PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number:
C07K 15/06, 15/28, A61K 37/02	A1	WO 94/04570
		(43) International Publication Date: 3 March 1994 (03.03.94)

(21) International Application Number: PCT/US93/07673

(22) International Filing Date: 19 August 1993 (19.08.93)

(30) Priority data:
07/934,371 21 August 1992 (21.08.92) US

(60) Parent Application or Grant
(63) Related by Continuation
US 07/934,371 (CIP)
Filed on 21 August 1992 (21.08.92)

(71) Applicant *(for all designated States except US)*: SCHERING CORPORATION [US/US]; 2000 Galloping Hill Road, Kenilworth, NJ 07033 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : HEATH, Andrew, W. [GB/US]; 135 O'Connor Street, Menlo Park, CA 94025 (US). HOWARD, Maureen [AU/US]; 12700 Viscaino Drive, Los Altos Hills, CA 94022 (US). LANIER, Lewis, L. [US/US]; 1528 Frontero Avenue, Los Altos, CA 94024 (US).

(74) Agents: DULAK, Norman, C. et al.; Schering-Plough Corporation, One Giralda Farms, M3W, Madison, NJ 07940-1000 (US).

(81) Designated States: AU, BB, BG, BR, BY, CA, CZ, FI, HU, JP, KR, KZ, LK, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CD40 LIGAND, ANTI CD40 ANTIBODIES, AND SOLUBLE CD40

(57) Abstract

The present invention provides materials and methods for inhibiting proliferation of malignant cells, including cells carrying a CD40 surface antigen. Reagents of the invention include ligands that bind CD40, including anti-CD40 antibodies, soluble CD40 molecules, and B cell CD40 ligands. Pharmaceutical compositions that include these reagents are also within the scope of the invention.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FR**	France	**MR**	Mauritania
AU	Australia	**GA**	Gabon	**MW**	Malawi
BB	Barbados	**GB**	United Kingdom	**NE**	Niger
BE	Belgium	**GN**	Guinea	**NL**	Netherlands
BF	Burkina Faso	**GR**	Greece	**NO**	Norway
BG	Bulgaria	**HU**	Hungary	**NZ**	New Zealand
BJ	Benin	**IE**	Ireland	**PL**	Poland
BR	Brazil	**IT**	Italy	**PT**	Portugal
BY	Belarus	**JP**	Japan	**RO**	Romania
CA	Canada	**KP**	Democratic People's Republic of Korea	**RU**	Russian Federation
CF	Central African Republic			**SD**	Sudan
CG	Congo	**KR**	Republic of Korea	**SE**	Sweden
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovak Republic
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LU**	Luxembourg	**TD**	Chad
CS	Czechoslovakia	**LV**	Latvia	**TG**	Togo
CZ	Czech Republic	**MC**	Monaco	**UA**	Ukraine
DE	Germany	**MG**	Madagascar	**US**	United States of America
DK	Denmark	**ML**	Mali	**UZ**	Uzbekistan
ES	Spain	**MN**	Mongolia	**VN**	Viet Nam
FI	Finland				

CD40 LIGAND, ANTI CD40 ANTIBODIES, AND SOLUBLE CD40

The present invention relates to materials and methods for modulating an immune response of an animal to neoplastic cells. More particularly, this invention provides reagents and methods for treating malignancies, whether they normally express CD40 or not.

BACKGROUND OF THE INVENTION

Leukemias, lymphomas, carcinomas, and other malignancies are described in, *e.g.*, *Harrison's Principles of Internal Medicine*, Wilson *et al.*, eds., McGraw-Hill, New York, pp. 1599-1612). As one example of such diseases, malignant lymphomas are neoplastic transformed cells that reside predominantly in lymphoid tissues (*see, e.g.*, Nadler, L.M. in *Harrison's, supra*). Ninety percent of non-Hodgkins lymphomas, of which approximately 30,000 new cases occur each year in the U.S., are B cell lymphomas. Less than 25% of these cases are cured. Another example is leukemia, of which approximately 30,000 new cases occur annually in the U.S. Thus, a need exists for a more effective treatment for B cell lymphomas, leukemias, carcinomas, and other malignancies.

Growth of normal resting B cells (also referred to as "B lymphocytes") involves two distinct steps. First, the resting cells are activated to pass from the G_0 to G_1 phase of the cell cycle (*see, e.g.*, Alberts *et al.*, eds. (1989) *Molecular Biology of the Cell*, Garland Publ., NY; and Darnell *et al.* (1990) *Molecular Cell Biology*, Freeman, NY). Next, the activated cells are induced to proliferate (*see, e.g., Fundamental Immunology*, Paul, ed. (1989) 2nd Ed., Raven Press, NY).

Several factors have been identified that induce growth of B cells, including B-cell growth factors, interleukin-1 (IL-1), and interleukin-2 (IL-2). In addition, antibodies against

certain B cell surface molecules have been demonstrated to promote B cell proliferation. T cells (also referred to as "T lymphocytes") are also induced to proliferate by certain factors, which include phytohemagglutinin, anti-T cell receptor monoclonal antibodies, anti-CD3 monoclonal antibodies, and other agents.

Numerous studies implicate the CD40 molecule as a growth factor receptor and an important regulator of human B cell proliferation and development. B lymphocytes are activated by the interaction of CD40 molecules on the surface of the B lymphocytes with a ligand that is transiently expressed on activated helper T cells. See, e.g., Armitage *et al.*, *Nature 357*:80 (1992).

CD40 is a membrane-associated glycoprotein expressed on normal B lymphocytes and B cell malignancies, interdigitating cells, follicular dendritic cells, thymic epithelial cells, and some carcinomas. Human CD40 was identified by 1987 as the epitope recognized by a monoclonal antibody. CD40 is expressed almost exclusively on B lymphocytes, and therefore is a useful marker for B cells. The human CD40 antigen is a 45-50 Kd glycoprotein.

Anti-CD40 antibodies provide a potent co-stimulatory signal for B cell proliferation induced by either phorbol myristic acetate (PMA), anti-CD20 antibodies, or anti-immunoglobulin antibodies. The addition of anti-human CD40 antibodies plus IL-4 to activated B cells also causes numerous effects, including short-term replication, induction of IgE synthesis, and long-term proliferation when cultures are further supplemented with CD32 transfected L cells.

T cells are known to express a CD40 ligand on their cell surface. See Armitage *et al., supra.* This ligand, which is referred to herein as the "T cell CD40 ligand," mediates B cell

proliferation. No counterpart or equivalent ligand has previously been found on B cells.

Moreover, CD40$^-$ malignancies have escaped the immune system's immune surveillance and continue to proliferate. Stimulation of an immune response to better recognize these cells may lead to effective therapeutic treatment of this class of malignancies.

Because CD40 is involved in the proliferation of certain CD40$^+$ malignant cells, including B lymphomas, there is a need for materials and methods for inhibiting the action of CD40 to control these B cell malignancies. There is also a need for better methods to treat CD40$^-$ malignancies.

SUMMARY OF THE INVENTION

The present invention fills these needs by providing such materials and methods.

More particularly, this invention provides a soluble form of CD40. This soluble form substantially lacks the cytoplasmic and/or transmembrane domain of wild-type CD40.

This invention further provides an isolated B cell CD40 ligand and isolated DNA encoding this ligand.

The present invention still further provides a method for inhibiting proliferation of CD40$^+$ malignant cells comprising contacting CD40$^+$ malignant cells with an effective amount of a soluble form of CD40 and/or a ligand that binds CD40.

This invention still further provides a method for rendering CD40$^-$ malignant cells more immunogenic to T cells comprising transforming CD40$^-$ malignant cells with DNA encoding human CD40, whereby human CD40 is expressed on the surface of the cells.

The present invention still further provides a method for reducing activity by T cells against CD40+ cells comprising:

(a) contacting CD40+ cells with an effective amount of a ligand that binds CD40, and/or

(b) contacting T cells with an effective amount of a soluble form of CD40.

