A SYSTEM FOR THE PRODUCTION OF HUMAN MONOCLONAL ANTIBODIES

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science, Graduate Department of Immunology, University of Toronto

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Abstract

The ability to maintain antibody secreting human B lymphocytes in culture is important for studies into B cell repertoires and for monoclonal antibody production. The CD40 culture system stimulates B lymphocytes to allow for in vitro expansion and immunoglobulin secretion (17). We have used a CD40 ligand expressing (CD40L+) fibroblast feeder layer which we have transfected with cytokine genes (IL-10, IL-6, IL-4 and IL-2). EBV-transformed lines grown on the cytokine-transfected feeder layers demonstrated similar patterns of immunoglobulin secretion as cells grown on the CD40L+ line with exogenously added cytokines. IL-10 and IL-6 were able to increase immunoglobulin production of four EBV-transformed cell lines, whereas IL-2 and IL-4 did not have any effect at the concentrations used in either the cytokine-transfected lines or with the exogenous cytokine. Thus these cytokine secreting CD40L+ feeder cell lines will be a valuable tool for the in vitro culture of human B lymphocytes.
Introduction

Human monoclonal antibodies are important for therapy, diagnosis and understanding disease mechanisms. One of the most interesting uses of monoclonal antibody technology is in the study of B cell repertoires. Immortalizing cells from individuals recovering from infections would provide a wealth of information about the immune response to that agent and information on the antibodies which are protective against the particular organism. Examining the B cell repertoire and antibody secretion patterns of autoimmune individuals would provide valuable information on the ontogeny, progression and hopefully therapy of such diseases. A comprehensive review of the uses of human monoclonal antibodies has been described by James and Bell (1).

The preparation of mouse monoclonal antibodies has been very successful and many cell lines and different specificities are available. However, despite tremendous effort, there are still many difficulties with the preparation of human monoclonal antibodies. The following section will summarize some of the current technologies for human monoclonal antibody production and some of the difficulties associated with them.

Hybridomas

In 1975, Kohler and Milstein described a cell line secreting anti-sheep red blood cell antibodies which was made from the fusion of mouse myeloma and spleen cells from an immunized mouse (40). This hybridoma technology has been widely used in the preparation of mouse monoclonal antibodies. However, the success with human hybridomas has been more limited and the number of lines that have been produced with desired antibody specificities is relatively low in comparison (7). In brief, production of
hybridomas (8) involves fusing two types of cells together: the first an antibody producing cell from a human or immunized animal and the second an immortalized fusion partner. The B lymphocyte produces the antibody of the desired specificity and the fusion partner (a myeloma cell in most mouse fusions) provides immortality to the combination. This immortalized cell carries a drug selection marker which facilitates the selection of the fused cells. For example, a commonly used method is selection under HAT (hypoxanthine, aminopterin and thymidine) medium. Myeloma cells that are HGPRT (enzyme required to survive under HAT selection) negative are used for fusion. B lymphocytes which express the HGPRT enzyme are able to survive under HAT selection, but if they are not immortalized, they will die after a few days of in vitro culture. Unfused myeloma cells, which lack the enzyme to survive, will be killed in HAT medium. Thus, only the fused hybridoma cells will contain both the enzyme required to survive HAT selection and the properties of the immortalized cell line. The cells which grow out in culture can be screened by ELISA to select the cells producing the desired antibody. These wells can be cloned by limiting dilution to achieve a cell line producing monoclonal antibodies (Figure 1).

Human hybridoma technology has allowed for the production of a variety of IgM secreting lines. However, in contrast to the success with mouse hybridomas, there are still many problems associated with human hybridoma technology. One of these difficulties is priming of the B lymphocytes with the desired antigen (8). With mice, one is able to follow a convenient schedule of immunization with a variety of infectious agents, cancer cells or other agents. This is obviously unethical and impractical with humans. It is possible to use cells from individuals that have been naturally immunized against selected pathogens however, the number of pathogen specific cells in the circulation is low and decreases with time (8). In vitro culture conditions have been used to prime the B lymphocytes, however it has been difficult to obtain an IgG response from the cells with this method, and a low number of antigen specific cells are produced (8). Recently an in
isolate B lymphocytes

antibody producing cells

fusion with PEG

selection under HAT

hybridoma cells

screen for desired specificity

Figure 1. The production of hybridoma cells
vitro culture system was described in which peripheral blood was depleted of CD56+ (Natural killer and some cytotoxic T cells) and cultured with autologous donor serum, IL-2 and Pokeweed mitogen (PWM). When these cells were fused with mouse myeloma cells, IgG producing heterohybridomas were established. However, these were short lived (9). Cells have been primed by inoculating SCID mice with cancer tissues and regional lymph node cells obtained from a lung cancer patient, allowing for an in vivo stimulation of the lymph node cells. The SCID-hu spleen cells were incubated with Epstein-Barr Virus (EBV) to produce lymphoblastoid cell lines. These cell lines secreted IgG monoclonal antibodies, some of which were shown to be reactive to lung cancer cells (10). However, these results are still preliminary and the technology is relatively new. It also requires the use of SCID mice, whose maintenance may not be practical in labs not already set up for such conditions, as well as human tissue from the site of the individual tumor and lymph nodes.

It appears that not all stages or sources of B lymphocytes are able to fuse with the immortalized fusion partner. This conclusion is based on the observation that mouse spleens are much easier to fuse than cells found in peripheral blood (8). It was therefore postulated that peripheral blood does not contain enough cells at the "appropriate" stage of differentiation and proliferation (8). The appropriate stage is unknown. It is difficult to obtain human spleen and therefore, peripheral blood is the only human source that is consistently available. There has been some success in increasing fusion efficiency by stimulating cells with mitogens and using viral transformation on murine B lymphocytes.(8,11). However, such protocols have been less successful when applied to human cells. At 50μg/ml, LPS stimulation had no effect on the ability to fuse human PBL, although this concentration was used successfully with mouse cells (10).

There are well established mouse myeloma lines available to be used as fusion partners, however, identification of suitable human fusion partners has been much more difficult. One review lists 38 different fusion partners and heteromyelomas used in human
monoclonal antibody production (1) and many others have been described (41). On the other hand, a few good mouse fusion partners were found early and have come to dominate. Most lines are derivatives of P3-X63, Sp2/0 or NS-1 (42,43). Because of the poor growth of many myelomas in cultures, many of the human fusion partners are lymphoblastoid lines, mainly derived from EBV infection (1). Although lymphoblastoid cell lines are much easier to grow in culture, they may not be at the most desirable stage of differentiation and the resultant hybridomas secrete less immunoglobulin than fusions with myelomas (1). One method to improve upon the human fusion partners has been to use heterohybridoma lines. These are lines generated by the fusion of a lymphoblastoid cell and a human or mouse myeloma cell (12). A fusion partner created by the fusion of a lymphoblastoid line and a mouse myeloma has been able to produce more stable hybrids than the original myeloma was able to alone. However, heterohybrids tend to reject human chromosomes (1).

**EBV Transformation**

An alternate method of producing human antibodies is by directly immortalizing B lymphocytes through viral transformation using EBV (13). EBV is a herpesvirus which has been implicated in Burkitt's lymphoma, infectious mononucleosis and nasopharyngeal carcinoma (14). It is a double stranded DNA virus, which is linear in the viral particle, but circularizes once it has infected a host cell (15). It remains as an episome in the cell, but can on occasion insert into the genome (1). EBV infects a host cell through the CR2 (CD21) complement receptor (15). Transformation causes the production of immortalized lymphoblastoid lines that can be grown in tissue culture.

