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Long-Term Human B Cell Lines Dependent on Interleukin-4 and Antibody to CD40

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CD40 is a 45- to 50-kilodalton transmembrane glycoprotein expressed on B lymphocytes, epithelial cells, and some carcinoma cell lines. Human resting B lymphocytes entered a state of sustained proliferation when incubated with both the mouse fibroblastic Ltk$^-$ cell line that had been transfected with the human Fc receptor (FcγRII/CDw32) and monoclonal antibodies to CD40. In combination with interleukin-4, factor-dependent long-term normal human B cell lines were generated that were consistently negative for Epstein-Barr viral infection. Thus, cross-linking of CD40 is likely to represent an important phenomenon in the clonal expansion of B cells.

ALTHOUGH FACTOR-DEPENDENT human T cell lines and clones have been available for more than a decade, all attempts to reproducibly generate factor-dependent human B cell lines have failed (*1*), and combinations of agonistic antibodies and cytokines have essentially resulted in short-term proliferation of B cells (*2*). Here we show that interleukin-4 (IL-4) and monoclonal antibodies (MAbs) to CD40 (anti-CD40), presented in a cross-linked fashion by transfected mouse Ltk$^-$ cells that are stably expressing human FcγRII/CDw32 (*3*), permit establishment of factor-dependent long-term human B cell lines that were consistently free of Epstein-Barr virus infection.

Resting T cells strongly proliferate in response to MAbs to CD3 (anti-CD3) presented on irradiated fibroblastic mouse Ltk$^-$ cells expressing FcγRII/CDw32 (*3*) (CDw32 L cells). This proliferation results from the cross-linking of CD3 molecules on the surface of T cells after the Fc portion of the MAb binds with CDw32 (*4*). A variety of MAbs specific for various B cell surface antigens were tested for their capacity to induce the proliferation of highly purified human resting B lymphocytes cultured in the presence of irradiated CDw32 L cells (Table 1). Monoclonal antibodies 89 and G28-5, both specific for CD40, induced B cell proliferation, whereas MAbs to CD19, CD21, CD23, CD24, and CD37 did not. Monoclonal antibody 89–induced proliferation was dependent on the expression of CDw32 by L cells, since untransfected L cells or L cells transfected with HLA class I antigens did not allow MAb 89 to induce B cell proliferation (*5*). B cell proliferation occurred over a wide range of MAb 89 concentration and was maximal at 30 ng per milliliter ($\approx 10^{-10}$ M). Proliferation of B lymphocytes induced by MAb 89 + CDw32 L cells was sustained for at least 10 days after the onset of culture (Fig. 1). Thus, anti-CD40, in addition to well-known costimulatory effects on anti–immunoglobulin M (IgM)–activated B cells (*6*), can exert a direct proliferative effect on B cells when appropriately presented. Because resting B cells barely proliferate in response to soluble MAb 89, cross-linking of the CD40 antigen is probably required for B cells to proliferate. The present procedure, which is based on the use of CDw32 expressing fibroblast cells, is particularly efficient in inducing B cell proliferation, and immobilization of MAb 89 on the solid phase or cross-linking of MAb 89 with a goat antibody to mouse Ig (*5*) induced a less intense cell proliferation.

Addition of recombinant IL-4 to cultures strongly enhanced MAb 89–induced [^{3}H]thymidine incorporation (Fig. 1). In contrast, IL-2 only weakly enhanced anti-CD40–induced proliferation (*5*). Monoclonal antibody 89 induced multiplication of cultured B cells (Fig. 2A). In contrast, without MAb 89 a few viable B cells could be

