murine T cells. The exact nature of the smaller band is unclear. PCR experiments (our unpublished data) have not provided any evidence for alternate mRNA splicing. There is only a single AATAAA polyadenylation consensus sequence in the 3' noncoding region (Fig. 1). However, the sequence AATAAG appears at position 989 and might possibly serve as an alternate polyadenylation signal.

**Cellular Distribution of the hCD40-L.** RNA was obtained from CD3 mAb-stimulated PB T cells and a number of cell lines of myeloid, lymphoid, fibroblastic, and epithelial origin, and examined for hCD40-L transcripts. The following cell types were found negative by Northern blot analysis using 5 μg poly(A)<sup>+</sup> RNA per lane: Blin, CB23, Raji, Jurkat, PBM, monocytes, Thp-1, U937, HeLa, A549, placenta, and dermal fibroblasts. After hybridization with the <sup>32</sup>P-labeled antisense probe described above, no hCD40-L-specific mRNA could be detected in any cell type other than T cells or their derivatives. In addition, we examined a commercially available Northern blot containing total RNA derived from a variety of human tissues (Fig. 4). On this blot, the radiolabeled hCD40-L probe hybridized with RNA derived from lung tissue. This was somewhat surprising based on the



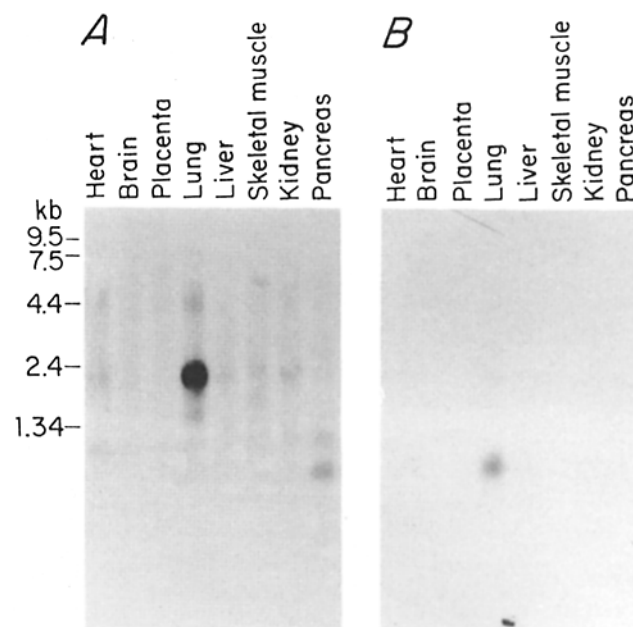
**Figure 2.** Precipitation of hCD40-L protein using soluble CD40.Fc. COS cells expressing the hCD40-L protein or CD3 mAb-stimulated PB T cells were radiolabeled as described in Materials and Methods. Lysates were precipitated using CD40.Fc and protein A-Sepharose and analyzed under reducing conditions on an 8–16% polyacrylamide SDS gradient gel. Lanes: (a) molecular weight markers; (b) recombinant hCD40-L protein derived from transfected cells; (c) cells transfected with empty vector; (d) hCD40-L protein from stimulated PB T cells; and (e) unstimulated PB T cells.



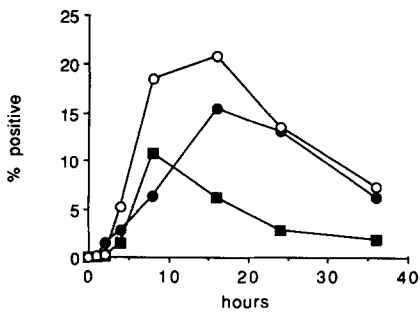
**Figure 3.** Northern blot analysis of hCD40-L-expressing T cells. Total RNA from stimulated PB T or tonsil T cells was analyzed on Northern blots using a <sup>32</sup>P-labeled antisense hCD40-L probe. Lanes: (a) unstimulated PB T cells; (b) CD3-stimulated PB T cells; and (c) PMA plus Con A-stimulated tonsil T cells.

Northern blot analysis of specific cell RNAs. However, it was possible that the hCD40-L-specific mRNA in this lung sample was due to contaminating T lymphocytes. To address this, we rehybridized this tissue blot with a <sup>32</sup>P-labeled antisense probe derived from the δ chain of the TCR (Fig. 4 B). This experiment resulted in the detection of a band of the expected size which was present exclusively in the lung-derived sample. Therefore, it appears that within the cell types examined thus far, expression of the hCD40-L is restricted to T lymphocytes.