Although EBV can infect all B lymphocytes, only a fraction of the infected cells are actually transformed. Many cell lines have been produced through this method, however most secrete only IgM. It has been suggested that differences in the distribution
of viral receptors among developmental subpopulations of B lymphocytes was the cause of this preference (16). To address this, a B lymphocyte population was implanted (using a membrane solubilization and a co-reconstitution techniques with mosaic membrane vesicles containing receptors) with viral receptors and subsequently infected with EBV. Infection of such cells lead to an increase in the total number of lymphocytes secreting immunoglobulin and an increase in the proportion of cells secreting IgG (16).

This method however is not without problems. In many cases the transformed lines may grow well for 1-2 months and then suddenly the secreted antibody titre decreases and the transformed line may stop producing immunoglobulin completely. This especially seems a problem in the case of human IgG lines. These lymphoblastoid lines are also found to be extremely difficult to clone. Many of the lines that were created which secreted useful antibodies were lost due to poor cloning efficiencies (1).

**Transformation with oncogenes**

Another method which has been described, is the use of oncogenes to transform B lymphocytes. Most successes reported with this method have used murine lymphocytes (36,38,39) with oncogenes inserted into the cells using retroviral vectors. Murine pre-B cells have been transformed by retroviral vectors containing v-Ha-ras and v-myc however, they were unable to transform mature B cells (38). A v-abl containing vector was able to transform murine B cell lines. These lines were LPS-dependent but did secrete IgG (36). Another report showed immortalization of murine B cells with a v-myc and v-raf/mil containing retrovirus (39). These cells were able to respond to B cell mitogens by proliferation and IgM secretion. They could be induced to switch to IgG production after incubation with staphylococcal enterotoxin B activated TH2 cells. Although this technology looked promising, similar experiments using human cells have not been as encouraging. There has been one report of human lymphoblastoid lines
transformed with the ras oncogene (37). Data showed enhanced antibody secretion that was comparable with a hybridoma line created with the same parental lymphoblastoid line. These studies were not done on naive cells but on B cell lines that had been grown in culture for many years. It was possible that other significant chromosomal changes had occurred during this time. Our lab has used v-myc, ras and bcl-2 containing retroviruses to infect an IgM producing EBV cell line (Ge, unpublished data). However, there was not a significant increase in antibody production seen with any of these lines. Also, some preliminary work was done using these same retroviral vectors to infect human lymphocytes purified from peripheral blood. We were not able to generate any immortalized cell lines from cells transformed with single or double combinations of these genes. However, we were not able to demonstrate that these genes were being expressed from these cells. Thus, to date, the success of this method with respect to the human system has been limited.

**Genetically Engineered Antibodies**

One approach that has been described for monoclonal antibody production bypasses the need to immortalize B lymphocytes. The phage display library involves amplifying total mRNA using multiple heavy (H) and light (L) chain specific primers (2,3). RT-PCR is used to generate individual libraries of the H and L chains. These libraries are combined in a phage vector, which therefore contains one H and one L chain gene. This creates a random combinatorial library which contains the genes required to produce the antibody binding fragments. The H chain gene of the antibody is fused with the bacteriophage coat protein. After infection into bacteria, the H and L chain proteins assemble in the phage. The antibody-fusion-protein is expressed on the phage coat and can be selected by screening for binding to antigen. Phage particles that are positive for
Figure 2. Production of genetically engineered antibodies
binding are isolated and purified. These antibodies can be cloned and then amplified and joined to the invariant portion of the antibody molecule to form a complete antibody. The reconstructed antibody genes are transfected into appropriate host cell lines which will secrete the selected antibody (Figure 2).

The screening procedure is limited by the antigen which is being studied. The antigen must be available in significant purified quantities to allow for screening. This causes a problem in isolating antibodies for which the antigen has not yet been identified, or is difficult to extract, purify, or obtain in large enough quantities. This method can also generate H and L combinations that may not exist in vivo and it is necessary to screen a large combinatorial library to recover original pairs (4). This makes it less useful in studies where the in vivo combinations are important such as in exploring B cell repertoires of individuals. This technology has however, allowed for the production of "humanized" mouse monoclonal antibodies. This is achieved by linking mouse variable regions with human constant regions (5). This reduced the immunogenicity of the antibody, however some immunogenicity was retained by the variable region (6). This method has been very successful for producing human antibodies for therapy, but was not useful in exploring natural antibody repertoires that may be involved in disease.

**CD40 system**

The CD40 system was first described by Banchereau et al in 1991 (17,18). It allowed for the in vitro maintenance of B lymphocytes through stimulation of CD40 and recombinant cytokines. The CD40 antigen is a member of the TNF receptor superfamily. It is found on the surface of B lymphocytes, B malignancies, B cell precursors, dendritic cells, follicular dendritic cells, hematopoietic progenitor cells, epithelial cells and carcinomas, but not on plasma cells (19). Ligation of CD40 with its ligand (CD40L) or cross linking of CD40 with immobilized anti-CD40 causes B lymphocyte proliferation.
Activation via CD40-CD40L interaction in the presence of exogenous cytokines allowed for "long-term" growth of B lymphocytes, immunoglobulin production and the generation of memory B cells (18,20,21). CD40 ligation was also shown to prevent germinal centre B lymphocytes from undergoing apoptosis (22). Recently, it has been shown that cross-linking of CD40 and immunoglobulin, together with the addition of Th2 type cytokines is the most efficient method of inducing a germinal centre B cell phenotype in vitro (52).

The system initially described involved incubating B lymphocytes with a mouse fibroblast cell line which had been transfected with the human Fc receptor which allowed for the immobilization of CD40 monoclonal antibodies on the cells (18) (Figure 3). Subsequent reports have used fibroblast cell lines directly transfected with CD40 ligand instead of using immobilized anti-CD40 antibodies (21). B lymphocytes grown in this system could be kept in culture for up to 3 weeks, unlike B lymphocytes stimulated with soluble anti-CD40 which gave a poor response (18). Lymphocyte cultures grown in the CD40 system produced low levels of IgM, IgG and IgA but no IgE. They further explored the system by culturing the B cells with immobilized CD40L and various recombinant cytokines. IL-4 induced B cell proliferation and allowed for the long-term growth of these cultures. Lines were generated from tonsil, spleen, peripheral blood and cord blood isolated B lymphocytes and such lines were kept for up to 10 weeks and could be both frozen and thawed. The addition of IL-10 was also able to induce B cell proliferation. However, the effect of IL-10 decreased after 10 days and stopped by 2-3 weeks. The cytokines added to the cultures of CD40L+ fibroblast lines affected immunoglobulin production. IL-4 augmented the secretion of IgM, IgG and large amounts of IgE. A combination of IL-2 and IL-4 augmented production of IgM and IgA, however the IL-2 did not alter the IL-4 induced proliferation. IL-10 caused the production of large amounts of IgG, IgA and IgM but no IgE. Thus, this system allowed for the growth of factor dependent B cell lines which secreted specific antibody.
Figure 3. The CD40 system for culturing B lymphocytes
The CD40 system has also been used to culture B cell malignancies that proliferated poorly in culture (23). Chronic lymphocytic leukemia (CLL) and hairy cell leukemia (HCL) cells were grown in this system (23). The proliferation of CLL cells were increased 2-4 fold, however no change in differentiation was seen as measured by immunoglobulin production. HCL, B-PLL, immunocytoma and multiple myeloma cells were also cultured using this system.