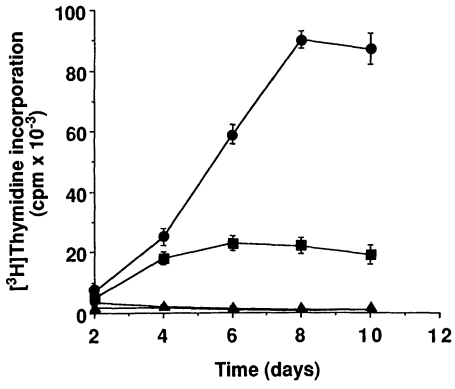

Fig. 1. Anti-CD40–induced proliferation of normal B lymphocytes is long lasting and enhanced by IL-4. Purified spleen B cells (2.5×10^4, >97% CD20+) were cultured in flat-bottom microwells for up to 10 days on 2.5×10^3 irradiated CDw32 L cells without MAb 89, without IL-4 (□); without MAb 89, with IL-4 (100 U/ml) (▲); with MAb 89, without IL-4 (■); with MAb 89, with IL-4 (100 U/ml) (●). IL-4 is a purified recombinant protein (specific activity; 10^7 U/mg) obtained from Schering-Plough Research. [^{3}H]Thymidine pulses (16 hours) were performed daily. Results are means ± SD of triplicates.

Table 1. Resting human B cells proliferate in response to MAbs to CD40 presented by CDw32-transfected L cells. Quiescent human B cells were purified by negative selection and Percoll density sedimentation from tonsils as described (*14*). B cells (10^5, 98% CD20$^+$) were cultured on irradiated (7500 rads) CDw32 L cells (10^4) in medium (*14*). Monoclonal antibodies (all IgG1, which react best with CDw32) were added to cultures at increasing concentrations (0.01 to 10 μg/ml). Only results obtained with 1 μg/ml are shown, as they are typical of the experiment. Monoclonal antibodies G28-5 and 8F1 were provided by E. A. Clark and J. F. Cantaloube, respectively. B4 was from Coulter (Hialeah, Florida) and IOB3 and IOB1 were from Immunotech (Marseilles, France). A 16-hour [^{3}H]thymidine pulse (1 μCi/well) was performed on day 3. Results are means ± SD of triplicates. ND, not determined. Results are representative of three experiments.

MAb	CD	[^{3}H]Thymidine incorporation (cpm × 10^{-3})	
		Exp. 1	Exp. 2
0		2.2 ± 0.4	1.3 ± 0.1
MAb 89	40	68.2 ± 1.4	15.0 ± 1.5
G28-5	40	ND	17.8 ± 1.3
B4	19	ND	1.3 ± 0.1
8F1	21	2.4 ± 0.5	ND
MAb 25	23	1.9 ± 0.4	ND
IOB3	24	ND	1.3 ± 0.1
IOB1	37	ND	1.2 ± 0.1

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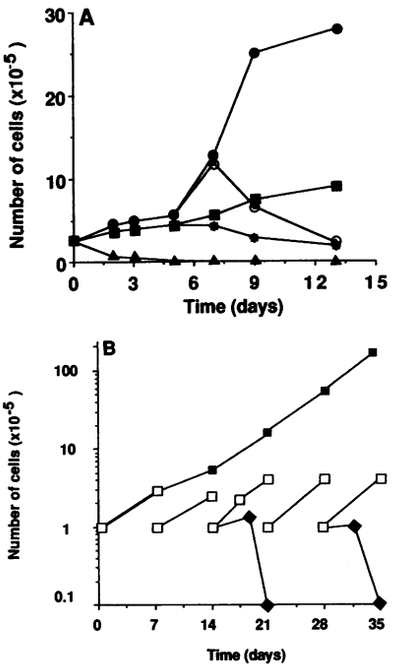
Fig. 2. Normal human B cell lines dependent on anti-CD40 and IL-4. (**A**) Purified spleen B cells (2×10^5) in 500 μl of complete culture medium were seeded in wells of 48-well microplates containing irradiated CDw32 L cells (2.5×10^4) with MAb 89 (1 μg/ml) with or without IL-4 (100 U/ml). Cultures performed with either MAb 89 or MAb 89 and IL-4 were divided at day 5 into two wells plated with irradiated CDw32 L cells (2.5×10^4) with or without the original stimulant. Enumeration of viable B cells (trypan blue exclusion) was done with a hemocytometer. No activator (▲); MAb 89, whole culture (■); MAb 89, first 5 days (*); MAb 89, IL-4, whole culture (●); MAb 89 + IL-4, first 5 days (○). (**B**) Purified tonsillar B cells were cultured at 10^5 per well on 2.5×10^4 irradiated CDw32 L cells with MAb 89 (1 μg/ml) and IL-4 (100 U/ml) in 500 μl. Cells were enumerated at the indicated time and cultures were divided every week at 10^5 per well on freshly irradiated CDw32 L cells with fresh medium and activators (□). Black squares (■) represent the theoretical number of generated B cells. At day 14 and 28, cell portions were recultured on CDw32 L cells without MAb 89 and IL-4 (◆). The two experiments presented here have been reproduced in more than 30 long-term normal human B cell lines.