The ligands that bind CD40 mentioned above can be an antibodies against CD40, preferably monoclonal antibodies.

Pharmaceutical compositions comprising the soluble form of CD40 or antibodies against CD40, and a pharmaceutically acceptable carrier, are also provided by this invention.

DESCRIPTION OF THE INVENTION

All references cited herein are hereby incorporated in their entirety by reference.

As used herein, the term "CD40- malignant cells" is defined to mean malignant cells which do not express the CD40 antigen on the cell surface. The term "CD40+ malignant cells" is defined to mean malignant cells that do express CD40 on the cell surface. Preferred types of such cells, the proliferation of which can be inhibited by the materials and methods of this invention, are B lymphoma cells.

It has surprisingly been found that proliferation of CD40+ malignant cells, e.g., B lymphoma cells, but not normal B cells, is markedly inhibited by soluble CD40 molecules and by ligands that bind CD40. The present invention exploits this fact by providing reagents, compositions, and methods for treating such CD40+ malignant cells.

The reagents include soluble CD40 molecules that substantially lack a transmembrane domain, antibodies against CD40, and proteins that are substantially identical to a CD40 ligand that is found on the surface of B cells. The reagents and methods are useful for treating $CD40^+$ malignant cells. Typically, these $CD40^+$ cells are functionally affected by the presence of a T cell CD40 ligand.

T cells are known to express a CD40 ligand on the cell surface [Armitage *et al.*, *Nature 357*:80 (1992)]. This ligand, referred to herein as "T cell CD40 ligand," mediates B cell proliferation, and T cell proliferation and cytotoxicity. Surprisingly, it has been found that B cells express a different ligand (or counterstructure) for CD40. This ligand is referred to herein as "B cell CD40 ligand." Methods for isolating and characterizing this B cell CD40 ligand are disclosed below.

Normal growth of $CD40^+$ malignant cells, e.g., B lymphoma cells, apparently reflects an autocrine interaction between a CD40 molecule on one cell and a CD40 ligand/counterstructure molecule on a neighboring B lymphoma cell. This interaction leads to transduction of a growth signal, presumably at the CD40 molecule (known to be a receptor for B cell growth induction signals), and possibly also at the CD40 ligand. Blocking this interaction using antagonists (such as anti-CD40 antibodies) or molecules that bind to the CD40 ligand (such as soluble CD40 or functional analogs thereof) suppresses growth of the B lymphoma cells. Alternatively, stimulation of the cell by anti-CD40 antisera leads to a negative signal being transduced through CD40.

In one embodiment of the present invention directed to $CD40^-$ malignancies, transformation of the malignant cells with a nucleic acid encoding CD40 causes the transformed cells to be more readily recognized by T cells. Endogenous T cells will interact with such cells, to initiate a more effective response to eliminate the malignant cells. These $CD40^-$ malignant cells

may be transformed using standard techniques, either *in vitro* or *in situ.*

The anti-CD40+ malignancy reagents of the present invention are tested for efficacy by determining their ability to inhibit proliferation of malignant CD40+ cells *in vitro.* For example, the cells are cultured in flat bottomed 96-well tissue culture plates (3072, Falcon Labware) at various cell densities in cRPMI medium. Additional growth stimulants such as growth factors may be added.

Proliferation is evaluated, *e.g.*, via a 4 hour pulse of ^{3}H-thymidine (Amersham, Arlington Heights IL) added at 48 hours after culture initiation. Proliferation is said to be "inhibited" by a reagent if cells grown in the presence of the agent incorporate, *e.g.*, about 20% less ^{3}H-thymidine than cells grown in the absence of the agent, and preferably about 30% less ^{3}H-thymidine than cells not treated with the reagent.

Contacting cells *in vitro* using the reagents of this invention also provides a system which can be used for the rational design of other agents that can be used to inhibit CD40+ malignant cell proliferation. The anti-CD40+ malignancy reagents of the invention can also be tested *in vivo* by using mouse models of CD40+ malignancies. This provides a basis for predicting clinical efficacy.

Soluble CD40 Molecules

Soluble CD40 (sCD40) molecules can be prepared by expressing a CD40 cDNA that has been modified to substantially delete the coding region for a transmembrane domain. Murine and human [Stamenkovic *et al., EMBO J.* *8*:1403 (1989)] CD40 cDNAs have previously been cloned.

Techniques for nucleic acid manipulation, such as are used to modify CD40 cDNA coding sequences to delete a transmembrane domain and to express resulting soluble CD40

molecules, are described generally in, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2nd ed.), Vols. 1-3, Cold Spring Harbor Laboratory, and Ausubel *et al., Current Protocols in Molecular Biology*, 1987 and periodic supplements, Greene/Wiley, New York.

Other useful techniques such as subcloning nucleic acid sequences encoding polypeptides into expression vectors, labelling probes, DNA hybridization, and so on are also described in, *e.g.*, Sambrook *et al.* Truncation of CD40 cDNA may be performed, for example, by oligonucleotide-directed deletion mutagenesis. Oligonucleotide-directed *in vitro* mutagenesis is described, for example, by Kunkel *et al.* (1987) *Meth. Enzymol.* 154: 367-382. *See also, Current Protocols in Molecular Biology*, Ausubel *et al.*, eds., Greene Publishing and Wiley-Interscience, New York (1987 and periodic supplements).

The nucleotide sequences used to transfect the host cells can be modified according to standard techniques to yield CD40 molecules with a variety of desired properties. The CD40 molecules of the present invention can be readily designed and manufactured utilizing various recombinant DNA techniques. Many techniques are well known to those skilled in the art, and are provided in the cited references.

For example, the CD40 molecules can vary from the naturally-occurring sequence at the primary structure level by amino acid insertions, substitutions, deletions, and the like. Protein fusions may also be utilized that may confer new activities or combination activities with the CD40 ligand. These modifications can be used in a number of combinations to produce the final modified protein chain.

The amino acid sequence variants can be prepared with various objectives in mind, including facilitating purification and preparation of the recombinant polypeptide.

The modified polypeptides are also useful for modifying therapeutic half life, improving therapeutic efficacy, and lessening the severity or occurrence of side effects during therapeutic use. The amino acid sequence variants are usually predetermined variants not found in nature but exhibit similar proliferation-inhibiting activity as native-sequence CD40. For instance, polypeptide fragments comprising only a portion (usually at least about 60-80%, typically 90-95%) of the primary structure can be produced. Alternatively, synthetic methods can be used to prepare peptides. *See, e.g.*, Merrifield (1986) *Science* 232: 341-347; Atherton *et al.*, *Solid Phase Peptide Synthesis: A Practical Approach*, IRL Press, Oxford).

In general, modifications of the sequences encoding the CD40 molecule can be readily accomplished by a variety of well-known techniques, such as site-directed mutagenesis [*see*, Gillman *et al.*, *Gene 8*: 81 (1979), and Roberts *et al.*, *Nature 328*: 731 (1987)]. Most modifications are evaluated by routine screening in a suitable assay for the desired characteristic.

For instance, the effect of various modifications on the ability of the polypeptide to inhibit $CD40^+$ malignant cell proliferation can be determined using the proliferation assays, described below. Modifications of other properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolysis, or the tendency to aggregate are all assayed according to standard techniques.

The term "substantially identical", when referring to polypeptides, indicates that the polypeptide or protein in question is at least about 70% identical to an entire naturally occurring protein or a portion thereof, typically at least about 80%, more typically at least about 90%, and preferably at least about 95% identical.

When referring to nucleic acids, the term "substantially identical" indicates that the sequences of two nucleic acids, or subsequences thereof, when optimally aligned and compared, are identical, with appropriate nucleotide insertions or deletions, in at least about 80% of the nucleotides, usually at least about 90% to 95%, and more preferably at least about 98 to 99.5% of the nucleotides. Alternatively, substantial nucleic acid sequence identity exists when a nucleic acid segment will hybridize under selective hybridization conditions, to a complement of another nucleic acid strand.