More recent work with the CD40 system has looked at the bidirectional regulation that CD40-CD40L interactions have on B lymphocytes. This means that CD40 ligation can have both a positive or negative effect on immunoglobulin production. Low concentrations of CD40L enhanced immunoglobulin production by B cells, and high concentrations of CD40L reduced immunoglobulin production (53,54). The higher concentration of CD40L did not suppress the proliferation of B cells (53). The stage of differentiation of the B lymphocyte also affected the outcome of CD40 ligation. IgD⁺ naïve B lymphocytes were found to be less susceptible to the suppression by higher CD40L concentrations than were IgD⁺ memory B lymphocytes (53).

The goal of my project was to establish a CD40L⁺ and cytokine expressing feeder cell layer and explore the response of EBV-transformed cell lines in this system. I transfected a CD40L expressing fibroblast cell line with IL-10, IL-6, IL-4 or IL-2 to achieve a cell monolayer that presented CD40L and locally produced the cytokines at the site of cell-cell interactions. I have used these lines to enhance growth of EBV-transformed cell lines and to increase the amount of specific antibody produced.
Materials and Methods

Reagents

Recombinant human IL-10 and recombinant human IL-4 were purchased from PharMingen Canada. Recombinant human IL-2 was obtained from Cetus (Emeryville, USA). Recombinant human IL-6 was a generous gift from Dr. J. Gauldie (McMaster University, Hamilton). Affinity purified goat anti-human IgG(\gamma) was purchased from Kirkegaard and Derry Laboratories Inc. (Maryland, USA). Affinity purified goat anti-human IgM, alkaline phosphatase-conjugated goat anti-human IgM and alkaline phosphatase-conjugated AffiniPure goat anti-human IgG were purchased from Jackson ImmunoResearch Lab. Inc. (Westgrove, USA). Human IL-10pBluescriptSK (ATCC 107480) and human IL-4pBluescriptSK (ATCC 105495) were purchased from the American Type Culture Collection (Rockville, MD). The pHIP6 vector (24) containing human IL-6 and the pCaIL-2 vector (25) containing human IL-2 were generous gifts from Dr. Robert Hawley (Toronto General Hospital).

Cells and Culture Conditions

Peripheral blood was obtained from healthy donors and the peripheral blood mononuclear cells (PBL) were separated using a Ficoll-Pacque (Pharmacia Biotech) density gradient. PBL were washed two times with calcium and magnesium free PBS and resuspended in complete media (CM) consisting of RPMI 1640 (Gibco BRL, Gaithersburg, MD) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (Cansera, Rexdale, ON) and 100 U/ml penicillin/ 100 \mu\text{g}/ml streptomycin (Gibco BRL, Gaithersburg, MD). EBV-transformed cell lines used were previously created in our laboratory and maintained in complete media (26,27). Murine NIH 3T3 fibroblast cells transfected with human CD40 ligand were a generous gift from Joachim Schultze (Harvard Medical School) (28,29). The fibroblast cells were maintained in Iscove's
modified Dulbecco's media (IMDM) (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum and 100U/ml penicillin/100 μg/ml streptomycin. HT-2 (CRL-1814) indicator cells were obtained from the American Type Culture Collection (Rockville, MD). They were maintained at 3.5 x 10^4 cells/ml in RPMI 1640 supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10mM HEPES, 1 mM sodium pyruvate, 10% fetal bovine serum and 200 U/ml recombinant IL-2. B9 indicator cells were maintained in IMDM supplemented with 5% fetal bovine serum, 50 μM B-mercaptoethanol and 2% IL-6 conditioned medium (gift from T. Hawley, Toronto General Hospital). All cell cultures were grown in a 37°C humidified incubator with 5% CO₂. All cells were fed 2-3 times weekly. Adherent fibroblast lines were split by aspirating media, washing once with PBS and addition of 0.25% trypsin (Gibco BRL, Gaithersburg, MD). Cells were washed off with fresh media and split onto new tissue culture dishes.

Construction of cytokine expression vectors

The mammalian expression vector, pRep10 (Invitrogen, Carlsbad, CA) containing the hygromycin B resistance gene was used for expression of all cytokines. Human IL-10 and human IL-4 were excised from the IL-10pbluescriptSK phagemid by restriction endonuclease digestion (30) of the KpnI and BamHI sites contained in the multiple cloning site of the pbluescript vector. The fragments were separated on a 1% agarose gel containing ethidium bromide. The band corresponding to approximately 1600 bp (IL-10) or 650 bp (IL-4) were cut out of the gel and recovered by centrifugation through glass wool and precipitated with ethanol. The fragments were ligated into KpnI/BamHI digested pRep10. Human IL-2 was excised from pCaIL-2 (25) by digestion with BamHI and Sal I. The fragment of approximately 470 bp (IL-2) was purified as above and ligated into BamHI/XhoI digested pRep10. Human IL-6 was excised from pHIP6 by digestion
with Hind III. The 5' overhangs were filled in with Klenow in the presence of dNTP's (30). The fragment of approximately 700 bp (IL-6) was purified as above and digested with BamHI. The fragment was ligated into PvuII/BamHI digested pRep10.

The plasmids were transformed by heat shock into competent DHα cells prepared by calcium chloride treatment (30). Transformed cells were plated on LB agar plates with 50 μg/ml ampicillin. Plasmids were purified from overnight cultures by the alkaline lysis method (30). Plasmids were analyzed for correct insertion of the desired fragment by restriction endonuclease mapping.

**Transfection of fibroblast lines**

Murine NIH 3T3 human CD40L transfected cells were washed with PBS and resuspended at 2 x 10⁶ cells/ml in PBS. 0.8 ml of cells were mixed with 20 μg of StuI or ClaI digested plasmid DNA and added to an electroporation cuvette. Cytokine containing pRep10 plasmids were transfected by electroporation at 250 volts and 950 μF using a Electro Square Porator (T820, BTX Corporation, San Diego, CA). The suspension was added to a 25cm² flask with 5 ml media and incubated for 2 days. Cells were transferred into 10cm tissue culture plates containing IMDM supplemented with 10% FBS, and 200 μg/ml Hygromycin B (Rose Scientific, Edmonton, Alb.). Cells were grown under selection for a minimum of 2-3 weeks before cloning. Cultures were subcloned at 0.3 cells/well in 96-well tissue culture plates. Wells that were positive for cell growth were expanded and then re-cloned again at 0.3 cells/well. Positive wells were expanded into larger tissue culture plates.

Transfected cell lines were screened for mRNA expression by reverse transcriptase - polymerase chain reaction (RT-PCR). Total RNA was extracted from the transfected cells using the RNeasy Blood Minikit (Qiagen, Santa Clara, CA) as per manufacturer's instructions with the following changes: cells were trypsinized, washed
two times with PBS and resuspended in 600 μl of RLT lysis solution. The sample was added to the Qiashredder spin column and the protocol was continued from step number seven of the manufacturer's instructions. The RNA was reverse transcribed into cDNA using "Ready To Go T-Primed First-strand kit" (Pharmacia Biotech) as per manufacturer's instructions. Polymerase chain reaction was performed using "Ready To Go PCR Beads" (Pharmacia Biotech). PCR was performed using 2.5 μl of cDNA and 1 μg of each primer (0.5 μg for G3PDH primers). Primers used were as follows:

