stable in neurons than in glial cells. Another possibility is that the virus may be transferred from one cell type to another, as has been described for rabies and herpes viruses (*12*).

The use of adenovirus vectors provides a method to study the function of cloned genes, which is complementary to that of transgenic animals. For instance, infection of the hippocampus would be useful for the study of integrated phenomena such as long-term potentiation. The possibility of selecting the time at which a particular gene is to be expressed is important when the expression of a transgene in early development is deleterious to the animal. In the context of degenerative diseases, it may also be possible to express neurotransmitters or growth factors locally as an alternative to the grafting of fetal cells.

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13. The SCG were removed from 2-day-old Wistar rats, dissociated, plated on 16-mm collagen-coated dishes, and cultured as described (*7*). Cytosine arabinofuranoside (10 μM) was added during the first week of culture to prevent proliferation of ganglionic non-neuronal cells. After 6 days in culture, the cells were inoculated with 10^6 PFU of Ad.RSVβgal in culture medium or, as a control, exposed only to culture medium. Twenty-four hours later, the virus was removed, and the cells were maintained for 2 days in culture medium. After washing and paraformaldehyde fixation, β-gal–expressing cells were characterized with X-gal histochemistry.
14. Cells were plated in 35-mm-diameter plastic dishes and grown in supplemented Dulbecco's modified Eagle's medium for 5 days. The cells in each dish were then inoculated with 2 μl of the adenoviral solution (titer, 10^8 PFU/ml) for 24 hours. Histochemical staining was processed as in (*13*).

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CD40 Ligand Gene Defects Responsible for X-Linked Hyper-IgM Syndrome

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The ligand for CD40 (CD40L) is a membrane glycoprotein on activated T cells that induces B cell proliferation and immunoglobulin secretion. Abnormalities in the CD40L gene were associated with an X-linked immunodeficiency in humans [hyper-IgM (immunoglobulin M) syndrome]. This disease is characterized by elevated concentrations of serum IgM and decreased amounts of all other isotypes. CD40L complementary DNAs from three of four patients with this syndrome contained distinct point mutations. Recombinant expression of two of the mutant CD40L complementary DNAs resulted in proteins incapable of binding to CD40 and unable to induce proliferation or IgE secretion from normal B cells. Activated T cells from the four affected patients failed to express wild-type CD40L, although their B cells responded normally to wild-type CD40L. Thus, these CD40L defects lead to a T cell abnormality that results in the failure of patient B cells to undergo immunoglobulin class switching.

Human hyper-IgM immunodeficiency is a rare disorder characterized by normal or elevated serum concentrations of polyclonal IgM and markedly decreased concentrations of IgA, IgE, and IgG (*1*, *2*). Reports of X-linked, autosomal recessive, autosomal dominant, and acquired forms of the disorder indicate genetic heterogeneity and that several different pathologic mechanisms may be responsible (*2*, *3*). In the X-linked form of hyper-IgM syndrome, affected males usually experience the onset of recurrent infections in the first year of life. Affected males have normal numbers of circulating B and T lymphocytes, although lymph node hyperplasia with an absence of germinal centers is common (*1*, *2*). This condition is lethal in the absence of medical intervention; however, patients typically respond well to a maintenance therapy consisting of intravenous treatment with γ globulin.

The cellular abnormalities that underlie the various forms of hyper-IgM syndrome are unclear. Studies of patterns of X chromosome inactivation in obligate carrier females indicate a randomized pattern of X chromosome usage in either B or T lineage cells, which suggests that the defect does not alter maturation of these cells by cell autonomous mechanisms (*4*). Some studies have suggested that the affected phenotype is likely a result of B cell dysfunction insofar as patient B cells treated with polyclonal B cell activators, such as pokeweed mitogen, could not be induced to switch to IgG or IgA production (*5*). Other reports suggest that the

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intrinsic immunoglobulin heavy chain class switch mechanism is intact and, further, that patient B cells can be induced to undergo isotype switching when cultured in vitro with a specific T cell line (*4*, *6*).

A number of studies have focused on the elucidation of the mechanisms involved in immunoglobulin isotype switching. The CD40 cell surface antigen, which is important in B cell proliferation and differentiation, acts as a receptor capable of transmitting a signal in B cells (*7*, *8*). The combination of interleukin-4 (IL-4) and CD40 antibody can induce B cells to switch, resulting in the production of IgG and IgE (*9*).