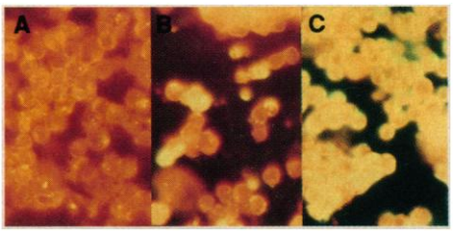


Fig. 3. Epstein-Barr virus–free long-term B cell proliferation with anti-CD40– and CDw32-transfected L cells. Epstein-Barr nuclear antigen staining (*15*) of (**A**) day 28 B cells, (**B**) day 28 B cells infected with B95-8 EBV for 36 hours, and (**C**) day 28 B cells infected with B95-8 EBV for 7 days. Yellow-green fluorescence indicates the presence of EBNA proteins.

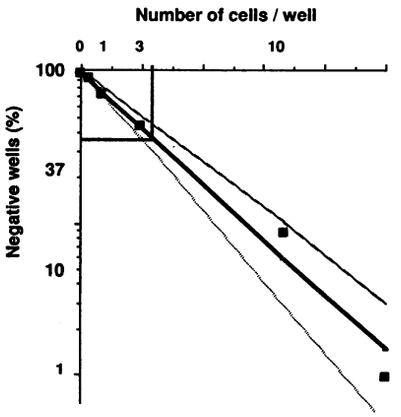


Fig. 4. Anti-CD40 and CDw32-transfected L cells induce the clonal expansion of 1 of 3.5 B cells. Wells (96) were seeded with either 0.3, 1, 3, 10, 30, 100, or 300 B cells with 2.5×10^3 irradiated CDw32 L cells with MAb 89 (1 μg/ml) and IL-4 (100 U/ml) in 100 μl. Complete medium (50 μl) was added at days 3 and 5, and 100 μl of medium was replaced by new medium at day 10. Clone frequency was scored at day 15, and linear regression analysis was used to calculate the best slope (*16*). Confidence intervals around the slope were calculated by Student's *t* test, and conformance of the data to linearity was tested by using a χ^2 analysis, which indicated a single hit curve, thus demonstrating activity on a single limiting cell type.

recovered in cultures after 5 days. The addition of IL-4 to anti-CD40 cultures resulted in continuous cell growth, representing, at day 9, a 10- to 15-fold increase of the B cell input. Thus IL-4, in addition to being a T cell growth factor (*7*), acts as a B cell growth factor. Phenotypic analysis on proliferating cells confirmed their B lineage, as they express CD19 and CD20, but not CD3, and they secrete immunoglobulins (*8*). Cultures of proliferating cells could be divided and further proliferation could be observed (Fig. 2B). Within 5 weeks, the total B cell population expanded theoretically 150 to 400 times. Such B cell lines could be frozen and thawed, maintained for up to 10 weeks (*9*), and generated from B cells originating from peripheral blood, cord blood, spleen, and tonsils (*9*). These B cell lines, which grew in clumps, did not express the EBNA2 (Epstein-Barr nuclear antigen 2), as determined either by polymerase chain reaction or by immunofluorescence analysis (Fig. 3). These B cell lines could be infected by Epstein-Barr virus (EBV) and consequently expressed EBNA antigens. Seeding these anti-CD40 + CDw32–activated B cells on CDw32 L cells without MAb 89 and IL-4 (Fig. 2, A and B) halted cell proliferation and resulted in cell death, demonstrating the factor dependency of these B cell lines. Cell cycle analyses with acridine orange (*10*) have shown that at least 70 to 80% of B cells enter into the G1 phase of cycle and 50 to 60% into the S phase on stimulation with MAb 89 and CDw32 L cells (*5*). Limiting dilution studies have shown that 20 to 30% of the resting B cells stimulated by this procedure can generate B cell clones of 50 to a few hundred cells after 15 days of culture (Fig. 4). B cell lines cultured for 20 days presented a normal karyotype.