IL-10 sense: 5' - ATG CCC CAA GCT GAG AAC CAA GAC CCA - 3'  
IL-10 anti-sense: 5' - TGG GAT AGC TGA CCC AGC CCC TTG AGA - 3'  
IL-6 sense: 5' - ATG AAC TCC TTC TCC ACA AGC - 3'  
IL-6 anti-sense: 5' - CCA GCC TGA GGG CTC TTC GGC AAA TG - 3'  
IL-4 sense: 5' - ATG GGT CTC ACC TCC CAA CTG C - 3'  
IL-4 anti-sense: 5' - AAC GGC TCG CAG GAA CCT C - 3'  
IL-2 sense: 5' - GCA CTA AGT CTT GCA CTT G - 3'  
IL-2 anti-sense: 5' - CTG AAC AGA TGG ATT ACC - 3'  
G3PDH sense: 5' - TGA AGG TCG GAG TCA ACG GAT TTG GT - 3'  
G3PDH anti-sense: 5' - GTG GTG GAC CCT CAT GGC CCA CAT G - 3'

Conditions were as follows: denaturation at 94 °C for 40 seconds, annealing at 57 °C (IL-10 and IL-2), 55 °C (IL-6) or 53 °C (IL-4) for 50 seconds and extension at 72 °C for 1 minute 10 seconds for 30 cycles followed by a final extension of 5 minutes. PCR products were analyzed on a 1% agarose gel containing ethidium bromide.
Assay for cytokine production

IL-2

Indicator assays were performed in IMDM supplemented with 10% fetal bovine serum, 50 μM β-mercaptoethanol and 100 U/ml penicillin/100 μg streptomycin. IL-2 production was measured using the HT-2 indicator cell line (31). Supernatant from a confluent plate of IL-2 transfected or untransfected fibroblast line was harvested and 100 μl added in serial two-fold dilutions to a 96 well plate. HT-2 cells were washed two times with PBS (to remove IL-2 containing culture media), resuspended in culture media and added at 10^4 cells/well. The total volume per well was 200 μl. The plates were incubated for 18 hours and then each well was pulsed with 1 μCi of 3H-thymidine (Amersham, Arlington Heights, IL). Plates were incubated for a further 6-8 hours and harvested using a cell harvester onto glass fibre filter paper (Inotech, Rockville, MD). 3H-thymidine uptake was measured using a liquid scintillation counter (Beckman, Fullerton, CA).

IL-4

IL-4 production was examined by measuring the proliferation of EBV-transformed cell lines or PBL. IL-4 transfected or untransfected lines were irradiated at 5000 rads and added at 10^4 cells per well in a 96 well microtitre plate. EBV-transformed cell lines were resuspended at 10^4 cells per well and PBL at 10^5 cells/well in RPMI complete media and 100 μl was added in the appropriate well (total volume 200 μl). Plates were incubated 3 or 7 days and pulsed with 3H-thymidine. After an overnight incubation, plates were harvested as above and radioactivity measured by scintillation counting.
A human IL-4 ELISA kit (Endogen, Woburn, MA) was used to determine the amount of cytokine in cell supernatants. The ELISAs were carried out as per manufacturers instructions.

**IL-6**

IL-6 production was measured using the B9 indicator cell line (32). The assay was performed as per the HT-2 cells except 5000 indicator cells were added per well and plates were allowed to incubate for 48 hours before the ^3^H-thymidine pulse.

**IL-10**

IL-10 production was examined by measuring the proliferation of EBV-transformed cell lines or PBL. IL-10 transfected, or untransfected lines were irradiated at 5000 rads and added at 10^4^ cells per well in a 96 well microtitre plate. EBV-transformed cell lines were resuspended at 10^4^ cells per well and PBL at 10^5^ cells/well in RPMI complete media and 100 μl was added in the appropriate well (total volume 200 μl). Plates were incubated for 3 or 7 days and pulsed with ^3^H-thymidine. After an overnight incubation, plates were harvested as above and radioactivity measured by scintillation counting.

A human IL-10 ELISA kit (Endogen, Woburn, MA) was used to determine the amount of cytokine in cell supernatants. The ELISAs were carried out as per manufacturers instructions.

**CD40 system**

The CD40 system adapted from Banchereau et al (18,33), was set up using five different EBV-transformed cell lines. Cytokine transfected or untransfected CD40L+ fibroblast lines were irradiated at 5000 rads and 100 μl of a 10^5^ cells/ml suspension was
added per well. Recombinant human cytokines were added in some wells containing untransfected CD40L+ fibroblast lines at the following concentrations: rIL-10 at 30 ng/ml, rIL-6 at 1 ng/ml, rIL-4 at 3 ng/ml and rIL-2 at 0.3 ng/ml. EBV-transformed cell lines were added at $10^3$ cells/well with a final volume of 200 μl per well. Plates were incubated for 7 days, after which supernatant was removed for immunoglobulin measurement (see below) and $^3$H-thymidine was added for measurement of proliferation (as described earlier).

**ELISA**

Total IgM and IgG were measured using an ELISA assay (34). Wells were coated with goat anti-human IgG (1:1000) or IgM (1:2000) and blocked with 1% BSA/PBS. Dilutions of sample supernatants or pooled sera at known concentrations were added at 100 μl per well followed by alkaline phosphatase-conjugated goat anti-human IgG or IgM (diluted 1:5000). Plates were developed with p-nitrophenyl phosphate and the optical density read at 410 nm on a Spectra Max 250 ELISA reader. A standard curve was prepared using the pooled sera and the concentration of the test supernatant was calculated using SoftmaxPro version 2.1.0 (Molecular Devices Corp., Menlo Park, CA).

**Statistical Analysis**

Student t-tests were performed using Statview, version 5 (SAS Institute Inc., Santa Clara, CA).
Results

Construction of cytokine containing vectors

Each of the four cytokine genes were cloned into the pRep10 expression vector. pRep10 is a mammalian episomal expression vector which contains an ampicillin resistance gene for selection in prokaryotic systems and the hygromycin B resistance gene for selection in mammalian cells. Genes inserted into the multiple cloning site are driven at a high level of transcription by the Rous Sarcoma Virus long terminal repeat enhancer/promoter. The 468 bp fragment containing the IL-2 coding region was purified from the pCaIL-2 vector, the 618 bp fragment containing the IL-4 coding region was purified from IL-4pbluescript, the 700 bp fragment containing the IL-6 coding region was purified from the pHIP6 vector and the 1601 bp fragment containing the IL-10 coding region was purified from the IL-10pbluscript vector. The purified fragments were inserted into the multiple cloning site of pRep10 (Figure 4). Each construct was analyzed by restriction endonuclease digestion (data not shown) to ensure proper insertion and correct orientation of the gene.

Expression of cytokine message from CD40L+ NIH 3T3 cells after transfection

The CD40L+ NIH 3T3 cells were transfected with IL-10, IL-6, IL-4 or IL-2 containing vectors by electroporation. After selection for 2-3 weeks in the presence of Hygromycin B (200 µg/ml), cells were tested for cytokine mRNA expression by RT-PCR (Figure 5). Primers used for PCR were designed for the coding region of the genes (appendix illustrates primer locations and portions of genes inserted). Cytokine expression in the transfected lines were compared to the untransfected CD40L+ line.
Figure 4. Construction of cytokine expression vectors. Cytokine genes were cut and purified from previously constructed vectors and inserted into the multiple cloning site of pRep10. All constructs were analyzed by restriction endonuclease digestion.
Figure 5. PCR products obtained from the RT-PCR reaction on transfected or untransfected CD40L+ NIH 3T3 cell lines. Products were run on a 1% ethidium bromide agarose gel. Lanes 1 and 13 contain a 100 bp DNA ladder. Expected sizes of PCR products are as follows: IL-10: 352 bp, IL-6: 650 bp, IL-4: 351 bp, IL-2: 490 bp and G3PDH: 997 bp.
Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used for a housekeeping gene as a control for the amount of cDNA present. PCR products of expected size were generated for all transfected lines: 352 bp for IL-10 (lane 2), 630 bp for IL-6 (lane 4), 351 bp for IL-4 (lane 6), 400 bp for IL-2 (lane 8) and 997 bp for G3 (lanes 3, 5, 7, 9, 15). These PCR products were not seen in the untransfected CD40L+ line (lanes 10-14).