**Induction of hCD40-L on PB T Cells.** The induction of hCD40-L expression on PB T cells was examined over 36 h after activation with CD3 antibody, PMA, or a combina-

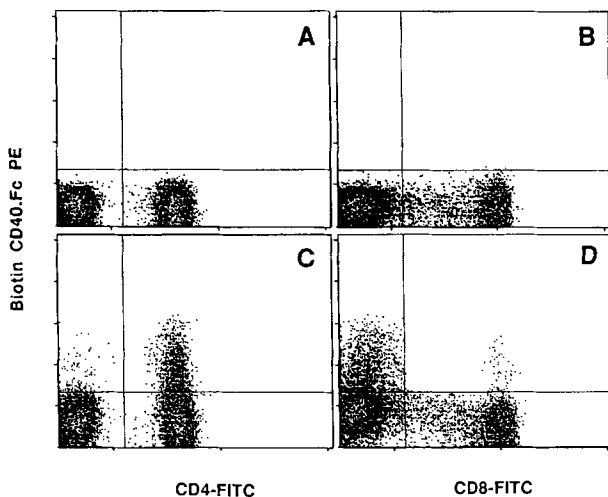


**Figure 4.** Northern blot analysis of tissue-specific RNAs. Poly(A)<sup>+</sup> RNA from the indicated tissues was hybridized with antisense riboprobes as described in Materials and Methods. (A) Northern blot hybridized with a radiolabeled probe corresponding to the coding region of the hCD40-L. (B) The same Northern blot, partially stripped and rehybridized with a probe specific for the δ chain of the TCR.



**Figure 5.** Induction of CD40 ligand expression on PB T cells. Purified peripheral blood T cells were activated for up to 36 h in the presence of 5  $\mu\text{g}/\text{ml}$  immobilized CD3 mAb (●), 5 ng/ml PMA (■), or a combination of CD3 mAb plus PMA (○). Cells were harvested at the time indicated, stained with 5  $\mu\text{g}/\text{ml}$  biotinylated CD40.Fc and streptavidin-PE, and analyzed by flow cytometry. Results are expressed as the percent of cells positive for biotinylated CD40.Fc binding compared to cells stained with control biotinylated IL-4R.Fc.

tion of the two (Fig. 5). Unstimulated T cells expressed virtually undetectable levels of hCD40-L, as determined by flow cytometric analysis of cells stained with biotinylated CD40.Fc. Treatment with CD3 mAb induced detectable hCD40-L by 4 h, with maximal expression at 8–16 h, and with significant expression still detected at 36 h. Stimulation with PMA resulted in lower levels of ligand expression but similar kinetics of induction. A combination of CD3 mAb and PMA induced detectable hCD40-L by 2 h and resulted in a higher level of ligand expression at 16 h than seen with either factor alone. In contrast to CD3 mAb, PHA was not an effective inducer of hCD40-L, but in combination with PMA resulted in levels of ligand comparable to those seen with a combination of CD3 mAb and PMA (data not shown).

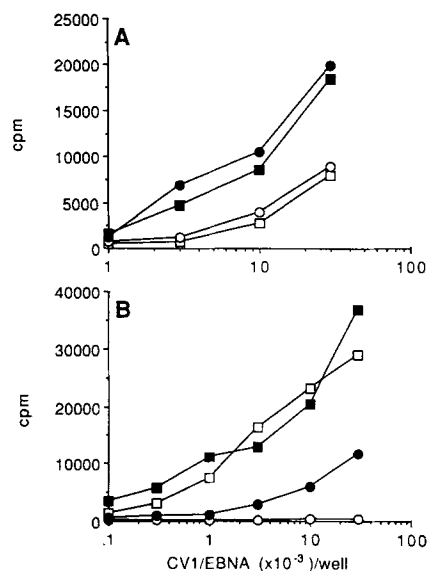


**Figure 6.** CD40 ligand is induced predominantly on CD4<sup>+</sup> T cells. Purified PB T cells were cultured for 16 h in medium (A and B) or activated with 5  $\mu\text{g}/\text{ml}$  immobilized CD3 mAb (C and D) and stained with biotinylated CD40.Fc and streptavidin-PE and either FITC-conjugated CD4 (A and C) or CD8 (B and D) mAb. Results are representative from one of six experiments performed.