To express the soluble CD40 molecules from the modified coding regions, the truncated nucleic acids can be operably linked to signals that direct gene expression. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame.

The gene encoding the soluble CD40 molecule can be inserted into an "expression vector", "cloning vector", or "vector," terms which usually refer to plasmids or other nucleic acid molecules that are able to replicate in a chosen host cell. Expression vectors can replicate autonomously, or they can replicate by being inserted into the genome of the host cell, by methods well known in the art. Vectors that replicate autonomously will normally have an origin of replication or autonomous replicating sequence (ARS) that is functional in the chosen host cell(s). Often, it is desirable for a vector to be usable in more than one host cell, *e.g.*, in *E. coli* for cloning and construction, and in a mammalian cell for expression.

The particular vector used to transport the genetic information into the cell is also not particularly critical. Conventional vectors used for expression of recombinant proteins in prokaryotic or eukaryotic cells can be used.

The expression vectors typically have a transcription unit or expression cassette that contains all the elements required for the expression of the DNA encoding the soluble CD40 molecule in the host cells. A typical expression cassette contains a promoter operably linked to the DNA sequence encoding a soluble CD40 molecule, and signals required for efficient polyadenylation of the transcript. The promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

The DNA sequence encoding the soluble CD40 molecule can be linked to a cleavable signal peptide sequence to promote secretion of the encoded protein by the transformed cell. Additional elements of the cassette can include selectable markers, enhancers, and, if genomic DNA is used as the structural gene, introns with functional splice donor and acceptor sites.

Enhancer elements can stimulate transcription up to 1,000 fold from linked homologous or heterologous promoters. Many enhancer elements derived from viruses have a broad host range and are active in a variety of tissues. For example, the SV40 early gene enhancer is suitable for many cell types. Other enhancer/promoter combinations that are suitable for the present invention include those derived from polyoma virus, human or murine cytomegalovirus, the long terminal repeat from various retroviruses such as murine leukemia virus, murine or Rous sarcoma virus, and HIV. *See, Enhancers*

and Eukaryotic Expression, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. 1983.

In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region can be obtained from the same gene as the promoter sequence or can be obtained from a different gene.

For more efficient translation of the mRNA encoded by the structural gene, polyadenylation sequences are also commonly added to the vector construct. Two distinct sequence elements are required for accurate and efficient polyadenylation: GU or U rich sequences located downstream from the polyadenylation site and a highly conserved sequence of six nucleotides, AAUAAA, located 11-30 nucleotides upstream. Termination and polyadenylation signals that are suitable for the present invention include those derived from SV40, or a partial genomic copy of a gene already resident on the expression vector.

The vectors containing the gene encoding the soluble CD40 molecule are transformed into host cells for expression. "Transformation" refers to the introduction of vectors containing the nucleic acids of interest directly into host cells, e.g., by well known methods. The particular procedure used to introduce the genetic material into the host cell for expression of the soluble CD40 molecule is not particularly critical. Any of the well known procedures for introducing foreign nucleotide sequences into host cells can be used. It is only necessary that the particular procedure utilized be capable of successfully introducing at least one gene into the host cell which is capable of expressing the gene.

Transformation methods, which vary depending on the type of host cell, include electroporation; transfection employing calcium chloride, rubidium chloride calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; infection (where the vector is an infectious agent); and other methods. *See, generally*, Sambrook *et al.*, (1989) *supra*, and *Current Protocols in Molecular Biology, supra*. Reference to cells into which the nucleic acids described above have been introduced is meant to also include the progeny of such cells.

Mammalian cell lines are often used as host cells for the expression of polypeptides derived from eukaryotes, such as the soluble CD40 molecules. Propagation of mammalian cells in culture is *per se* well known. *See, Tissue Culture*, Academic Press, Kruse and Patterson, ed. (1973). Host cell lines may also include such organisms as bacteria (*e.g., E. coli* or *B. subtilis*), yeast, filamentous fungi, plant cells, or insect cells, among others.

After standard transfection or transformation methods are used to produce prokaryotic, mammalian, yeast, or insect cell lines that express large quantities of the soluble CD40 molecule, the molecule is then purified using standard techniques. *See, e.g.*, Colley *et al., J. Biol. Chem. 64*:17619 (1989) ; and *Methods in Enzymology*, "Guide to Protein Purification", M. Deutscher, ed. Vol. 182 (1990). The recombinant cells are grown and the soluble CD40 molecule is expressed, after which the culture medium is harvested for purification of the secreted protein.

The medium is typically clarified by centrifugation or filtration to remove cells and cell debris and the proteins are concentrated by adsorption to any suitable resin such as, for example, CDP-Sepharose, Asialoprothrombin-Sepharose 4B, or Q Sepharose, or by use of ammonium sulfate fractionation,

polyethylene glycol precipitation, or by ultrafiltration. Other means known in the art are equally suitable.

Further purification of the soluble CD40 molecule can be accomplished by standard techniques, for example, affinity chromatography, ion exchange chromatography, sizing chromatography, or other protein purification techniques used to obtain homogeneity. The purified proteins are then used to produce pharmaceutical compositions, as described below.

Alternatively, vectors can be employed that express the soluble CD40 molecule intracellularly, rather than secreting the protein from the cells. In these cases, the cells are harvested, disrupted, and the soluble CD40 molecule is purified from the cellular extract, *e.g.*, by standard methods.

As used herein, the terms "isolated", "substantially pure" and "substantially homogeneous" are used to describe a protein that has been separated from components which naturally accompany it. Typically, a monomeric protein is substantially pure when at least about 60 to 75% of a sample exhibits a single polypeptide backbone. Minor variants or chemical modifications typically share the same or similar polypeptide sequence.

A substantially purified protein will typically comprise over about 85 to 90% of a protein sample, more usually about 95%, and preferably will be over about 99% pure, either by mass or by molecular numbers. Protein purity or homogeneity can be indicated by a number of means well known in the art, such as polyacrylamide gel electrophoresis of a protein sample, followed by visualizing a single polypeptide band on a polyacrylamide gel upon staining. For certain purposes high resolution will be needed and HPLC or a similar means utilized for purification.

B Cell CD40 Ligand

Evidence that B cells express a ligand or counterstructure for CD40 is provided by the ability of mouse L cells that have been transfected with murine CD40 to augment the anti-IgM-induced proliferative response of highly purified small dense murine B cells, even when added to responder cells cultured at cell densities as low as 10^3 cells/well. Untransfected cells do not augment B cell proliferation.

Similarly, highly purified preparations of the soluble murine CD40 extracellular domain (sCD40) augment the proliferative response of highly purified small dense murine B cells induced by sub-optimal concentrations of human B cell growth factor (BCGF). Soluble CD40 inhibits anti-CD40 antibody-induced proliferation of B cells.

The soluble murine CD40 extracellular domain specifically binds to the murine B lymphoma A.20, demonstrating that a CD40 ligand is present on the surfaces of the B lymphoma cells. This binding of soluble CD40 to the B lymphoma cells profoundly inhibits the *in vitro* growth of the lymphoma cells.

That A.20 B cells do not express the CD40 ligand expressed by activated T cells (Armitage *et al., supra*) has been indicated by PCR analysis. This was confirmed by demonstrating that a monoclonal antibody specific for the CD40 ligand expressed by activated T cells does not bind to A.20 cells. These data demonstrate that B lineage cells lack the CD40 ligand expressed by activated T cells, and instead express a different ligand that specifically interacts with CD40.

This B cell CD40 ligand can be characterized and produced using recombinant DNA methodology as described in the Example below, and purified by standard methods using the assay described.

For use as a pharmaceutical composition, the B cell CD40 ligand will most preferably be converted to a soluble form by using analogous methods to those used to produce the soluble CD40 molecule. First, nucleic acid sequences, *e.g.*, preferably a cDNA, that code for the ligand are isolated by standard methods. Thus, this aspect of the invention relies on routine techniques in the field of recombinant genetics, well known to those of ordinary skill in the art. A basic reference disclosing the general methods of use in this invention is Sambrook *et al.*, *supra*.