Lines that were shown to be positive for mRNA expression were cloned twice under limiting dilution conditions at 0.3 cells/well. Expanded clones were re-tested for mRNA expression. Cytokine transfected CD40L+ fibroblast clones given the designation IL-10T-7C, IL-6T-2B, IL-4T2-4C and IL-2T-2C were positive for mRNA expression and were used for subsequent experiments.

_Cytokine expression from transfected CD40L+ fibroblast lines_

a) IL-2

We used the HT-2 indicator cell line, which is a murine T helper cell-derived cell line that is responsive to human IL-2 to test for functional IL-2 production from IL-2 transfected fibroblast cell lines (Figure 6). Supernatant from a confluent plate of IL-2T-2C was added in sequential 2-fold dilutions and compared to the ability of both recombinant IL-2 and supernatant from untransfected CD40L+ fibroblasts to effect proliferation of HT-2 cells. Supernatant from untransfected CD40L+ fibroblasts consistently showed only a marginal effect on proliferation whereas supernatant from IL-2T-2C demonstrated a large increase in proliferation of the HT-2 cells. As expected, this level of proliferation was seen to decrease with serial dilutions of the IL-2T-2C supernatant. From the standard curve of the response of HT-2 cells to recombinant IL-2, the amount of IL-2 in the culture supernatant was determined. Supernatant from a confluent 6 cm dish contained 25 U/ml of IL-2.
Figure 6. IL-2 production by cytokine transfected cells. A) Supernatant from a confluent plate of IL-2 transfected (IL2T2C) or untransfected fibroblast line was harvested and 100 µl added in serial two-fold dilutions to a 96 well plate containing 10⁴ HT-2 cells/well. After 18 hours incubation, cells were pulsed with 1 µCi/well ³H-thymidine and incubated a further 6 hours before harvesting. Results shown are means of triplicate wells and representative of two independent experiments. B) Standard curve produced by adding known concentrations of recombinant IL-2 to the HT-2 cells and measuring proliferation.
b) IL-6

The ability of the IL-6 transfected CD40L+ fibroblast line (IL-6T-2B) to produce functional IL-6 was explored using the B9 indicator cell line (Figure 7). The B9 cell line is a murine B-cell hybridoma line that requires IL-6 at low levels for survival and proliferation. Supernatants from IL-6T-2B, untransfected CD40L+ fibroblasts and recombinant IL-6 were added to sequential 2-fold dilutions to the B9 indicator cells. The presence of supernatant from untransfected CD40L+ fibroblasts did not alter the basal level of B9 proliferation. Supernatant from IL-6T-2B cells as well as recombinant IL-6 significantly enhanced B9 proliferation. There was a dose-response decrease of B9 proliferation to serial dilutions of both. Recombinant IL-6 and IL-6T-2B supernatant both demonstrated a decrease in proliferation at the highest concentrations of IL-6. Using the standard curve of the response of B9 cells to recombinant IL-6, we determined that the supernatant from a confluent 6 cm dish contained 15 U/ml of IL-6.

c) IL-4 and IL-10

Indicator cell lines were unavailable to test the ability of the transfected cell lines to produce human IL-4 or IL-10. In the presence of CD40L, IL-4 and IL-10 are both able to enhance the proliferation of B lymphocytes (1). Thus, this method was used to indicate the production of IL-4 or IL-10 from the transfected CD40L+ cells. Irradiated IL-4T2-4C, IL-10T-7C or untransfected CD40L+ fibroblasts were incubated with four different EBV-transformed cell lines. The proliferative response of these cells were measured by 3H-thymidine uptake (Figure 8). The IL-4 transfected CD40L+ cell line, IL-4T2-4C, did not consistently cause any significant increase in proliferation compared to untransfected CD40L+ cells (p=0.42). The IL-10T-7C cells consistently caused an
Figure 7. IL-6 production by cytokine-transfected cells. A) Supernatant from a confluent plate of IL-6 transfected (IL-6T2B) or untransfected fibroblast line was harvested and 100 μl added in serial two-fold dilutions to a 96 well plate containing 5000 B9 cells/well. After 48 hours incubation, cells were pulsed with 1 μCi/well ³H-thymidine and incubated a further 6 hours before harvesting. Results shown are means of triplicate wells and representative of two independent experiments. B) Standard curve produced by adding known concentrations of recombinant IL-6 to the B9 cells and measuring proliferation.
Figure 8. Proliferative response of EBV-transformed lines to feeder layers. EBV-transformed line were added at $10^4$ cells/well to 96 well plates containing $10^6$ irradiated (5000 rads) CD40L-, IL-4 transfected CD40L+ or IL-10 transfected CD40L+ fibroblast cells. Plates were incubated for 7 days and $^3$H-thymidine uptake was measured. Results are representative of 3 experiments, each in triplicate. Stimulation index was calculated as the increase in proliferation as compared to cells growing on untransfected CD40L+ fibroblasts alone.
increase in proliferation in all cell lines compared to untransfected CD40L+ cells (p=0.01).

Supernatants from IL-4 and IL-10 transfected cells were both measured by ELISA. These assays did not detect the presence of either cytokine in the cell line supernatants (data not shown).

Transfected cells continue to produce functional protein after irradiation

The CD40L+ fibroblast cells must be irradiated at 5000 rads for use in the CD40 system, to ensure that the feeder layer does not proliferate and overgrow during the culture period. It was therefore necessary to see if the cytokine transfected fibroblasts were still secreting protein over the culture period. To do this, 5 x 10^4 IL-2T-2C cells, irradiated (5000 rads) or unirradiated were plated in 24 well culture plates. Every two days, culture supernatant was removed and replaced with fresh media. At the end of the culture period, all supernatants were assayed for IL-2 production using the HT-2 indicator cell line (Figure 9). The unirradiated IL-2T-2C cells caused an increase in proliferation of the HT-2 cells, showing an increase of IL-2 in the culture medium over each two day period. The irradiated cells showed a relatively constant production of IL-2 over the eight days tested.

The CD40 system can be used to culture EBV-transformed cell lines

Five different EBV-transformed cell lines were grown under the culture conditions outlined in the methods section. At the time of the assay two of the EBV-transformed cell lines secreted IgM (10C3 and 106), two secreted IgG (2G2 and 100C5) and one was not secreting (045). The production of total IgG and IgM by the five EBV-
Figure 9. Cytokine expression after irradiation of transfected fibroblast cells. CD40L- or IL-2T-2C fibroblast cells were irradiated at 5000 rads and cultured at $5 \times 10^4$ cells/well in a 24-well plate. Every two days, culture supernatant was removed and replaced with fresh media. At the end of the culture period, all supernatants were assayed for IL-2 production using the HT-2 indicator cell line.
transformed cell lines was examined under the following conditions: 1) culture of the lines in the presence of CD40L+ fibroblast lines and added exogenous cytokine, 2) culture of the lines with cytokine transfected CD40L+ lines and 3) the effect of added exogenous cytokine when cells were grown on CD40L+ feeder layers (Figure 10).