Our results show that normal human B lymphocytes can be induced to proliferate on cross-linking of the CD40 antigen; the continuous proliferation observed in the presence of IL-4 allowed us to generate specific factor-dependent human B cell lines. Soluble antibodies to CD40 can prevent the apoptosis of tonsil germinal center B cells that express surface IgG and it was thought that the CD40 cross-linking might represent an important phenomenon in the antigen-driven selection of B cells in germinal centers (*11*). Our present data therefore confirm and extend this observation. However, in other experiments we found that high-density resting B cells expressing surface IgD and IgM (mantle zone B cells) proliferate in response to CD40 cross-linking at least as well as low-density B cells that poorly express surface IgD and surface IgM (germinal center B cells), thus suggesting that the agonistic signals observed after cross-linking of CD40 may not be restricted to germinal center B cells. Thus, it is now important to determine whether the CD40 antigen is a receptor for either a membrane counterstructure expressed on cells interacting with B cells (for example, T cells and dendritic cells) or a cytokine, as suggested by the homology of CD40 with nerve growth factor receptor (*12*) and tumor necrosis factor receptors (*13*). In conclusion, we anticipate that these B cell lines will be most useful for the study of activation and regulation of B cell growth and differentiation. This novel approach may ultimately allow us to establish antigen-specific human B cell lines and clones.

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8. Cell lines cultured in the absence of IL-4 were found to secrete IgG, IgA, and IgM. Addition of IL-4

enhances IgG and IgM production and induces secretion of IgE [F. Rousset, E. Garcia, J. Banchereau, unpublished data].
9. Established cell lines were found to die out after 10 weeks for presently unexplained reasons. Induction of B cell proliferation with immobilized anti-CD40 turned out to be a very constant feature over 30 months and more than 30 B cell lines have been established from various sources. More than 100 experiments with 60 different tonsils, 18 experiments with cord blood, 30 experiments with peripheral blood, and 18 experiments with four different spleens have shown proliferation of B cells with anti-CD40 + CDw32 L cells.
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17. We thank K. Moore (DNAX) for providing CDw32 L cells and continuous interest through the present study, G. Lenoir for testing EBNA protein expression, J. M. Seigneurin for testing EBNA2 expression by polymerase chain reaction, A. M. Manel for karyotype analysis, A. Waitz, R. Callard, and H. Martinez-Valdez for reading the manuscript, and N. Courbière for expert editorial assistance. A.V. was supported by the Mérieux Fondation.

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Expression cDNA Cloning of the KGF Receptor by Creation of a Transforming Autocrine Loop

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An expression cloning strategy was devised to isolate the keratinocyte growth factor (KGF) receptor complementary DNA. NIH/3T3 fibroblasts, which secrete this epithelial cell–specific mitogen, were transfected with a keratinocyte expression complementary DNA library. Among several transformed foci identified, one demonstrated the acquisition of specific high-affinity KGF binding sites. The pattern of binding competition by related fibroblast growth factors (FGFs) indicated that this receptor had high affinity for acidic FGF as well as KGF. The rescued 4.2-kilobase complementary DNA was shown to encode a predicted membrane-spanning tyrosine kinase related to but distinct from the basic FGF receptor. This expression cloning approach may be generally applicable to the isolation of genes that constitute limiting steps in mitogenic signaling pathways.