Steps useful to clone a cDNA or genomic DNA coding for the B cell CD40 ligand and express soluble molecules suitable for the invention are described below, and include methods well known to one of skill in the art. In summary, the manipulations necessary for one method for preparing nucleic acid segments encoding the ligand involve 1) purifying the B cell CD40 ligand from the appropriate sources, 2) preparing degenerate oligonucleotide probes corresponding to a portion of the amino acid sequence of the purified protein, and 3) screening a cDNA or genomic library for the sequences which hybridize to the probes. Alternative methods for obtaining a nucleic acid encoding the B cell CD40 ligand, such as panning, are described below. Various expression cloning methods can be applied to B cell cDNA libraries, e.g., using a soluble CD40 as a labeled marker.

Methods for purifying desired proteins are well known in the art and can be applied using the assay described herein to monitor purification. For a review of standard techniques see, *Methods in Enzymology*, "Guide to Protein Purification", *supra*. For instance, the B cell CD40 ligand can be purified

using affinity chromatography, SDS-PAGE, and the like. Affinity chromatography using immobilized CD40 can be used to isolate the ligand from a preparation of B cell proteins. Soluble CD40 can be immobilized on a solid support using various methods, e.g., biotin-streptavidin coupling. The B cell protein preparation is applied to the column, which is then washed. Bound proteins are then eluted using high salt, low or high pH, or other techniques. Affinity chromatography techniques are described in, for example, *Practical Immunology* (Hudson & Hay, eds., Blackwell).

After isolation of the ligand, the amino acid sequence of, e.g., the N-terminus, is determined and degenerate oligonucleotide probes, designed to hybridize to the desired gene, are synthesized. Amino acid sequencing is performed and oligonucleotide probes are synthesized according to standard techniques as described, for instance, in Sambrook *et al., supra.*

Oligonucleotide probes useful for identification of desired genes can also be prepared from conserved regions of related genes in other species. For instance, probes derived from a gene encoding a B cell CD40 ligand from one species may be used to screen libraries for the gene encoding the ligand from a different species. Thus, B cell CD40 ligand can be isolated from other species, e.g., mouse and human.

Genomic or cDNA libraries are prepared according to standard techniques as described, for instance, in Sambrook, *supra.* To construct genomic libraries, large segments of genomic DNA are generated by random fragmentation and are ligated with vector DNA to form concatemers that can be packaged into the appropriate vector. Two kinds of vectors are commonly used for this purpose, bacteriophage lambda vectors and cosmids.

To prepare cDNA, mRNA from appropriate cells, *e.g.*, B cells or B lymphoma cells is first isolated. Eukaryotic mRNA has at its 3' end a string of adenine nucleotide residues known as the poly-A tail. Short chains of oligo d-T nucleotides are then hybridized with the poly-A tails and serve as a primer for the enzyme reverse transcriptase. This enzyme uses RNA as a template to synthesize a complementary DNA (cDNA) strand. A second DNA strand is then synthesized using the first cDNA strand as a template. The double-stranded cDNA is inserted into a plasmid or λ phage vector for propagation in *E. coli* by means of linkers, homopolymer tailing, or another method.

Identification of clones in either genomic or cDNA libraries harboring the desired nucleic acid segments is performed by either nucleic acid hybridization, or by immunological detection of the encoded protein if an expression vector is used. The bacterial colonies are then replica plated on solid support, such as nitrocellulose filters. The cells are lysed and probed with either oligonucleotide probes described above or with antibodies to the desired protein.

An alternative method for identifying a cDNA encoding the B cell CD40 ligand is a panning technique. For example, soluble CD40 may be bound to the wells of petri dishes. Transfected COS cells containing a B cell cDNA library in an expression vector are placed in the wells, and those cells expressing a B cell CD40 ligand cDNA bind to the immobilized CD40. The unbound cells, which do not possess the ligand on their surface and therefore do not bind, are removed by washing. The cDNA insert is isolated from cells which, by virtue of their surface ligand, bind to the dishes.

Another method for isolating the ligand is by sorting. An expression library is labeled, e.g., with soluble CD40, and sorted to select cells expressing a ligand for the soluble CD40.

Other methods well known to those skilled in the art can also be used to identify or characterize desired genes. For example, the presence of restriction fragment length polymorphisms (RFLP) between wild type and mutant strains lacking the B cell CD40 ligand can be used. Amplification techniques, such as the polymerase chain reaction (PCR) can be used to amplify the desired nucleotide sequence. U.S. Patents Nos. 4,683,195 and 4,683,202 describe this method. See also Innis *et al.*, eds. (1990) *PCR Protocols: A Guide to Methods and Applications*, Academic Press, NY). Sequences amplified by PCR can be purified from agarose gels and cloned into an appropriate vector according to standard techniques. Appropriate primer sequences can be selected from CD40 coding sequences, or those flanking these sequences.

After isolating a nucleic acid encoding the B cell CD40 ligand, a soluble form of the ligand can then be produced. Analogous techniques to those used to express the soluble CD40 molecule are used to construct a coding sequence and express a soluble form of the B cell CD40 ligand. The ligand molecules are then purified and used to produce pharmaceutical compositions, as described below.

The B cell CD40 ligand can also be immobilized on a solid support and used to isolate the soluble form of CD40 by affinity chromatography. Soluble forms of the ligand can also be labeled by standard methods, e.g., with a radioactive or fluorescent molecule, and used to quantify CD40 on cells. The B cell CD40 ligand can also be used as an immunogen, to make antibodies useful in inhibiting the proliferation of B lymphoma cells and other $CD40^+$ malignant cells.

Antibodies against CD40 or B Cell CD40 Ligand

Antibodies against either CD40 or the CD40 ligand present on B lymphoma cells are also useful for treating $CD40^+$ malignancies, e.g., B cell lymphomas, leukemias and

carcinomas. Thus, the multitude of techniques available to those skilled in the art for production and manipulation of various immunoglobulin molecules are suitable for antibodies of the present invention. See, e.g., Harlow and Lane, *Monoclonal Antibodies: A Laboratory Manual*, 1991, Cold Spring Harbor Press, Cold Spring Harbor, NY. For a discussion of immunoglobulin forms, *see, e.g., Fundamental Immunology, supra.*

Antibodies that bind to CD40 or the B cell CD40 ligand can be produced by a variety of means. The production of non-human monoclonal antibodies, *e.g.*, murine, lagomorph, equine, etc., can be accomplished by, for example, immunizing the animal with a preparation containing isolated CD40 or ligand or recombinantly produced polypeptides comprising at least a portion of the full length CD40 molecule or B cell CD40 ligand.

Antibody-producing cells obtained from the immunized animals are immortalized and screened, or screened first for the production of antibody which binds to CD40 or the B cell CD40 ligand, and then immortalized. For a discussion of general procedures of monoclonal antibody production see Harlow and Lane, *Antibodies: A Laboratory Manual,* Cold Spring Harbor Pubs., N.Y. (1988); Coligan et al. (1991 and periodic supplements) *Current Protocols in Immunology,* Greene Publ., NY.

Immunoglobulins from polyclonal serum can be produced by methods well-known to those skilled in the art. An example is chromatographic purification of polyclonal sera to produce substantially monospecific antibody populations.

In addition, fragments of the antibody molecule can also be used such as $F(ab')_2$ or Fab fragments. Methods for the production of these fragments are well known and are described for instance in Harlow and Lane, *supra.*

The invention also provides synthetic or recombinant immunoglobulins, including chimeric immunoglobulins and humanized antibodies. Chimeric immunoglobulins are typically the product of chimeric DNA, which is recombinant DNA containing genetic material from more than one mammalian species. Chimeric antibodies or peptides are typically produced using recombinant molecular and/or cellular techniques.