There was no statistically significant change in immunoglobulin production when lines were grown on the CD40L+ feeder layer with the addition of recombinant IL-2 (0.3 ng/ml) or when grown on the IL-2 transfected feeders (p > 0.05). Similarly, there was no change with recombinant IL-4 (3 ng/ml) or with the IL-4 transfected feeders.

The addition of recombinant IL-6 (1 ng/ml) to the CD40L+ culture conditions was able to cause an increase in both IgG and IgM secretion. The amount of immunoglobulin produced was on average 3.9 times greater in the IgG secreting lines (100C5 and 2G2) and 1.7 times greater in the IgM secreting lines (10C3 and 106) than with CD40 ligation alone. The IL-6 transfected CD40L+ feeder layers were also able to increase immunoglobulin secretion. The amount of immunoglobulin produced was on average 2.8 times greater in the IgG secreting lines and 1.6 times greater in the IgM secreting lines than with CD40 ligation alone.

The effect of IL-10 on EBV-transformed lines grown in the CD40L feeder layer varied depending on the cell line. When the EBV-transformed cell lines were grown on the CD40L+ feeder layer with the addition of recombinant IL-10 (30 ng/ml), immunoglobulin secretion was consistently enhanced in IgG lines (3.5 times on average). The effect on IgM lines was less consistent. In one experiment, IgM secretion was doubled however, in a subsequent experiment only a marginal increase in IgM secretion was observed. Enhancement of immunoglobulin secretion was isotype specific. The 10C3 and 106 lines (IgM secretors) did not show any significant increase in IgM production when grown on the IL-10 transfected CD40L+ feeder layer. However, the cell lines 2G2 and 100C5 (IgG secretors) had on average 3.1 and 1.6 times increase respectively in IgG production after growth on the IL-10 transfected CD40L+ feeder line.
The EBV-transformed cell line, 045 which was not secreting immunoglobulin, was not induced to secrete any isotype of immunoglobulin examined using any of the culture conditions.
Figure 10. Immunoglobulin production by EBV cells grown in the CD40 system. The CD40 system was setup as described in the Methods section using either recombinant cytokines and CD40L* fibroblast cells or cytokine transfected CD40L* fibroblast cells. The cell lines 10C3 and 106 are IgM producing lines and the lines 2G2 and 100C5 are IgG producing lines. IgG or IgM secretion was measured after 7 days. Stimulation index was calculated as the increase in immunoglobulin secretion as compared to cells grown on untransfected CD40L* lines.
Discussion

The CD40 system (17,19), allows for increased proliferation and immunoglobulin secretion by B lymphocytes. Previous studies have used CD40 ligand expressing feeder layers with the addition of recombinant cytokines to grow B lymphocytes. We were interested in transfecting the cytokine genes directly into the CD40 ligand expressing fibroblast cells to obtain a feeder layer that was presenting CD40L and expressing cytokines directly at the site of cell-cell interactions.

IL-10, IL-6, IL-4 or IL-2 were transfected into CD40L expressing NIH 3T3 fibroblast lines. We have shown that all transfected cell lines produced mRNA transcripts for their respective cytokines. These lines were cloned, expanded and supernatants were tested for cytokine protein expression. IL-6 and IL-2 transfected cell lines produced biologically active cytokine, as demonstrated by the results of culture with the appropriate indicator cell lines. Therefore, we have successfully produced stable, functional human IL-2 and IL-6. Indicator cell lines were not available to explore the production of IL-10 or IL-4, and therefore, we used two methods to look at expression of these cytokines. In the presence of CD40L, both IL-4 and IL-10 are able to enhance the proliferation of B lymphocytes (1). We found that IL-10 transfected CD40L+ cells caused a significant increase in the proliferation of EBV-transformed cell lines. The IL-4 transfected cell line was unable to cause a significant increase in the proliferation of these same cell lines.

IL-10 and IL-4 cytokine production was also explored using ELISA. Neither cytokine was shown to be secreted using this method. There is a possibility that the antibodies in the ELISA kits used recognized an epitope that was no longer present on the recombinant protein. This possibility is suggested by our demonstration of functional IL-10. Alternatively, since the cytokine vectors were not directly sequenced, there may have been base pair mutations within the cytokine sequences which may alter their
immunoreactivity (in the case of IL-10) or in the case of IL-4, it may have resulted in loss of function or immunoreactivity. These is also a possibility that there was a block in translation of the IL-4 protein. We have no indication that the IL-4 transfected cells are producing any functional cytokine, despite the demonstration of IL-4 mRNA. However, in the case of the IL-10 transfected cells, evidence of increased proliferation and increased immunoglobulin production by EBV lymphoblastoid cells grown on IL-10 secreting fibroblast lines, gave strong evidence of IL-10 protein secretion from this cell line. Therefore we have produced a stable CD40L presenting fibroblast cells line, secreting functional human IL-10. Further experiments are required to demonstrate the presence of functional IL-4.

The cytokine-transfected feeder layers must be irradiated to prevent proliferation and overgrowth during the culture period and it is desirable for the feeder cells to be continually producing cytokine. We looked at the ability of the IL-2 transfected line to produce biologically active cytokine up to eight days after irradiation at 5000 rads. As the unirradiated IL-2 transfected cells continued to divide, they showed an increase in IL-2 production over the eight day culture period as expected. The irradiated cells were unable to proliferate, but the produced cytokine should be biologically active. Thus a constant amount of IL-2 should be present in the cell supernatant every two days. Our results demonstrated that the transfected cell line continued to produce IL-2 at a constant level for at least eight days in culture.

In previous studies (18), B cells cultured in the CD40 system with exogenous cytokines were maintained in culture and were induced to proliferate and secrete immunoglobulin. IL-4 and IL-10 induced proliferation of B cells. IL-4 caused the secretion of IgM, IgG and large amounts of IgE. A combination of IL-2 and IL-4 caused production of IgM and IgA, while IL-10 caused the production of large amounts of IgG, IgA and IgM, but no IgE. As the first step in using this system for the production of human monoclonal antibodies, we examined the ability of these cytokines to affect
immunoglobulin production on established EBV-transformed cell lines. We have expertise using EBV-transformed lines and wished to test if the proliferation and immunoglobulin production of these lines could be enhanced by co-culture with the cytokine-transfected lines. We compared the effect of the standard CD40 system with added exogenous cytokine to the effect of our cytokine-transfected cell lines on EBV-transformed lines.

The addition of exogenous recombinant IL-2 alone to CD40L stimulated cells did not significantly change the immunoglobulin secretion patterns of any of the EBV-transformed cell lines tested. Similarly, EBV-transformed lines grown on our IL-2 transfected feeder layer did not alter their immunoglobulin secretion patterns. This is not surprising as IL-2 is able to cause the proliferation of activated B cells only in the presence of other factors such as IL-4. It is likely that to see an effect on immunoglobulin production with our IL-2 transfected line, we will have to introduce another cytokine gene or mix the feeder layers to allow for the cooperation between the cytokines.

We were unable to see any increase in immunoglobulin production when our EBV-transformed cell lines were grown on the untransfected layer in the presence of recombinant IL-4 or on the transfected feeder layer. These results were surprising as IL-4 is known to promote the proliferation and differentiation of B lymphocytes. Since we were unable to show that our IL-4-transfected lines were producing any immunoreactive cytokine, it may not be surprising that no effect was demonstrated. However, the fact that the recombinant IL-4 did not have any effect may be explained by our use of an insufficient concentration of IL-4 or it may be due to that fact that exogenous IL-4 does not have an effect on our immortalized lines. It is also possible that the cells were secreting an isotype that was not examined. In EBV stimulated B lymphocytes, IL-4 induces the production of IgE (50), not detected by our IgG and IgM assays. Therefore, further studies are required to demonstrate the presence of biologically active stable IL-4-transfected CD40L+ cells.
IL-6 was able to cause an increase in immunoglobulin production in all four cell lines tested. This is consistent with information showing that IL-6 is a growth factor for malignant B cells such as multiple myeloma (35). Both recombinant IL-6 and the IL-6 transfected feeder layer were able to cause an increase in immunoglobulin production, however, this effect was much more pronounced in the IgG secreting lines than in the IgM secreting lines.

