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Evidence That the Leukocyte-Common Antigen Is Required for Antigen-Induced T Lymphocyte Proliferation

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Summary

The leukocyte-common antigen (L-CA) is a family of large molecular weight glycoproteins uniquely expressed on the surface of all nucleated cells of hematopoietic origin. The glycoprotein consists of a heavily glycosylated exterior domain, a single membrane spanning region, and a large cytoplasmic domain that contains tyrosine phosphatase activity. To investigate the function of this family, we generated T cell clones that lacked L-CA (L-CA⁻). The expression of the $\alpha\beta$ T cell receptor, CD3, CD4, IL-2 receptor (p55), LFA-1, Thy-1, and Pgp-1 (CD44) was normal. The L-CA⁻ T cell clones failed to proliferate in response to antigen or cross-linked CD3; however, they could still proliferate in response to IL-2. An L-CA⁺ revertant was obtained and the ability to proliferate in response to antigen and cross-linked CD3 was restored. These data indicate that L-CA is required for T cells to enter into cell cycle in response to antigen.

Introduction

The leukocyte-common antigen (L-CA, CD45) is a family of tyrosine phosphatases uniquely and abundantly expressed by cells of hematopoietic origin (for review, see Thomas, 1989). Different cell types express family members in a precise manner that is controlled during both cell lineage differentiation and activation. L-CA is encoded by a single gene located in a syntenic region found on chromosome 1 in both humans and mice (Ralph et al., 1987; Hall et al., 1988; Saga et al., 1988; Seldin et al., 1988; Johnson et al., 1989). The family is generated by differential splicing of three consecutive exons that encode sequences near the amino terminus of the molecule. A total of eight possible mRNAs can be generated, of which six have been isolated as cDNAs (Barclay et al., 1987; Ralph et al., 1987; Saga et al., 1987; Streuli et al., 1987; Thomas et al., 1987).

The mature glycoprotein is composed of an amino-terminal external domain, a single membrane-spanning region, and a very large cytoplasmic domain (Thomas et al., 1985). The external domain, based on protein biochemistry, interspecies sequence comparison, and genomic structure, can be divided into four subdomains (Thomas, 1989). The region at the amino terminus is predicted to be a random protein structure containing O-linked carbohydrate sites. The O-linked carbohydrate region is followed by two separate cysteine clusters and then a short spacer region before the membrane-spanning region. The differential use of the three variable

exons results in changes in the O-linked carbohydrate region. Since the regulation of alternative splicing of L-CA mRNA is a highly regulated event, it appears likely that the carbohydrate structures are of functional importance.

It was recently demonstrated that the cytoplasmic domain of L-CA contains tyrosine phosphatase activity (Charbonneau et al., 1988; Tonks et al., 1988; Ostergaard et al., 1989). This activity is likely to be important for cellular function since sequence comparison between species shows a remarkable degree of conservation: 85% over 700 amino acids. The large cytoplasmic domain of L-CA is divided into two 300 amino acid tandem repeats that share 35% identical residues. Each subdomain is approximately 35% homologous to another tyrosine phosphatase, PTPase 1B (Charbonneau et al., 1988). This suggests that both cytoplasmic subdomains will have tyrosine phosphatase activity, but with different substrate specificity and perhaps different regulation.

The function of L-CA has been a long-standing puzzle. The recent demonstration of tyrosine phosphatase activity suggests that L-CA may be involved in the regulation of hematopoietic cell growth. This has been supported by studies using antibodies to L-CA that have implicated this family in the activation and proliferation of lymphocytes. The proliferative response induced by the lectin phytohemagglutinin or the cross-linked anti-CD3 antibody can be modulated using monoclonal antibodies to L-CA (Bernabeu et al., 1987; Martorell et al., 1987). Similarly, modulatory effects are also seen by cross-linking L-CA to other surface glycoproteins (Ledbetter et al., 1988). Anti-L-CA antibodies can inhibit cytotoxicity by NK cell or cytotoxic T lymphocytes; they can also inhibit B lymphocyte proliferation and antibody production (Seaman et al., 1981; Nakayama et al., 1982; Newman et al., 1983; Harp et al., 1984; Yakura et al., 1986; Mittler et al., 1987). Direct evidence, however, that L-CA is involved in leukocyte cell growth has been difficult to obtain. To investigate the function of L-CA, we generated mouse T cell clones deficient in their expression of surface L-CA (L-CA⁻). We report here that these clones are altered in their capacity to divide in response to antigen and provide evidence that L-CA is involved in the initiation of signals required for cell division.