Typically, chimeric antibodies have variable regions of both light and heavy chains that mimic the variable regions of antibodies derived from one mammalian species, while the constant portions are homologous to the sequences in antibodies derived from a second mammalian species. Methods for production of such antibodies are well known and are described in, for example, U.S. 4,816,397, EP publications 173,494 and 239,400.

In addition, "humanized" immunoglobulins comprising a human-like framework region and a constant region that is substantially homologous to a human immunoglobulin constant region can also be used. Hence, most parts of a human-like immunoglobulin, except possibly the CDRs are substantially homologous to corresponding parts of one or more native human immunoglobulin sequences. Production of humanized immunoglobulins are described in PCT Publication No. WO/90/07861 and Reichmann *et al.*, *Nature 332*:323 (1988).

Compositions and Methods for Treating CD40$^+$ Malignancies

The present invention also includes compositions and methods for inhibiting the growth of CD40$^+$ malignant cells, including B lymphoma cells, leukemias and carcinomas. The compositions and methods are also useful for augmenting T cell proliferation and T cell-mediated cytotoxicity against malignancies expressing CD40. The methods may be used to

treat these diseases in patients, *e.g.*, animals possessing an immune system, typically mammals, and preferably domestic animals or humans.

The methods involve treating a patient with a pharmaceutical composition that contains a reagent that inhibits the growth of $CD40^+$ malignant cells. Such reagents include the soluble CD40 molecules, anti-CD40 antibodies, antibodies against B cell CD40 ligand and, for gene therapy, nucleic acids encoding the reagents.

Soluble B cell CD40 ligand molecules that (except for substantially lacking a transmembrane domain) are similar to those CD40 ligands present on B lymphoma cell surfaces may also be useful therapeutically.

For pharmaceutical compositions, the reagents that inhibit proliferation of $CD40^+$ malignant cells as described herein are administered to an individual having a $CD40^+$ malignancy. Compositions containing the anti-$CD40^+$ malignancy reagents are administered to a patient in an amount sufficient to effectively block signalling between CD40 and the CD40 ligand, e.g., present on the surface of malignant cells, and thereby completely or partially arrest proliferation and its symptoms and/or complications. An amount adequate to accomplish this is defined as "therapeutically effective dose."

Amounts effective for this use will depend on, *e.g.*, the nature of the anti-malignancy composition, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician, but will generally range from about 0.01 mg/kg to about 100.0 mg/kg of anti-malignancy reagent per day, with dosages of from about 0.1 mg/kg to about 10.0 mg/kg of anti-malignancy reagent per day being more commonly used.

It must be kept in mind that the anti-malignancy reagents and compositions derived therefrom may be employed in serious disease states, that is, life-threatening or potentially life threatening situations. In such cases, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these compositions. Thus, where the reagent is an antibody, human monoclonal antibodies or substantially human antibodies are most preferred under these circumstances. These reagents can also be administered in combination with other reagents, *e.g.*, growth factors, cytokines, or other agonists or antagonists.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of anti-malignancy reagent of the invention sufficient to effectively treat the patient. Administration should begin at the first indication of a $CD40^+$ malignancy or shortly after diagnosis, and continue until symptoms are substantially abated and for a period thereafter. In well established cases of disease, loading doses followed by maintenance doses will be required.

The pharmaceutical compositions for therapeutic treatment are intended for parenteral, topical, oral, or local administration. Preferably, the pharmaceutical compositions are administered parenterally, *e.g.*, intravenously, subcutaneously, intradermally, or intramuscularly. Thus, the invention provides compositions for parenteral administration which comprise a solution of the anti-malignancy reagent dissolved or suspended in an acceptable carrier, preferably an aqueous carrier.

A variety of aqueous carriers can be used, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid, and the like. These compositions can be sterilized by conventional, well known sterilization techniques, or can be

sterile filtered. The resulting aqueous solutions can be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration.

The compositions can contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

The concentration of anti-malignancy reagent of the invention in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 1%, usually at or at least about 10-15% to as much as 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

Thus, a typical pharmaceutical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution and the appropriate amount of anti-malignancy reagent. Actual methods for preparing parenterally administrable compounds will be known or apparent to those skilled in the art and are described in more detail in, for example, *Remington's Pharmaceutical Science*, 18th ed., Mack Publishing Company, Easton, PA (1990); and *The Pharmaceutical Bases of Therapeutics*, Goodman *et al.*, eds. Permagon Press, NY).

The anti-malignancy reagents can also be administered via liposomes. The reagents can serve to target the liposomes to particular tissues or cells displaying the CD40 molecule or its ligand. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions,

lamellar layers, and the like. In these preparations the reagent to be delivered is incorporated as part of the liposome, alone or in conjunction with a molecule which is, for example, toxic to the target cells. A liposome suspension containing an reagent can be administered intravenously, locally, topically, etc. in a dose which varies according to, for example, the manner of administration, the peptide being delivered, and the stage of disease being treated.

For solid compositions of the anti-CD40$^+$ malignancy reagents of the invention, conventional nontoxic solid carriers can be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more anti-malignancy reagents, and more preferably at a concentration of 25%-75%.

Compositions and Methods for Treating CD40$^-$ Malignancies

The compositions and methods of this invention are also useful for augmenting T cell proliferation and T cell-mediated cytotoxicity against CD40$^-$ malignancies. These malignancies survive, in part, due to the low efficiency of T cell recognition and response to these cells. Were these undesirable cells to express CD40, i.e., to become CD40$^+$, T cells would better assist in mounting an appropriate and efficient immune response to eliminate the malignancy.

CD40$^-$ malignancies can be made to express CD40 by transforming the cells. Typically, this involves genetic transformation of the undesirable cells with CD40-encoding nucleic acids but may include CD40$^+$ cell or liposome fusions. The malignant cells, thus becoming CD40$^+$, are then exposed

to T cells *in vitro* or *in situ*, thereby stimulating the T cells and promoting the desired immune response. In particular, ballistic transformation may be valuable for *in situ* transformation.

Decreasing Immune Responses

Under some circumstances, the immune system can mount an inappropriate, excessive response through T cell-mediated mechanisms, e.g., as in the case of autoimmune diseases, delayed-type hypersensitivity reactions and transplantation graft-versus-host disease. These conditions often involve a signal utilizing CD40 which could be blocked using an appropriate reagent of this invention. For example, transplantation graft-versus-host disease can be treated by administering a composition which blocks the normal CD40 interaction, thereby minimizing a T cell-mediated response.

EXAMPLES

The present invention can be illustrated by the following, non-limiting Examples. Unless otherwise specified, percentages given below for solids in solid mixtures, liquids in liquids, and solids in liquids are on a wt/wt, vol/vol and wt/vol basis, respectively. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Sterile conditions were maintained during cell culture.

Example I: Preparation of Soluble Murine CD40

Materials and Methods

Construction of Soluble CD40 Gene

To construct a cDNA encoding the extracellular domain of CD40 (designated "soluble CD40"), polymerase chain reaction (PCR) was used to amplify the region of the mouse CD40 cDNA encoding the first 191 amino acids. The following oligodeoxynucleotide primers containing an *Xho*I site in both primers and a termination codon in the antisense primer were synthesized on an Applied Biosystems 380A DNA synthesizer (Foster City, CA) and used for the PCR amplification:

Sense: 5'-ACAGCTCGAGCCATGGTGTCTTTGCCTCGGCTGTG-3' (SEQ ID NO:1)

Antisense: 5'-GTAGCTCGAGCTCACCGGGACTTTAAACCACAGATG-3' (SEQ ID NO:2)

The amplified fragment was digested with *Xho*I and ligated into a *Xho*I-cleaved mammalian/bacterial expression vector, *e.g.*, pME18S. The cloned fragment was sequenced using the dideoxy method to confirm the sequence.