IL-10 is able to promote differentiation of B lymphocytes, induce the secretion of IgG, IgA and IgM and maintain viability in vitro culture systems (17). Recently, it has been suggested that it is IL-10 and not CD40 ligation that determines if B lymphocytes will differentiate into plasma cells (55). Using CD40L+ cells and exogenous recombinant IL-10, we were able to increase immunoglobulin production in both the IgG and IgM EBV-transformed cell lines. However, the results with the IL-10 transfected lines appeared to depend on the isotype being secreted. The IgG secreting lines were induced to secrete more IgG when grown on the IL-10 transfected feeder line, whereas the IgM secreting lines did not change significantly in their secretion pattern as compared to CD40L stimulation alone. Further experiments need to be performed to further investigate these results. Higher levels of IL-10 production by the transformed line may be necessary to affect the secretion of the IgM lines or the specific lines could be naturally less susceptible to IL-10 stimulation at that level. Our IL-10 transfected lines were likely secreting cytokine at a lower level than the added exogenous IL-10 used. It will be interesting to grow a larger variety of lines on this feeder layer to see if the pattern continues. An alternative explanation may be related to the fact that EBV-infected cells contain BCRF1, the viral interleukin 10 gene (44). This gene shares many functions with the human homologue and both are expressed in established EBV-transformed line (44). It is conceivable that the different EBV-transformed cell lines tested secreted various amounts of IL-10 and vIL-10. These variable amounts may vary the susceptibility of the lines to exogenous IL-10. It has been shown previously (51) that the endogenous cytokine
production by EBV-transformed lines affects their responsiveness to exogenous cytokines. It was observed that IgM-producing cells which did not secrete IL-6 was able to respond to recombinant IL-6 with a 5- to 10-times elevated IgM production, whereas lines which were capable of producing IL-6 showed only a minimal increase in immunoglobulin production when stimulated with recombinant IL-6 (51). Thus, it would be interesting to look at the endogenous cytokine secretion profile of our EBV-transformed cell lines to see if this could account for the differences in responsiveness to the cytokines tested. The 2G2 line did not respond as strongly to IL-10 then did the 100C5 line. In some preliminary RT-PCR experiments, it was observed that the 2G2 cell line demonstrated a strong IL-10 mRNA production as compared to the 045 line, however we do not have any data concerning endogenous IL-10 production by the 100C5 line.

Isotype switching is facilitated by CD40 ligation and directed by the cytokine profile. The ability of cytokines to cause a switch in the isotype being secreted is particularly important if using the system to culture PBL. PBL contain B lymphocytes which are at a spectrum of differentiation stages, many which would be receptive to switch factors. None of the cytokines tested were able to cause a switch in the isotype of immunoglobulin being secreted by the EBV-transformed lines. All lines were tested for IgG and IgM secretion and after stimulation they continued to secrete the same type of immunoglobulin as before stimulation. The only cell line tested which was not secreting IgG or IgM did not produce either isotype after stimulation with CD40L and the cytokines tested. It is possible that this cell line, after previous prolonged culture was no longer able to secrete immunoglobulin, was no longer receptive to class switching or it may have been secreting an isotype not tested. It is now necessary to examine the effect of our cytokine-transfected lines on PBL, which may be more receptive to class switching. This is especially relevant when looking at the antibody repertoire, since the antibodies produced in the CD40L system may not represent the isotype present in vivo due to class switching. Recently, it has been shown that it is memory B cells and not naïve B cells
which are induced to secrete immunoglobulin after CD40 ligation and cytokine stimulation (56). It was demonstrated that the naïve cells could switch to membrane IgG expression but would not differentiate past an IgD, IgG double positive stage when grown in the CD40 system with IL-4, IL-2, IL-10, IL-3 or combinations of these (56). Thus, the immunoglobulin secreting cells obtained from growing PBL in the CD40 system resulted from memory cells and not switched naïve cells.

It is important to note, that although the CD40 ligand presenting fibroblast cell line used was a murine line, the cytokines we transfected into this line were human. The fibroblast cell line may be producing murine cytokines in our cell cultures, however not all murine cytokines are biologically active on human cells. Human IL-2 can stimulate mouse T-cells at concentrations similar to mouse IL-2, however, mouse IL-2 stimulates human T cells at a much lower efficiency (46). Mouse IL-4 is inactive on human cells and human IL-4 is inactive on mouse cells (47). Human IL-6 is active on both human and mouse cells (48, 49). We did not find any effect of untransfected CD40L+ cells in our indicator systems. For our system to be effective for culturing human B lymphocytes, it was important that our feeder layer secreted human cytokines and this was done.

The effect of our IL-10 transfected line on the response of EBV-transformed cell lines was variable. Although, as previously stated, this may not be surprising. We may require other methods to demonstrate the biological activity of IL-10. There is an IL-10 responsive mast cell line now available that may provide the basis for a more sensitive assay to look at the cytokine secretion (45). Regarding IL-4, we will continue to attempt to demonstrate functional IL-4. When the transfected cell lines were generated, many clones were produced, but only one clone from each cytokine line was used in our experiments. We still have numerous clones to screen. The IL-4 clones can be screened to find an IL-4 producing feeder line. In the future, we will look at the other transfected cell lines for each cytokine and screen for high or low cytokine secretion.
In conclusion, we have been able to produce three feeder cell lines which present CD40L and secrete functionally active human cytokines. We have been able to use these lines to enhance proliferation of human EBV-immortalized B lymphocytes and to increase immunoglobulin production. Future experiments will be directed to examine the effect of these cell lines on B cells freshly isolated from peripheral blood. Peripheral blood lymphocytes cultured in the presence of our cytokine-transfected feeder cell lines could then be infected with EBV to increase the number of stable IgG lines. Our primary interest is in examining the B cell repertoire and antibody secretion patterns of autoimmune individuals and these lines are likely to be an important tool to allow for the production of stable, immortalized IgG secreting cell lines.
Appendix 1

Nucleotide sequences of cytokine gene inserts and G3PDH mRNA and location of primers used in PCR. Underlined sequences indicate the location of the primers.
Human interleukin 10 (IL10) mRNA, 1601 bp