GROWTH FACTOR SIGNALING PATHways have critical roles in normal development and in the genetic alterations associated with the neoplastic process (*1*). The isolation of cDNAs for important components of these pathways has generally involved difficult and time-consuming protein isolation and purification. Cloning methods based on transient expression assays (*2*) also require laborious screening procedures, sensitive biologic assays, or the availability of immunologic reagents capable of recognizing the gene product. In certain cases, naive cells have been shown to inherently express all of the necessary intracellular components required for effective mitogenic signaling if a given receptor can be artificially expressed and the appropriate ligand can be provided (*3*). Moreover, completion of an autocrine loop involving expression of a ligand and its receptor by the same cell can be associated with uncontrolled proliferation and malignant transformation (*4*). We reasoned that the stable ectopic expression of cDNA clones might result in the transformed phenotype if an autocrine loop were created, for example, by introduction of a growth factor receptor cDNA into cells expressing only its ligand. We have described a directional cDNA library expression vector that has all of the properties likely to be useful in testing this approach, including the capacity for plasmid rescue, a selectable marker, and a strong constitutive promoter (*5*). The ability of the method to synthesize long cDNAs with complete coding sequences has also been shown (*6*).

Keratinocyte growth factor (KGF) has potent mitogenic activity for a wide variety of epithelial cells but lacks detectable activity on fibroblasts or endothelial cells (*7*). Its synthesis by stromal fibroblasts in a large number of epithelial tissues has suggested that KGF is an important paracrine mediator of normal epithelial cell proliferation (*8*). Studies have further indicated specific KGF binding to keratinocytes but not fibroblasts (*9*). Thus, we sought to test the feasibility of our expression cloning strategy in the search to identify and functionally clone the receptor for this new growth factor.

We prepared a cDNA library (4.5×10^6 independent clones) from BALB/MK epidermal keratinocytes (*10*) in an improved vector, λpCEV27 (*11*), and transfected NIH/3T3 mouse embryo fibroblasts (*12*), which synthesize KGF (*13*). We detected 15 transformed foci among a total of 100 individual cultures. Each was shown to be resistant to G418, indicating that it contained integrated vector sequences. Three representative transformants were chosen for more detailed characterization based on differences in their morphologies. Several plasmids were isolated from each transformant after plasmid rescue (*14*). A single cDNA clone rescued from each transformant was found to possess high-titered transforming activity ranging from 10^3 to 10^4 focus-forming units per nanomole of DNA. Transfectants induced by the individual plasmids containing these epithelial cell-transforming cDNAs (designated *ect*1, *ect*2, and *ect*3) were used in subsequent analyses.

To investigate the possibility that any of the three genes might encode the KGF

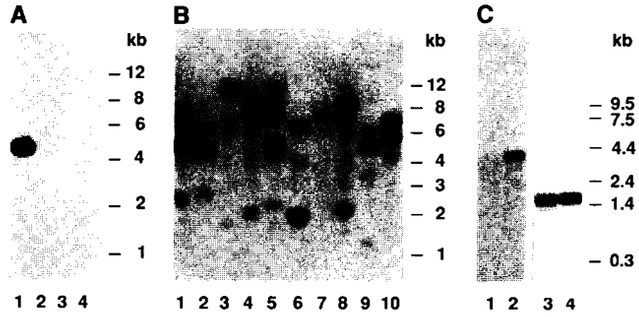

Fig. 1. Genomic analysis of *ect*1 DNA and comparative RNA expression. (**A**) Southern analysis of the Sal I–digested DNAs from NIH/3T3 and its transformants. The blot was probed (*28*) with the entire *ect*1 cDNA insert. Lane 1, NIH/*ect*1; lane 2, NIH/*ect*2; lane 3, NIH/*ect*3; lane 4, NIH/3T3. (**B**) Southern analysis of Eco RI–digested DNAs of different animal species (Clontech Labs, Inc.). The blot was probed (*28*) with the 5′-half of the *ect*1 cDNA insert (Fig. 2B) and washed under reduced stringency conditions. Lane 1, human; lane 2, rhesus monkey; lane 3, mink; lane 4, cat; lane 5, mouse; lane 6, cow; lane 7, chicken; lane 8, dog; lane 9, guinea pig; lane 10, pig. (**C**) Northern analysis of NIH/3T3 and BALB/MK RNA. The blot was probed (*29*) with the 5′-half of the *ect*1 cDNA (lanes 1 and 2) or a β-actin cDNA (lanes 3 and 4) and washed under stringent conditions. Lanes 1 and 3, NIH/3T3; lanes 2 and 4, BALB/MK.

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