A naturally occurring ligand for CD40 (CD40L) has recently been cloned (*10–12*). This ligand is a membrane-bound protein expressed in large amounts on activated $CD4^+$ T cells (*11*). The CD40L induces B cell proliferation in the absence of any costimulus and can also induce the production of IgE and other immunoglobulin isotypes in the presence of cytokines (*11*, *13*). This stimulation is specific and can be blocked by excess soluble CD40.

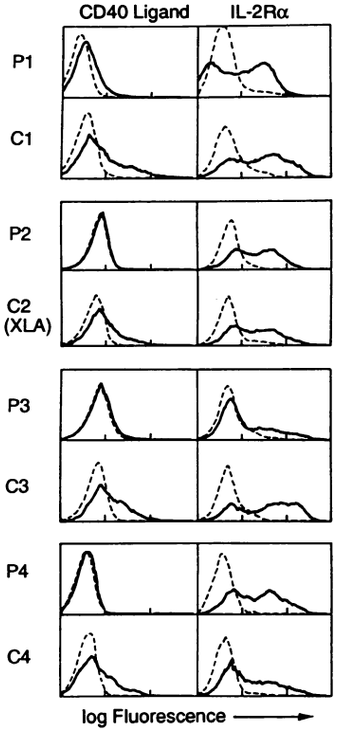

Fig. 1. Activated hyper-IgM patient T cells do not express CD40L. Peripheral blood T cells were purified from hyper-IgM patients (P1 through P4) and normal (control) donors (C1 through C4) as described (*30*) and cultured for 16 hours in the presence or absence of immobilized CD3 MAb. Cells were then stained with a biotinylated soluble construct of CD40 (CD40.Fc; 5 μg/ml) and streptavidin-phycoerythrin or with fluorescein isothiocyanate (FITC)–conjugated antibody to IL-2Rα (CD25) and analyzed by flow cytometry. For both CD40L and IL-2Rα expression, the staining of cells stimulated with CD3 is shown by a solid line and the staining of unstimulated cells shown by a dashed line. Stimulated cells from both patients and controls showed no detectable binding of biotinylated IL-4R.Fc or FITC-conjugated isotype-matched antibody used as specificity controls.

Using a cDNA corresponding to the coding region of the murine CD40L gene, we mapped the chromosomal locations of the murine CD40L gene (*CD40l*) to the proximal region of the murine X chromosome, linked to *Hprt* (*14*). *HPRT* maps to the q26 region of the human X chromosome, which suggests that the human homolog of the CD40L would also map to this region. This has recently been confirmed by Graf *et al.* (*12*) and by our own in situ hybridization studies (*15*). The in vitro biological data on the CD40L together with its chromosomal location suggested that this gene may function in X-linked hyper-IgM immunodeficiency syndrome.

The CD40L gene was examined in four patients whose clinical and laboratory findings are consistent with primary X-linked hyper-IgM syndrome. Three of these patients represent sporadic cases with no similarly affected male relatives. The fourth patient belongs to a family with a documented three-generation pedigree showing a classic X-linked inheritance of the disease.

The expression of CD40L was examined directly on peripheral blood T cells purified from the four hyper-IgM patients. Peripheral blood leukocytes (PBLs) were purified from heparinized whole blood from patient or control donors by separation over Ficoll-Hypaque. T cells were activated with immobilized CD3 monoclonal antibody (MAb) and analyzed by flow cytometry in the presence of a soluble form of CD40. This soluble CD40 protein (CD40.Fc) has been described and is a chimeric protein containing the extracellular domain of human CD40 fused to the Fc region of human IgG1 (*16*) (Fig. 1). All experiments included control cells from normal adult donors (*17*) and, in the case of patients 1, 3, and 4, from age-matched unaffected males. Control cells for patient 2 included an age-matched, race-matched male diagnosed with X-linked agammaglobulinemia (XLA), an unrelated immunodeficiency. In contrast to T cells from control donors, T cells from patients 2, 3, and 4 failed to express any detectable CD40L upon activation by CD3 antibody. In some experiments, activated T cells from patient 1 appeared to weakly bind CD40. The activation of patient T cells did result in the expression of the α chain of the IL-2 receptor (IL-2Rα), a common T cell surface activation marker, in amounts comparable to those seen on T cells from control donors. In addition, T cells from hyper-IgM patients showed normal proliferative responses to phytohemagglutinin A (PHA) or CD3 MAb plus IL-7 (*18*).