1  aaaccacaag acagacttgcc aaaaagaagcc atgcacagct cagcactgct ctgttgccctg
61  gtctctctctgta ctggggtgag gcgcagccca gcggccagggca cccagttcga gaacagctg gc
121  acccacttcc caggcaacct gcttaaacatg ctctgagatc ttcctagcag cttcagcaga
181  gtaagaagcttacctctaat gtaagatcag cttggaacact tgttgttaaa ggatgctcttg
241  ctggaggact ttaaggtgga cttgggcttg gc aagcctttgt ctggagatgat ccagtctttac
301  ctggaggagg tggatcctgcca aacgctgacg gacagttagaataac gtcgctgac gcagagcc
361  tccctgggga aagaacctga aacgctcagg ctgaggtcact gcggcgtgtca tggatctcttt
421  cccgtgaaac acaagagcct gccgctttgg gaggcttgaag atgccttttaa taagctccaa
481  gaaaaagcct ttccttaaagc atcaggtgag ttggacatct tcatcaacta cataagaacc
541  ttcctcagaa tgaagatagc aacagctgac acctgggcttg cggactctata gaactctagga
601  cataaattag aagttctccaa aacgagcatct ggggcttccttgg gatcgcctgac ccaggccctt
661  gagaaccttt attgtacctc tcctatatga atttttattac ctctgacaccc tcaacccccca
721  ttacaatactct ggacctgacct gcaagatccat gcccttttaa aagagccc aataatttaat
781  tttaaatatttt ttcctataatttt ctcacaacct tctttgaggt tggagactag ggacacacta
841  tggatttttga gtgttgraatt ataattattat gttatcataaa gggagggaa aaaaaatttct
901  tggggaggcc aacagagacct tcctacctcc ccagtttttc ggcggcctgac ctgctgctcc
961  tttttcctga cttctctctta atttatcttg tctctgctctt gggggtctcc taactgtctac
1021  aaatactctt attgagagca accagagctc tctcaatcag attaaatttc acctcagttg
1081  ctcggaggg gttcttcctaac tcttttaacc aaccctttca ttttggagaa cgtggtcagtt
1141  cttgcttaatt ataacaacct aaatttggtt ctggccttga cgcggtgctg caagccttga
1201  atcccaagcc tttggaaggc tgaggccgggt ggtcactcctgct aggtcagag ttctcaacca
1261  gcctgttgccaa catggtgaa aacccggctcct acctaaaaata caaaaatttag ccgggcatgg
1321  tgggctgcac ctgtaacctt acgtctttgg gaggctgtag ccagagactt gctggaaccc
1381  agggatgga agttgcagtg acgctgtatc atggccctgtt actccagcct ggtgagcaga
1441  gcaagacttc tctctcttaaat aatataataa aatatatatg tggctcctaat gagaatcaagt
1501  ttttaactagg gattttggaa aatgttaata tttttgtctgt tttctcatagc
1561  agatattaat tttgaaatat ttaaatgtatc ttattcctat

Insert size: 1601 bp
PCR product: 352 bp
Human interleukin 6 (IL-6), 1128 bp

1  attctgcccct ccagccccacc gggaagcggaa gagaagctct atctccccct caggagccca
61  gctatatcctctctcaagcccttc ggtccagttg cctttctcccc ggggctgctc
121 cttggtgttgc ctgtgctcct ccctgcccccag ttgacacgaa tcagagatc acaaatgta
181 gcccgcacc acagacagcc actcactcct tcagagcggaa ttgacaaaaca aatcgggtac
241 atcctcgcag gcatctcagc cctgagaaag gagacatgtga acaagagtaa catgtgtaaa
301 agcagcaaaag aggcactggc ctgagccttc ccaagagcggaa ttgacaaaaca aatcgggtac
361 ggtgtgttgc ctggtgttgcc cttgagcttcc caatgttagatt caatgttagatt caatgttagatt
421 cctgtaatacagcgagtttcccgctctgctct cctgagaaag cctgcccccag gggaagcggaa gagaagctct atctccccct caggagccca
Human interleukin 4 (IL-4) mRNA, 614 bp

1  gatcgttagc ttcctcgtat aaactaattg cctcactattg tcaetgcaa tgcacaccta
ttaatgqqr tcaacctcca acggtttcc cccctctgttcttt tcctgctagc atgtgccggc
121 aactttgtcc acggacacaa gcggagatate accttacagg agatcatcaa aactttgaac
181 agcctcacag agcagaagac tctgtgcacc gagggtaccg taacagacat ctttgcgtgcc
241 tccaagaaca caactgagaa ggaacacctc tgcatggtctg cagctgtgct ccgactgttcc
301 tacagccacc atgagaagga cactcgctgc ctgggtgca ga ctgcacagca ttcacacagg
361 cacaagcagc tgatcggatt cctgaeaagcg etecagcaga acctetgggg cctgcgcgggc
421 ttgaattct gccctgtgaa ggaagccacag agagtacgt tggaaaactt cttggaaggg
481 ctaaagacca tcagagagaa gaaatattt caatgggttca agatggaatatt ttaatttatg
541 agctttggat agcccttttt ttaaatttt tatatatattta taacctcatca taaaataaag
601 tatatatagc atctaa

Insert size: 618 bp

PCR product: 351 bp
Human interleukin-2 (IL-2), 812 bp

1 atcactctct ttaatcacta ctcacagtta cctcaacctc tgccacaatg tacaggatgc
61 aactcctgtc ttgcattgca ctaaggctttg cactttcctac acacatgtgca cctacttcaa
121 gttctacaaa gaaaaacag ctcacactgg aagcttctgt gctggattta cagatgattt
181 tgaatggaat taataattac aagaatccca aacctcaccag gatgcctcaaa ttaaagtttt
241 acgtgcccaaa gaaggccaca gaactgaacac atcttcagtg tctagaagaa gaactcaaac
301 ctctggagga agtgctaaaat ttgctaaaa gcaaaaaactt tcactaaga cccagggact
361 taatcagcgtatcctacgta atagttctgg gactaaaggg atctgaacaca acattcatgt
421 gtgaatatgc tgatgagaca gcaaccattg tagaatttt ctgaacagatgg attacaccttttt
481 gtccttgaactgttcaca ctaacctgat aattaagtgc tttccactta aacatatca
541 ggcctttctat ttatattaaa atatatttt tatatattt attctagatgt gttgattgta tggtttgcta
601 cctattgttaa ctattatctt taattctaaa actataataa ttgatctttt attgattctttt
661 tgtaagcccc tagggtctct taaatggttt cacttatatta tcccaaaata tttattatta
721 tgttgaatgt aataatatag atctatgtat atgggttagtatactatttt atataatttg
781 ataataataaa aaaaaaaaaa caaaaaaaaaa aa

Insert size: 468 bp
PCR product: 400 bp
Human glyceraldehyde-3-phosphate dehydrogenase mRNA, 1237 bp

1 gtcgccagcc gagccacact gcczagaaac ca cctatgggga aggtagaaggt cggaggtcaac
61 ggatattggct gtattgggcg cctggctacc agggcgtgct ttaactcttg taaaagtggat
121 atgttgccca tcaatgaccc cttcattgac ctcaactaca tggtttacat gttccaatata
181 gatctccaccc atggcaaaatt ccattgacacc gtcagggctg agaagagggaa gcttggtgatc
241 aatggaaaat ccatcaccat cttcaggagag cgagactcct ccaaatctca aatgggccgat
301 gctgggctgtg agtacgctgt ggagtccact ggcgtcttca ccaccatgga gaagggctggg
361 gctcatttgc aaggggggac caaaaggggac atcatctcctg cccctctctg tgaagggccc
421 atgtttcgctca tggtgtgaa ccattgagaag tatgacaaaa gcctcagat gattcagcata
481 gcctcctgca ccaccaactg ccatgacacc cttggccaaagg tcatccatga caactttaatgtt
541 atgcgtgcaag gactcagcac cacagtcctat gccatcactg ccacccaaag gaagtctggat
601 ggcgtcccctcg ggaaaaacttg ggcgtgctggc cgcgggggctc tccagaaagat cattccttggcc
661 tctactggtgc ctggccaggg ctgtggcaagag tgcagctcctg ctgtggaacgg gagaacctcctg
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961 cttcattttctc ggtatgcaaca cgaatgtgggcc tttttttttttt tcagcactca cttctccagagt
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1201 cctgtgctatg tacctcaata aagtaccttg gccttacc

PCR product: 997 bp
References