Expression of Soluble CD40 in COS7 Cells

Plasmids carrying soluble CD40 cDNA were transfected into COS7 cells by electroporation, as described by Galizzi *et al.* [*Int. Immunol.* 2: 669 (1990)]. Briefly, 0.75 ml of COS7 cell suspension in serum-free Dulbecco's Minimal Essential (DME) medium (Gibco/BRL Catalog, Gaithersburg, MD) (10^7 cells/ml) were incubated with 50 µl of plasmid solution (20 µg plasmid) at room temperature for ten minutes. The mixture were then electroporated using a Bio-Rad gene pulser (Richmond, CA) at 960 µF and 220 volts. Ten minutes after electroporation, the COS7 cells were cultured for three days in four 10 cm dishes. For the purification of soluble CD40, the medium was changed, one day after electroporation, to RPMI 1640 supplemented with HB101 (HANA Biologics, Alameda, CA) that lacks phenol red.

Soluble CD40 was purified by anion exchange chromatography using standard procedures. Linear NaCl gradients were used to elute the soluble CD40 from the column. The eluted protein was then analyzed by Western blotting using rabbit anti-CD40 antiserum.

Metabolic Labeling of Soluble CD40

COS7 cells ($3x10^6$ cells) were transfected with 20 µg plasmid containing the soluble CD40 cDNA, and cultured in two 10 cm dishes. After two days of culture, dishes were washed twice with serum-free RPMI 1640 and incubated for 15 minutes with 5 ml of serum-free metabolic labeling medium (methionine-free RPMI 1640) at 37°C. Fifty µl (0.5 mCi) of ^{35}S-cysteine and 80 µl (0.8 mCi) of ^{35}S-methionine were then added to the dishes. Supernatants were collected after a 16-18 hour incubation and analyzed by Tricine-based SDS polyacrylamide gel electrophoresis (Novex, San Diego, CA). Gels were dried and subjected to autoradiography at -70°C.

Western Blotting

Soluble CD40 was purified by ion exchange chromatography on anion exchange columns using standard procedures. The protein was eluted from the column using a linear NaCl gradient and analyzed by Western blotting using a rabbit antiserum against a CD40 peptide. The antiserum was prepared by standard methods. *See, e.g.*, Ausubel (1987 and Supplements) *supra.*, and Sambrook *et al.*, *supra.*

Immunofluorescence

Cells were washed and incubated for 30 minutes on ice at 2 x 10^5 cells/well in 50 µg/ml rat anti-(mouse CD40) antiserum, or a normal rat serum control, diluted 1/400 in Hank's buffered saline solution with 3% BSA and 0.01% sodium azide (HBSS-BSA). Cells were washed three times with HBSS-BSA and incubated with a 1/250 dilution of goat anti-rat

IgG Kirkegard & Perry Labs, Gaithersburg, MD). Following three further washes, cells were analyzed using a FACScan analyzer (Becton Dickinson, Milpitas CA). Dead and aggregated cells were excluded on the basis of forward and side angle light scatter, or by propidium iodide uptake.

Results

A cDNA clone encoding murine CD40 was isolated by cross-hybridization using human CD40 probes and a library prepared from lipopolysaccharide (LPS)-stimulated murine B cells. This cDNA encodes a putative transmembrane protein consisting of a 172 amino acid extracellular domain, a 22 amino acid transmembrane domain, and a 99 amino acid intracellular domain. A truncated cDNA encoding the extracellular domain of murine CD40 was produced by PCR, using primers corresponding to nucleotide sequences 8-31 and 559-581 of the mouse CD40 cDNA.

COS7 cells were transfected with an expression vector for expression of the truncated CD40 cDNA. Metabolic labeling of the transfected revealed a specific and predominant band of approximately 26 Kd, consistent with the predicted molecular weight of a 172 amino acid extracellular domain following glycosylation.

The soluble CD40 extracellular domain expressed by COS7 cells was further visualized by Western blotting using a rabbit anti-CD40 peptide antiserum raised against an amino acid N-terminal peptide derived from the predicted amino acid sequence of recombinant CD40. Using this assay, soluble CD40 was purified to high specific activity and purity from supernatants of transfected COS7 cells using two rounds of ion-exchange chromatography.

The soluble CD40 cDNA was also expressed in baculovirus. Polymerase chain reaction (PCR) was used to construct a gene encoding soluble mouse CD40. A first primer was designed to introduce a *Bam*HI restriction site immediately 5' to the ATG start codon of the mouse CD40 open reading frame. The second primer introduced an arginine codon, followed by six histidine codons, two stop codons, and an *Xba*I restriction site immediately 3' of the DNA sequence that encodes the mouse CD40 extracellular domain.

After amplification by PCR, the resultant fragment was cloned into an expression vector. The resulting vector was transfected into *Spodoptera frugiperda* (Sf-9) insect cells along with wild-type *Autographa californica* nuclear polyhedron virus, using the protocol of Summers *et al.* (1987) "A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures," *Tex. Agric. Exp. Stn. Bull.*, Coll. Stn. no. 1555). Recombinant virus was purified by a plaque assay as described by Summers *et al., supra.* See also O'Reilly, Baculovirus Expression Vectors: A Laboratory Manual, 1992, Freeman & Company.

Soluble CD40 protein (mCD40E-H6) was produced in one liter spinner flasks by infection of Sf-9 cells with recombinant virus supernatants in serum-free medium (ExCell 401, JRH Scientific), followed by incubation for 3-4 days at 27°C. Supernatants were separated from the cells by centrifugation and sterile filtered.

Approximately 99.5% pure mCD40E-H6 protein was obtained by virtue of the affinity of the engineered histidine tail to a metal chelating resin charged with Ni^{2+}. After extensive washing, pure mCD40E-H6 was eluted using either a step gradient of 250mM imidizole (pH 7.5) or a continuous gradient of 0-300 mM imidizole (pH 7.5). Purity was determined by SDS-polyacrylamide gel electrophoresis,

followed by silver staining. Protein content was determined using the Bradford assay.

Example II: Production of a Specific Anti-murine CD40 Antiserum

Materials and Methods

Animals and Cell Lines

Eight-week-old female Lewis rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Eight week old female BALB/c mice were obtained from Simonsen Laboratories (Gilroy, CA) and used at 8-12 weeks. The A.20 murine B lymphoma was obtained from the American Tissue Culture Collection (ATCC Accession No. TIB208, Rockville, MD).

Hybridoma Production

Rats were immunized intraperitoneally with 10 μg of soluble CD40 in complete Freund's adjuvant, followed by boosts of 10, 10, 10, and 50 μg of soluble CD40 in incomplete Freund's adjuvant at 3, 4.5, 6, and 8.5 weeks, respectively. A final boost in saline was injected at 12 weeks. Test bleeds were evaluated for anti-CD40 antibody content by enzyme-linked immunosorbent assay (ELISA).

Hybridomas producing monoclonal rat anti-(mouse CD40) antibodies were then prepared by usual methods (see, *e.g., Antibodies: A Laboratory Manual, supra*). Antibodies were purified using standard methods.

Preparation of Stable Transfectants Expressing Murine CD40

Stable transfectants expressing murine CD40 were prepared by co-transfecting mouse L cells with two plasmids. One plasmid contained the CD40 cDNA (pMe18S-FLCD40), and the other contained a neomycin resistance gene (pME18S-neo).

The plasmids were added in a 5:1 ratio at a final concentration of 20 μg/ml.

Cells were transfected by plating 10^6 L cells into a 10 cm petri dish in 10 ml of Dulbecco's Minimal Essential Medium (DMEM) containing penicillin/streptomycin, 1X glutamine, 1X sodium pyruvate, and 10% fetal calf serum (FCS). Cells were transfected 24 hours after plating, with fresh medium having been added 2 hours before transfection.

The calcium phosphate precipitation protocol as described in *Current Protocols in Molecular Biology, supra*, was used for transfection.