Purified PBLs were incubated overnight with immobilized CD3 antibody or PHA. RNA was extracted from the stimulated cells and used to generate cDNA, which served as a template for polymerase chain reactions (PCRs) (*19*). In these experiments, control cells were provided by a normal adult donor or, in one case, a male child diagnosed with X-linked lymphoproliferative disorder (XLP), an unrelated immunodeficiency, who was included as a control for patient 1. Nucleotide sequence analysis of the resultant cDNAs indicated that single point mutations occurred in the CD40L of three of the four hyper-IgM patients. To ensure that these changes were not artifacts introduced during PCR amplification, we performed all reactions, including the initial cDNA synthesis reaction, at least in duplicate. Each nucleotide change was unique, and all occurred in the extracellular domain of the CD40L. These changes resulted in the following amino acid changes, numbered from the initiating methionine: a Gly to Val change at position 227 in patient 1; a Leu to Pro change at position 155 in patient 2; and a Thr to Asp change at position 211 for patient 3. The CD40L cDNA generated from patient 4 did not appear to contain any nucleotide changes within the coding region. Because the biological analysis performed on this patient clearly indicates a

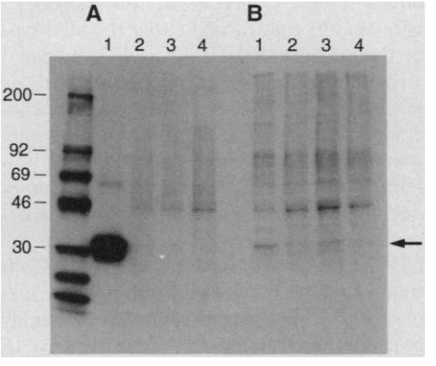

Fig. 2. Immunoprecipitations of recombinant wild-type and mutant CD40L. Cells were transfected with wild-type or mutant CD40L cDNAs with DEAE-dextran. On day 3 after transfection, cultures were incubated with ^{35}S Trans-label (100 μCi/ml) for 3 hours and then lysed in phosphate-buffered saline plus 1% nonionic detergent. Lysates from cells that expressed wild-type CD40L (lanes 1), mutated CD40L corresponding to patient 1 (lanes 2), mutated CD40L corresponding to patient 2 (lanes 3), or vector alone (lanes 4) were precipitated with CD40.Fc protein (**A**) or a polyclonal antiserum to CD40L (**B**). Precipitates were analyzed on 8 to 16% gradient polyacrylamide-SDS gels. The arrow indicates the position of the CD40L. Molecular weight markers are shown on the left in kilodaltons.

Table 1. Mutagenized recombinant CD40Ls are not biologically active. [^{3}H]Thymidine incorporation by 1 × 10^5 purified tonsil B cells was determined after 4 days of culture. IgE secretion by 1 × 10^5 purified tonsil B cells was measured after 10 days of coculture with IL-4 (5 ng/ml). Individual cell cultures were performed with CV-1/EBNA cells transfected with vector alone or wild-type or mutagenized CD40Ls (*28*).

Transfected CV-1/EBNA cells (*n*)	Transfection							
	Vector alone		Wild-type		CD40L mutation 1		CD40L mutation 2	
	[^{3}H]Thymidine incorporation (cpm)	IgE (ng/ml)	[^{3}H]Thymidine incorporation (cpm)	IgE (ng/ml)	[^{3}]Thymidine incorporation (cpm)	IgE (ng/ml)	[^{3}H]Thymidine incorporation (cpm)	IgE (ng/ml)
3 × 10^4	425 ± 93	<0.3	6463 ± 911	25.0 ± 2.8	375 ± 122	<0.3	407 ± 92	<0.3
1 × 10^4	450 ± 38	<0.3	4553 ± 405	5.7 ± 2.3	496 ± 170	<0.3	367 ± 64	<0.3
3 × 10^3	414 ± 54	<0.3	1710 ± 171	1.4 ± 0.4	398 ± 143	<0.3	501 ± 116	<0.3

lack of functional CD40L, another mechanism, possibly involving the 5′ or 3′ noncoding sequences, must be responsible for the absence of the CD40L on T cells in patient 4. No nucleotide changes were found in the CD40L cDNAs from any control samples.