**Expression of hCD40-L on T Cell Subpopulations.** CD4<sup>+</sup> and CD8<sup>+</sup> PB T cells were examined for the expression of hCD40-L after 18-h stimulation with CD3 mAb (Fig. 6). In the absence of stimulation, CD4<sup>+</sup> and CD8<sup>+</sup> T cells expressed no detectable hCD40-L as determined by binding of biotinylated CD40.Fc (Fig. 6, A and B). After treatment with CD3 mAb, hCD40-L expression was detected primarily on the CD4<sup>+</sup> population (Fig. 6 C). However, a reproducible finding from experiments performed on T cells from many donors was that a small proportion of CD8<sup>+</sup> T cells was also induced to express hCD40-L (Fig. 6 D), although this expression was consistently lower than that seen on CD4<sup>+</sup> cells.

**Induction of Proliferation with CD40-L.** The human and murine CD40-L were compared for their ability to mediate B cell proliferation. Purified human tonsil B cells were induced to proliferate in the absence of added cytokines when cultured with fixed CV1/EBNA cells transfected with either human or murine CD40-L (Fig. 7 A). This effect appeared to be dose dependent. Addition of IL-4 to cultures enhanced the proliferative response of human B cells to both human and murine ligand.

The effects of hCD40-L on proliferation of murine splenic B cells was also examined (Fig. 7 B). As shown previously, mCD40-L was mitogenic in a dose-dependent manner for murine B cells cultured in the absence of costimulus (14). In contrast, under the same culture conditions, CV1/EBNA cells expressing the hCD40-L had virtually no effect. However, the addition of IL-4 to cultures containing the hCD40-L resulted in the proliferation of murine B cells. Although IL-4



**Figure 7.** Comparative proliferative responses of B cells to human and murine CD40 ligand. (A) 10<sup>5</sup> purified human tonsil B cells were cultured for 6 d with a titration of CV1/EBNA cells transfected with human (●, ○) or murine (■, □) CD40 ligand, in the presence (filled symbols) or absence (open symbols) of 5 ng/ml hIL-4. (B) 10<sup>5</sup> purified murine splenic B cells were cultured for 3 d with CD40 ligand as described above in the presence or absence of 10 ng/ml mIL-4. Results are expressed as mean incorporated cpm from triplicate cultures.

enhanced the response of human B cells to mCD40-L (Fig. 7 A), no such enhancement was observed when IL-4 was added to murine B cell cultures containing mCD40-L (Fig. 7 B). In both human and murine proliferation assays, CV1/EBNA cells transfected with vector alone had no effect (13, data not shown).

The two forms of CD40-L were compared for their ability to induce IgE secretion from IL-4-activated B cells. Human and murine CD40-L showed comparable activity in the induction of human IgE secretion from purified tonsil B cells (Fig. 8 A). As was seen in the proliferation assays, this effect also appeared dose dependent. Similarly, both human and murine forms of CD40-L induced IgE from IL-4-stimulated murine splenic B cells (Fig. 7 B), although the human ligand appeared to exhibit lower activity than the murine ligand in this assay. No secreted human or murine IgE was detected in the absence of IL-4 or if CV1/EBNA cells transfected with vector alone were used (14, data not shown).

## Discussion

Cell surface molecules expressed on activated T cells that provide contact-dependent help to B cells have been the subject of intense investigation. Recently, a molecule expressed on CD3-activated murine T cells that induces B cells to proliferate in the absence of a costimulus and stimulates IgE secretion from IL-4-activated B cells was cloned and identified as a murine ligand for CD40 (14). Using crosshybridization techniques, we have isolated a human ligand for CD40 that exhibits significant homology (78% identity at the amino

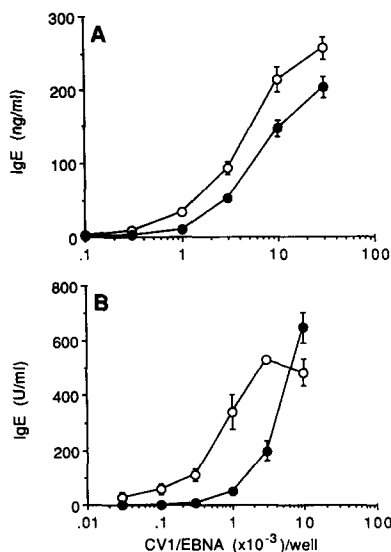
acid and 83% at the nucleotide levels) to its murine counterpart. This level of homology suggested that the hCD40-L would be active on both murine and human B cells in a manner similar to that described for mCD40-L (14). In terms of IgE secretion, this was indeed the case, although the hCD40-L displayed lower potency than the mCD40-L on murine B cells. However, with regard to induction of proliferation, we report significant differences between the murine and human CD40-L. Using murine B cells, hCD40-L requires IL-4 for induction of proliferation, whereas mCD40-L does not. On the other hand, in the human B cell culture system both hCD40-L and mCD40-L are capable of inducing significant proliferation in the absence of IL-4. Moreover, IL-4 augments the response of human B cells to hCD40-L and mCD40-L.