Results

Derivation and Characterization of L-CA-Deficient T Cell Clones

Mutational analysis has been a powerful tool in deciphering protein functions. We directed these methods toward the understanding of the function of L-CA. We chose to mutate mouse T cell clones because they maintain normal physiology and do not display a transformed phenotype. T cell clones were mutated with N-methyl-N'-nitro-N-nitrosoguanidine, and L-CA⁻ cells were selected by treating with antibody directed against a common L-CA determinant and rabbit complement. In one experiment, one

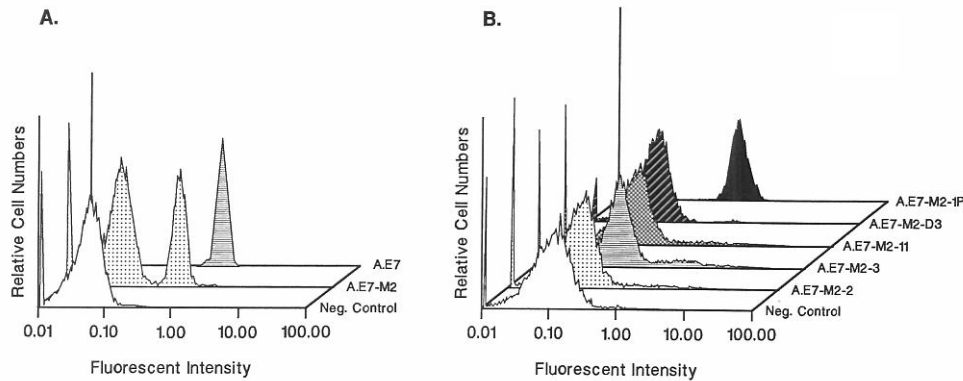


Figure 1. Flow Cytometry Analysis of L-CA⁻ Clones

(A) Cells were stained with anti-L-CA antibody I3/2.3 and a secondary antibody of fluorescein-conjugated goat anti-rat immunoglobulin. The negative control was A.E7-M2 stained with secondary antibody alone.

(B) Analysis of clonally isolated populations of cells. A.E7-M2-2, -3, and -11 were isolated from negatively sorted cells, and A.E7-M2-1P was isolated from positively sorted cells. A.E7-M2-D3 was cloned directly from A.E7-M2 population. Cells were stained with anti-L-CA I3/2.3 and a fluoresceinated second antibody. Each line was stained with secondary antibody alone as a negative control. The negative control for A.E7-M2-2 is shown. Cells were analyzed on a Becton-Dickinson FACS 440.

culture of a T cell clone, A.E7 (I-E^k restricted and specific for pigeon cytochrome c), contained a cluster of cells that appeared to be abnormal in morphology and growth. This culture, termed A.E7-M2, was expanded and examined by flow cytometry (Figure 1A). It is apparent that approximately half of the population of cells failed to react with anti-L-CA antibody I3/2.3. Individual T cells were isolated and expanded either by limiting dilution cloning directly from the A.E7-M2 line or by cloning after positive and negative selection by cell sorting. The clones isolated by positive cell sorting, for example, A.E7-M2-1P (Figure 1B), appeared to be identical to the parent line by morphology, growth parameters, and cell surface molecules (data not shown). Six L-CA⁻ clones were isolated by cloning directly from the A.E7-M2 line and 11 clones were isolated by cloning after negative selection. One clone from the di-

rect cloning, A.E7-M2-D3, and three clones from the negatively sorted cloning, A.E7-M2-2, -3, and -11, were examined further. As shown in Figure 1B, these cells were completely negative for L-CA as determined by flow cytometry using the antibody I3/2.3. Identical results were obtained with either 30F11.1 or M1/9.3.4HL.2 monoclonal antibodies, which also recognize common epitopes on L-CA. Also, the anti-allotypic antibody 104-2, which recognizes the Ly-5.2 determinant, failed to stain (data not shown). This indicates that the lack of L-CA detection was not merely due to the loss of the antigenic epitope.