To test whether these nucleotide changes affected the expression of the CD40L or its ability to bind to CD40 and were not simply normally occurring gene polymorphisms, we introduced the two nucleotide changes found in patient 1 and patient 2 into a mammalian expression vector that contained the complete coding region for the human CD40L (*20*). The introduction of the appropriate nucleotide change was confirmed in the actual expression vector by sequence analysis of the entire coding region. The human embryonic kidney cell line, 293, was transfected with vectors that carried either the wild-type or the mutagenized CD40Ls, and on day 3 after transfection, the cells were radioactively labeled with ^{35}S Trans-label (ICN Radiochemicals, Irvine, California). Cell lysates were examined for expression of CD40L protein by precipitation with a polyclonal serum directed against CV-1/EBNA cells that expressed the human CD40L or with the CD40.Fc chimeric protein (Fig. 2). Cells transfected with the wild-type CD40L expressed a 33-kD protein that can be readily precipitated with the CD40.Fc protein. In contrast, cells transfected with either mutant form of CD40L did not express a protein that is recognized by the CD40.Fc chimera. Immunoprecipitation of identical lysates with the polyclonal antiserum, however, resulted in the recognition of a 33-kD protein from cells that have been transfected by mutant as well as wild-type CD40Ls. This protein comigrated with the CD40L protein recognized by the CD40.Fc and was not present in lysates transfected with vector alone. Consistent with these results, Northern (RNA) blot analysis showed similar amounts of CD40L-specific RNA in both wild-type– and mutant-transfected cells (*21*).

Transfected CV-1/EBNA cells were also examined by flow cytometric analysis. Consistent with the data shown in Fig. 2, cells transfected with either mutant form of CD40L were completely negative for CD40.Fc binding, whereas cells that expressed the wild-type CD40L showed strong CD40.Fc binding (*22*). To address the biological activity of the mutant CD40L proteins, we examined cells transfected with wild-type or mutagenized CD40Ls for their ability to induce proliferation and IgE secretion from purified tonsil B cells cocultured with IL-4 (Table 1). In contrast to cells transfected with wild-type ligand, cells that expressed either form of mutagenized CD40L were unable to induce B cell proliferation or IgE secretion, which confirms the absence of functional CD40L on their cell surfaces.

To address the ability of X-linked hyper-IgM B cells to respond to wild-type CD40L, we performed proliferation and isotype secretion assays. T cell–depleted PBLs (B cell–enriched populations) were analyzed 4 days after culture with recombinant CD40L, and counts per minute (cpm) from [^{3}H]thymidine incorporation indicated no significant differences between patient and control cultures. Representative data derived from patient 1 and control 1 showed 12,436 ± 1,037 and 14,878 ± 1,178 cpm, respectively (*23*).

The culture of single-donor PBLs in the presence of IL-4 results in the production of IgE (*16*, *24*). PBLs from normal donors (controls 1, 3, and 4) produced measurable amounts of IgE when cultured with IL-4 (Table 2). In contrast, no IgE production was detected from any of the four hyper-IgM patients' PBLs cultured under the same conditions. Significantly, in three out of four cases (patients 1, 2, and 4), the addition of recombinant CD40L or the CD40 MAb G28-5 to cultures that contained the PBLs of hyper-IgM patients restored their ability to secrete IgE. Similarly, the T cell–depleted PBLs (B cell–enriched cultures) from these three patients and from all controls examined secreted IgE in the presence of IL-4 plus either recombinant CD40L or G28-5 antibody (Table 2). In the case of patient 3, no IgE was detected in PBLs cultured with IL-4 and recombinant CD40L or G28-5 antibody. The reason for these results is unclear. Additional PBLs were not available from this patient;

Table 2. PBLs from hyper-IgM patients can secrete IgE. IgE secretion from 1 × 10^5 unfractionated or T cell–depleted PBLs was measured after 10 days of coculture with IL-4 (5 ng/ml), together with G28-5 (200 ng/ml) or with CV-1/EBNA cells transfected with CD40L (*29*). Control 2 is an XLA individual.