Experiments performed in our laboratory (unpublished data) indicate that human and murine B cells differ in the time at which the maximum proliferative response to either hCD40-L or mCD40-L is observed. In murine B cell cultures, the maximal proliferative response is seen at day 2, whereas human B cells show the highest rate of proliferation at day 5, regardless of whether hCD40-L or mCD40-L is used as a stimulus. Because hCD40-L and mCD40-L have comparable activity on human B cells, the inability of hCD40-L to induce the proliferation of murine B cells in the absence of IL-4 is intriguing. One possible explanation for this disparity could be a decreased affinity of hCD40-L compared to mCD40-L for murine CD40.

The predicted amino acid sequence for the murine CD40 molecule is 62% identical to the human CD40 (2). This level of homology might suggest that the binding of both mCD40-L and hCD40-L to either form of CD40 would be comparable. In support of this, we have previously shown that mCD40-L binds to human CD40 with high affinity (21). However, additional binding studies using hCD40-L and mCD40-L will be necessary to further define this interaction.

Another possible explanation for the disparity in the activity of the two ligands is that hCD40-L may not induce proliferation of murine B cells in the absence of costimulus unless the CD40 receptor molecule is being expressed at some required threshold level. It is known that CD40 is constitutively expressed on nonactivated human B cells at high levels relative to those observed on murine B cells (1, 2, 22, and our unpublished observations). The addition of IL-4 to activated murine B cells enhances CD40 mRNA expression (2), which could result in an increased surface expression sufficient to allow the observed proliferative effects. Human B cells also respond to IL-4 by upregulating CD40 expression. However, if a minimum number or relative density of CD40 is required for optimal signaling, the comparable activity of hCD40-L and mCD40-L on human B cells in the absence of IL-4 may simply reflect the increased level of CD40 expression. In contrast to the proliferation assays performed in the absence of a costimulus, the observation that hCD40-L and mCD40-L induce similar levels of IgE from murine B cells is likely due to the absolute requirement for IL-4 in secretion of this isotype.

The conformation of the ligands and the CD40 receptors no doubt play an important role in defining their relative



**Figure 8.** Human and murine IgE secretion induced by CD40 ligand. (A)  $10^5$  purified human tonsil B cells were cultured for 10 d with a titration of CV1/EBNA cells transfected with human (●) or murine (○) CD40 ligand, in the presence of 5 ng/ml hIL-4. (B)  $10^5$  purified murine splenic B cells were cultured for 7 d with CD40 ligand as described above in the presence of 10 ng/ml mIL-4. Results are expressed as mean values  $\pm$  SEM from triplicate cultures.

affinities for one another. Fine structure epitope mapping using panels of mAbs will be useful, however, as of yet, such reagents are unavailable. Recently, Lederman et al. (23) described a mAb directed against a 30-kD activated T cell surface antigen which resembles the hCD40-L in its functional effects. This antibody inhibits the B cell proliferation and CD23 expression induced by activated CD4<sup>+</sup> T cells. However, the kinetics of induction of the T cell surface antigen recognized by this antibody differ from that seen in our laboratory for the hCD40-L. In addition, reactivity of this antibody is restricted to CD4<sup>+</sup> T cells, whereas hCD40-L can also be induced on a small percentage of CD8<sup>+</sup> T cells. Further studies will be necessary to determine the exact identity of the T cell antigen recognized by this antibody and its relationship to the hCD40-L.

The significance of the induction of the hCD40-L on a small proportion of CD8<sup>+</sup> PB T cells is unclear. Attempts to induce the expression of hCD40-L on human T cell clones (our unpublished observations) have thus far been successful only in CD4<sup>+</sup> cells, despite the fact that several CD8<sup>+</sup> clones have also been examined. Studies are currently underway to determine whether CD8<sup>+</sup> cells expressing hCD40-L play a unique role in the normal immune response in vivo.

In addition to B cells, the cellular distribution of CD40 includes follicular dendritic cells and epithelial cells. Given the diversity of the response to B cells to the CD40-L, it will be of interest in the future to determine the role of the CD40-L in the biology of other cell types.

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