To confirm the flow cytometry data, immunoprecipitation of surface-labeled cells was performed. Although analysis of the immunoprecipitate by SDS-PAGE showed no detectable surface-labeled L-CA from the L-CA⁻ clones, a single band at approximately 180,000 M_r is im-

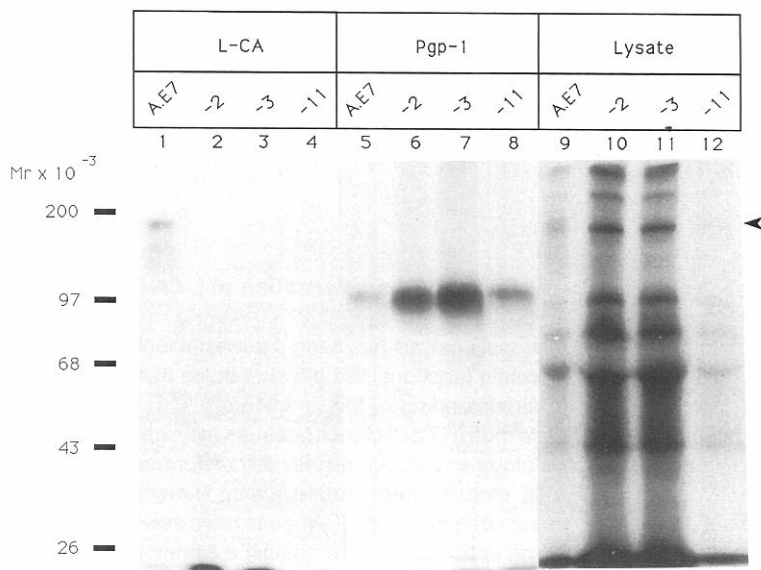


Figure 2. SDS-PAGE of Immunoprecipitates from Surface-Labeled A.E7 Parent and L-CA Mutant T Cell Clones

Cells were labeled with Na^[125I] by a lactoperoxidase catalyzed reaction, and lysates were prepared and immunoprecipitated. The antibodies used were: anti-L-CA (I3/2.3) (lanes 1-4); anti Pgp-1 (IM7.8.1) (lanes 5-8); and total cell lysates (lanes 9-12). The cells were: A.E7 (lanes 1, 5, and 9); A.E7-M2-2 (lanes 2, 6, and 10); A.E7-M2-3 (lanes 3, 7, and 11); and A.E7-M2-11 (lanes 4, 8, and 12). The arrow marks the position of the high molecular weight protein seen in lane 9 and not found in lanes 10-12. Lanes 1-8 were exposed overnight; lanes 9-12 were exposed for 7 days.

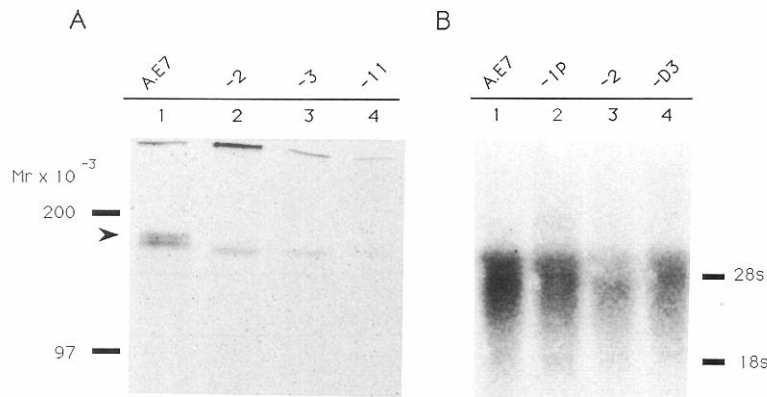


Figure 3. Biosynthesis and RNA Analysis of A.E7 and Mutant T Cell Clones

(A) SDS-PAGE of immunoprecipitates from lysates of [³⁵S]methionine-labeled cells. The cells were: A.E7 (lane 1); A.E7-M2-2 (lane 2); A.E7-M2-3 (lane 3); and A.E7-M2-11 (lane 4). The arrow denotes the position of the higher molecular weight band found in the A.E7 parent but not the mutant cell lines. The gel was exposed for 7 days. (B) Northern blot analysis. Total RNA was prepared and 5 μ g was electrophoresed per lane. The gel was blotted onto Zetaprobe (BioRad) and hybridized with a full-length L-CA cDNA (Thomas et al., 1987). The cells were: A.E7 (lane 1); A.E7-M2-1P (lane 2); A.E7-M2-2 (lane 3); and A.E7-M2-D3 (lane 4). The filter was exposed overnight.