Cells containing the neomycin resistance gene were selected by growing cells in medium containing G418 for two to three weeks. Neomycin-resistant cells were analyzed for CD40 expression. $CD40^+$ cells were sorted using a fluorescence-activated cell sorter (FACS). After three rounds of growing and sorting, stable CD40-transfected cell lines were cloned, and the clone expressing the highest amount of CD40 was used for further analysis.

B Cell Proliferation Assays

Small dense B cells from unstimulated mouse spleens were prepared as described previously [Umland *et al.*, *J. Immunol. 142*:1528 (1989)]. Briefly, spleens were teased into complete RPMI (cRPMI) containing 5% FCS (J.R. Scientific, Woodland, CA), 5 x 10^{-5} M 2-mercaptoethanol (Polysciences, Inc,, Warrington, PA), 2 mM glutamine (J.R. Scientific) and 25 mM HEPES buffer (Irvine Scientific, Santa Ana, CA), 100 U/ml penicillin and 100 μg/ml streptomycin (Irvine).

Red blood cells were lysed using 0.83% ammonium chloride, pH 7.4. T cells were removed using two successive treatments with anti-mouse Thy 1.2 mAb (New England Nuclear, Boston, MA) and anti-L3T4 antibody (RL172.4

hybridoma, a kind gift from Dr. H.R. MacDonald, Ludwig Institute, Epalinges, Switzerland) for 20 minutes on ice followed by complement (1:10 dilution of rabbit low-toxicity complement, Cedarlane Laboratory, Ontario, Canada) for 30 minutes at 37° C.

Small dense B cells were then isolated by density gradient centrifugation using a discontinuous gradient composed of 75%, 65%, and 50% PERCOLL (Pharmacia Fine Chemicals, Uppsala, Sweden) at 2,500 x g for 25 minutes at 4°C. Cells collected from the interface between 65% and 75% percoll were shown to be small dense B cells by a variety of criteria, including staining for B220 (specific for B cells), MAC-1 (macrophages/granulocytes, surface IgM (B cells), and Lyt2 and L3T4 (T cells) and were used in subsequent experiments. Large *in vivo* activated B cells collected from the 65% and 50% interface were used in some experiments.

B cells were cultured in flat bottomed 96-well tissue culture plates (3072, Falcon Labware) at various cell densities in cRPMI medium. Additional growth stimulants were added where indicated. Proliferation was evaluated via a 4 hour pulse of ^{3}H-thymidine (Amersham, Arlington Heights, IL) added at 48 hours after culture initiation.

Results

Lewis rats were immunized with several preparations of soluble CD40 in adjuvant, as described in Materials and Methods. Antisera were tested for strong specific binding in an ELISA utilizing microtiter plates coated with highly purified recombinant soluble CD40. Anti-CD40 antiserum prepared in this way bound L cells that had been stably transfected with the full-length murine CD40 cDNA, but did not bind untransfected L cells. Control normal rat serum did not bind transfected L cells.

The specificity of the anti-CD40 antiserum was demonstrated by the specific inhibition of its binding to L cell transfectants expressing full-length CD40 by the addition of highly purified recombinant soluble CD40.

The addition of anti-CD40 antiserum to highly purified small dense B cells from unstimulated mouse spleens caused vigorous proliferation of these cells. The antiserum proved to have a high titer, inducing optimal proliferation of 10^5 B cells/well at a final dilution of 1/8000, and with an end-point titer in these same assays of 1/100,000 or higher. Anti-CD40-induced B cell proliferation could be inhibited with 10 μg/ml soluble CD40 even at super-saturating concentrations of anti-CD40 antiserum. Anti-CD40 antiserum induced vigorous proliferation of both small dense murine B cells and large *in vivo* activated murine B cells, and in both cases, the proliferative response was further augmented by the addition of exogenous IL-4.

Example III: Inhibition of B Lymphoma Cell Proliferation by Anti-CD40 Antiserum

Growth of A.20 B lymphoma cells *in vitro* was strongly suppressed by anti-CD40 antiserum. Interestingly, this antiserum potently induces normal B lymphocyte proliferation. Anti-CD40 mediated inhibition of A.20 cell growth could be substantially reversed by prior inclusion of soluble CD40 in the assay, demonstrating that the effect is specific for CD40.

Anti-CD40-induced inhibition of A.20 cell growth was observed over a wide cell dose titration range, and was most prominent when A.20 cells were in log phase growth. The apparent inhibition of A.20 growth did not reflect an anti-CD40 mediated acceleration of lymphoma growth resulting in early exhaustion of the cultures, since kinetic analyses showed that anti-CD40 antibodies caused either suppression or no

effect on cell growth at all cell densities and time-points tested.

Anti-CD40 antiserum also suppressed the *in vitro* growth of numerous other B lineage malignancies, *e.g.*, CH31, WEHI 231 (ATCC Accession No. CRL 1702), and 70/Z (ATCC Accession No. TIB 158).

Example IV: Inhibition of B Lymphoma Cell Proliferation by Soluble CD40 Molecules

To evaluate the ability of soluble CD40 molecules to inhibit proliferation of A.20 B lymphoma cells, soluble CD40 was added to cells in tissue culture medium and incubated for two days. The growth of the cells was determined by measuring incorporation of ^{3}H-thymidine as described in, *e.g.*, *Current Protocols in Immunology, supra.* The growth of the A.20 B lymphoma cells was thereby found to be inhibited by the soluble CD40.

Example V: T cell CD40 Ligand is Involved in Stimulating T Cell Proliferation

P815 Transfection

A murine mastocytoma cell line, designated P815, was obtained from the American Type Culture Collection (ATCC, Rockville, MD). An expression vector containing human CD40 cDNA (obtained from R. de Waal, DNAX Research Institute) was transfected into P815 cells by electroporation. Cells expressing CD40 were selected by flow cytometry. P815 cells transfected with an expression vector containing human B7 cDNA, ELAM-1 cDNA, and untransfected P815 cells were used as controls.

Preparation of T Lymphocytes

Human T lymphocytes were prepared essentially as described by Azuma *et al.* [*J. Exp. Med. 175*:353 (1992)].

Proliferation Assay

Proliferation of T cells co-cultured with irradiated P815, $CD40^+$ P815, $B7^+$ P815, and $CD72^+$ P815 cells was measured as described by Azuma *et al., supra.* Briefly, $3x10^4$ cells/well were plated in 96 well microtiter plates. Anti-CD3 mAb was added to the cells. After 30 minutes at room temperature, responder T cells (10^5 cells/well) were added and the cultures were incubated at 37°C with 5% CO_2 in a humidified atmosphere for 72 hours.

Cultures were labelled for the final 18 hours with 1 μCi/well ^3H-thymidine and were harvested on a 96-well plate harvester (LKB Instruments Inc., Gaithersburg, MD). Incorporated radioactivity was measured triplicate determinations, in a beta plate scintillation counter (LKB Instruments Inc.). It was thereby found that proliferation of human T cells was co-stimulated by anti-CD3 mAb and $CD40^+$ P815 cells.

Example VI: Cloning of a B Cell CD40 Ligand

One method for the isolation of a B cell CD40 ligand involves (a) preparation of a soluble CD40 probe, (b) purification of mRNA from B cells, (c) construction of a CDNA library, and (d) screening the library using the soluble CD40 probe as a marker. Some specific cells, plasmids and reagents are suggested below, although others could of course be used instead.

B Cell Lines and Probes

An appropriate B cell lymphoma line, i.e., one which expresses the ligand, can be used as a source of mRNA. Such lines include, e.g., the A20, 70Z and WEHI231 mouse cell lines. The A20 line [Kim *et al., J. Immunol. 122*:549 (1979)] grows very fast and gives consistent staining results.

To identify the CD40 ligand, a labeled soluble CD40 probe is produced, expressed and purified using methods analogous to those described above. One such probe was constructed which consisted of the extracellular domain of mouse CD40 (mCD40) linked to the constant domain (Fc) of human IgG1 (CD40-Fc). Using labeled anti-Fc antibodies for detection, this probe was found to bind to COS7 and L cells transfected with cDNA encoding T cell CD40 ligand.