Stimulus	IgE (ng/ml)							
	Patient 1	Control 1	Patient 2	Control 2	Patient 3	Control 3	Patient 4	Control 4
				Unfractionated PBLs				
Vector alone	<0.3	8.6 ± 1.2	<0.3	<0.3	<0.3	16.3 ± 2.9	<0.3	22.9 ± 3.7
CD40L	43.3 ± 4.8	73.4 ± 6.7	51.9 ± 12.4	<0.3	<0.3	48.1 ± 4.4	33.5 ± 6.0	25.7 ± 4.0
G28-5	76.4 ± 6.8	87.1 ± 9.8	54.7 ± 12.5	<0.3	<0.3	87.2 ± 5.8	55.0 ± 8.6	84.4 ± 8.2
				T cell–depleted PBLs				
Vector alone	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
CD40L	39.2 ± 4.4	128.4 ± 13.6	22.6 ± 3.1	<0.3	<0.3	55.1 ± 4.6	23.1 ± 6.3	80.2 ± 8.3
G28-5	69.4 ± 6.2	146.0 ± 12.2	34.4 ± 11.9	<0.3	<0.3	93.5 ± 6.1	45.5 ± 6.0	98.0 ± 9.5

thus, it was not possible to determine whether this lack of response was reproducible or a result of experimental variation.

Some of the immune pathologies associated with hyper-IgM syndrome support what has already been suggested (*7, 8, 11, 13*) as an expanded role for CD40 and CD40L in immune development. Galy and Spits (*8*) have recently published experiments that show that antibody to CD40 in the presence of IL-1 and interferon-γ results in an increase of granulocyte-macrophage colony-stimulating factor secretion. It is possible, therefore, that the frequent neutropenia seen in hyper-IgM patients is an indirect result of diminished CD40L expression. Likewise, the absence of lymph node germinal centers sometimes seen in these patients is compatible with data from Liu *et al.*, who have shown that centrocytes, precursors of antigen-specific B cells abundant in germinal centers, can be rescued from apoptosis by activation through their surface CD40 molecules (*25*).

Owing in part to the rarity of the hyper-IgM disorder, the lack of distinction between primary and acquired forms of the disease, and the necessity of assessing patients who receive different forms of supportive therapies, the published clinical descriptions of hyper-IgM patients vary widely. It is generally accepted, however, that patients with this syndrome do not express IgE. This strongly implicates the CD40L as being required (in conjunction with IL-4) for production of IgE in vivo and suggests that it likely helps induce switching to all other isotypes. In addition, recent results suggest that signaling through CD40 may be critical for the production of all isotypes other than IgM. Whereas IgG4 and IgE secretion appears to depend on CD40L and IL-4 acting in concert (*9, 11*), the production of other IgG isotypes and IgA is achieved by the triggering through CD40 in the presence of IL-2, IL-10, and in some instances transforming growth factor–β (*9, 13, 26*). Thus, it is tempting to speculate that defective expression of CD40L would result in the inability to rearrange all immunoglobulin heavy chain genes downstream from the μ chain and that a CD40L deficiency may manifest a broader spectrum of antibody defects than typically included in hyper-IgM syndrome. Although CD40 is expressed on a variety of cell types, the expression of the CD40L appears much more restricted, appearing primarily on the $CD4^+$ population of T cells and on a subpopulation of $CD8^+$ T cells (*11, 27*). This restricted gene expression may permit the development of a targeted gene therapy approach to treatment of hyper-IgM disease.