Construction of B Cell cDNA Library

Messenger RNA is prepared from a B cell lymphoma, and double-stranded cDNA is then prepared, preferably using a kit such as the BRL cDNA kit (with *Bst*XI adaptors instead of *Sal*I adaptors). The resulting cDNA is cloned into the *Bst*XI-*Not*I ends of the mammalian expression vector pJFE14 (low copy number plasmid) or pME18S (high copy number plasmid). These vectors, which contain the SV40 replication origin and the improved SV40 early promoter, SRα, are used for transfection of COS7 cells. B cells expressing the CD40 ligand can be enriched by fluorescence-activated cell sorting (FACS) before library construction.

cDNA Library Screening

By subdividing the cDNA library repeatedly and transfecting recipient COS7 cells over several cycles, the single cDNA responsible for the B cell CD40 ligand can be identified. The following methods, alone or in combination with one or more of the others, can be used for the screening:

(1) The cDNA library is subdivided and COS7 cells are transfected with each pool. COS7 cells expressing a surface form of the CD40 ligand are then screened for CD40-Fc binding using flow cytometry. A fraction of the cells expressing the ligand is collected and the plasmid DNA is recovered.

The procedure is repeated until a single cDNA clone gives positive staining after transfection.

(2) COS7 cells transfected with pools from the cDNA library are cultured in chamber slides. Transfectants are then stained using CD40-Fc followed by FITC-conjugated anti-human IgG Fc and visualized by immunofluorescence microscopy. The cDNA library pools producing positively-stained cells are subdivided and transfected into COS7 cells.

(3) Panning can also be used for screening. In this method, COS7 cells are transfected with the subgrouped cDNA library, and the transfected cells are then placed in tissue culture plates that have been pre-coated with the soluble form of CD40. Unattached cells are removed, and plasmid DNA from the attached cells is transformed into *E. coli*. Transfection and panning are repeated to enrich a set of specific cDNA clones. The individual cDNA is finally transfected into COS7 cells, and binding of CD40-Fc is confirmed as described above.

(4) Another method that can be used for cloning the CD40 ligand from B cells is the emulsion method, which invloves transfection of COS7 cells with a subgrouped cDNA library, culturing the cells on chamber slides, binding labeled soluble CD40 (usually ^{125}I labeled), and carrying out autoradiography with a photographic emulsion. Repetition of subgrouping and transfection of positive cDNA pools leads to a single cDNA clone.

(5) The B cell CD40 ligand can be isolated by protein purification techniques using an appropriate assay for binding to CD40, e.g., as described. Affinity chromatography techniques making use of immobilized CD40 are preferred. The isolated protein is characterized and sequenced allowing preparation of oligonucleotide primers. These oligonucleotide primers are used, e.g., using PCR or hybridization techniques, to isolate a cDNA encoding the B cell CD40 ligand.

DNA encoding B cell CD40 ligand identified using one of the foregoing methods can be expressed and the ligand can be purified, as described above.

Many modifications and variations of this invention can be made without departing from its spirit and scope, as will become apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Schering Corporation
(B) STREET: One Giralda Farms
(C) CITY: Madison
(D) STATE: New Jersey
(E) COUNTRY: U.S.A.
(F) POSTAL CODE (ZIP): 07940-1000
(G) TELEPHONE: 201-822-7375
(H) TELEFAX: 201-822-7039
(I) TELEX: 219165

(ii) TITLE OF INVENTION: CD40 Ligand, Anti CD40 Antibodies, and Soluble CD40

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Apple Macintosh
(C) OPERATING SYSTEM: Macintosh 6.0.5
(D) SOFTWARE: Microsoft Word 5.1a

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/934371
(B) FILING DATE: 21-AUG-1992

INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ACAGCTCGAG CCATGGTGTC TTTGCCTCGG CTGTG 35

INFORMATION FOR SEQ ID NO:2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTAGCTCGAG CTCACCGGGA CTTTAAACCA CAGATG 36

WHAT IS CLAIMED IS:

1. A soluble form of CD40 which substantially lacks the cytoplasmic and/or transmembrane domain of wild-type CD40.

2. A substantially pure B cell CD40 ligand.

3. An isolated DNA encoding B cell CD40 ligand.

4. A method for inhibiting proliferation of $CD40^+$ malignant cells comprising contacting $CD40^+$ malignant cells with an effective amount of a soluble form of CD40 and/or a ligand that binds CD40.

5. The method of claim 4 in which the malignant cells are B lymphoma cells.

6. A method for rendering $CD40^-$ malignant cells more immunogenic to T cells comprising transforming $CD40^-$ malignant cells with DNA encoding human CD40, whereby human CD40 is expressed on the surface of the cells.

7. A method for reducing activity by T cells against $CD40^+$ cells comprising:

(a) contacting $CD40^+$ cells with an effective amount of a ligand that binds CD40, and/or

(b) contacting T cells with an effective amount of a soluble form of CD40.

8. A pharmaceutical composition for inhibiting proliferation of $CD40^+$ malignant cells comprising a pharmaceutically acceptable carrier and a soluble form of CD40 which substantially lacks the cytoplasmic and/or transmembrane domain of wild-type CD40 or a ligand that binds CD40.

9. A method for the manufacture of a pharmaceutical composition for inhibiting proliferation of $CD40^+$ malignant cells comprising admixing a pharmaceutically acceptable carrier with a soluble form of CD40 which substantially lacks the cytoplasmic and/or transmembrane domain of wild-type CD40 or a ligand that binds CD40.

10. The use of a soluble form of CD40 which substantially lacks the cytoplasmic and/or transmembrane domain of wild-type CD40 or a ligand that binds CD40 for inhibiting proliferation of $CD40^+$ malignant cells.

11. The use of claim 10 in which the malignant cells are B lymphoma cells.

12. The method, pharmaceutical composition or use of any one of claims 4, 7, 8, 9 or 10 in which the ligand that binds CD40 is an antibody against CD40, preferably a monoclonal antibody.

INTERNATIONAL SEARCH REPORT

Interna[illegible]al application No.
PCT/US 93/07673

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C07K 15/06, C07K 15/28, A61K 37/02
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C07K, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EP, A2, 0555880 (BRISTOL-MYERS SQUIBB COMPANY ET AL), 18 August 1993 (18.08.93), see column 6, lines 1-10	1-12
P,X	WO, A1, 9308207 (IMMUNEX CORPORATION), 29 April 1993 (29.04.93)	2-5,7-12
X	EP, A1, 0434879 (LABORATOIRES UNICET), 3 July 1991 (03.07.91), see the abstract	2-3

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 November 1993	17 -12- 1993
Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer MIKAEL G:SON BERGSTRAND

INTERNATIONAL SEARCH REPORT

Internal application No.
PCT/US 93/07673

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE JOURNAL OF IMMUNOLOGY, Volume 149, No 2, July 1992, William C. Fanslow et al, "Soluble forms of CD40 inhibit biologic responses of human B cells" page 655 - page 660	1-12
X	Proc. Natl. Acad. Sci. USA, Volume 89, 1992, Randolph J. Noelle et al, "A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells" page 6550 - page 6554	1-12
X	NATURE, Volume 357, May 1992, Richard J. Armitage et al, "Molecular and biological characterization of a murine ligand for CD40" page 80 - page 83	2-5,7-12

INTERNATIONAL SEARCH REPORT

Information on patent family members

01/10/93

International application No.
PCT/US 93/07673

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0555880	18/08/93	AU-A- 3298893	19/08/93
WO-A1- 9308207	29/04/93	AU-A- 3122693	21/05/93
EP-A1- 0434879	03/07/91	AU-A- 6901791	18/07/91
		CA-A- 2071886	15/06/91
		EP-A- 0505397	30/09/92
		JP-T- 5503214	03/06/93
		WO-A- 9109115	27/06/91