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14. A description of the probes and restriction fragment length polymorphism for the loci linked to *Cd40l*, including hypoxanthine-guanine phosphoribosyl transferase (*Hprt*), bone morphogenic protein-2b2 (*Bmp-2b2*), and connexin-32 (*Cnx-32*), have been reported previously [M. E. Dickinson *et al.*, *Genomics* **6**, 505 (1990); J. A. Haefliger *et al.*, *J. Biol. Chem.* **267**, 2057 (1992)]. Recombination distances were calculated as described [E. L. Green, in *Genetics and Probability in Animal Breeding Experiments* (Oxford Univ. Press, New York, 1992), pp. 77–113].
15. In situ hybridization showed that of 115 sites of hybridization scored, 18 (16%) were located on the distal portion of the long arm of the X chromosome. The largest number of grains was at band q26, with no significant hybridization on other human chromosomes [J. D. Marth *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7400 (1986)]. Interestingly, X-linked hyper-IgM syndrome was recently mapped to Xq26, close to *HPRT* [M. Padayachee *et al.*, *Genomics* **14**, 551 (1992); E. J. B. M. Mensink *et al.*, *Hum. Genet.* **76**, 96 (1987)].
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17. Blood from normal adult donors was drawn in parallel with all patient samples, and we cultured and analyzed equivalent numbers of PBLs alongside all patient- and age-matched control samples to ensure that experimental normal values were consistent with those previously reported (**11**).
18. [^{3}H]Thymidine incorporation by patient and control PBT cells was determined after 3 days in the presence of immobilized CD3 MAb or 0.1% PHA and human IL-7 (10 ng/ml) as described [R. J. Armitage, A. E. Namen, H. M. Sassenfeld, K. H. Grabstein, *J. Immunol.* **144**, 938 (1990)].
19. RNA was extracted from stimulated PBLs with RNAzol (Biotecx, Houston, TX). Total RNA (5 to 10 μg) was used to generate cDNA for a template in PCR reactions. Two PCR reactions were performed to cover the entire cDNA. The sequence of primers used to amplify the 5′ portion of the cDNA were 5′-CCAGAAGATACCATTTC-3′ and 5′-AGCCCACTGTAACACAG-3′; for the 3′ portion of the cDNA, the two primers used were 5′-CATGTCATAAGTGAGGC-3′ and 5′-CATAAGGAGGATCCTAG-3′. PCR products were filled in with Klenow (Pharmacia) and ligated into Sma I–cut pTZ19R for sequencing.
20. Mutants were constructed with the process of gene splicing by overlap extension (SOEing) [R. M. Horton, Z. Cai, S. N. Ho, L. R. Pease, *BioTechniques* **8**, 528 (1990)]. The primer used to recreate the mutation found in patient 1 was 5′-TGCGGGCAACAATCCATTCACTTGGGAGTAGTATTTGAATTGCAA; the primer used for patient 2 was 5′-CCATGAGCAACAACTTGGTAACCCCGGAAAATGGGAAACAGC. The remainder of the necessary primers was generated from the published human CD40L sequence (*10–12*).
21. Northern blot analysis was performed essentially as described [M. K. Spriggs *et al.*, *J. Biol. Chem.* **265**, 22499 (1990)] except that 2 μg of total RNA was electrophoresed per well and a ^{32}P-labeled antisense RNA probe was generated corresponding to the complete coding region of the human CD40L gene.
22. Flow cytometric analysis of CV-1/EBNA cells was performed essentially as described (Fig. 1). Cells were stained with biotinylated CD40.Fc (5 μg/ml) or a control IL-4R.Fc protein and streptavidin-phycoerythrin.
23. Mean [^{3}H]thymidine incorporation values from T cell–depleted PBLs were determined from triplicate cultures. The mean counts per minute from the remaining patient studies were as follows: patient 2, 13,734 ± 2,069; control 2 (XLA), 195 ± 59; patient 3, 4,458 ± 776; control 3, 7,134 ± 926; and patient 4, 9,468 ± 724; control 4, 14,524 ± 1,216.
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28. Purified tonsil B cells (1×10^5) were cultured with fixed CV-1/EBNA cells transfected with vector alone, wild-type CD40L, or mutagenized CD40L either alone for the determination of proliferation or in the presence of IL-4 (5 ng/ml) for IgE secretion. Proliferation was determined after 96 hours of culture. Briefly, cells were pulsed for the final 6 hours of culture with [^{3}H]thymidine (1 μCi per well) and harvested, and incorporated counts per minute were determined by tritium-sensitive avalanche gas ionization detection on a Matrix 96 Direct Beta Counter (Packard, Meriden, CT). Secreted IgE was determined after 10 days of culture by enzyme-linked immunosorbent assay as described (*16*). Proliferation and IgE secretion results are expressed as the mean ± SEM of triplicate cultures.
29. IgE secretion from 1×10^5 unfractionated or T cell–depleted PBLs was determined after 10 days of culture with IL-4 (5 ng/ml), together with G28-5 (200 ng/ml) or 1×10^4 fixed CV-1/EBNA cells transfected with vector alone or human CD40L. The preparation of PBLs and the determination of secreted IgE concentrations were performed as described (*16*). Results are expressed as the mean ± SEM of triplicate cultures.
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