munoprecipitated from the A.E7 parent (Figure 2). The faint lower molecular weight species seen in the L-CA immunoprecipitate from the A.E7 parent is due to degradation. A 30-day exposure confirmed the lack of surface L-CA from the L-CA⁻ clones (data not shown). In contrast, approximately equivalent amounts of the surface protein Pgp-1 (CD44) were found on the mutant cells as well as the parent line (Figures 2 and 4). When total surface-

labeled proteins were examined by SDS-PAGE, the only visible difference appeared to be a high molecular weight protein (Figure 2). This high molecular weight protein is most likely L-CA since L-CA is a major cell surface glycoprotein of A.E7 cells.

L-CA⁻ cells were also examined by immunoprecipitation of biosynthetically labeled cells and by Northern blot analysis (Figure 3). Approximately equivalent amounts of

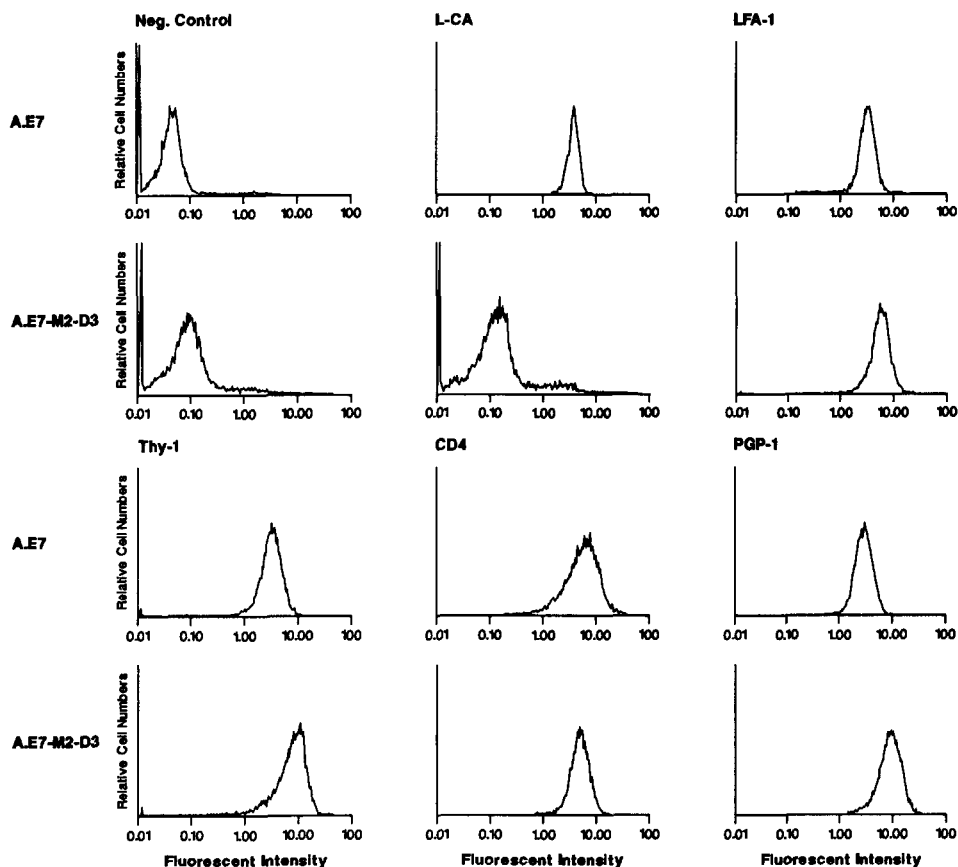


Figure 4. Flow Cytometry Analysis of A.E7 and A.E7-M2-D3 T Cell Clones

The negative control was cells stained only with the secondary fluorescein-conjugated antibody. Cells were analyzed on a Becton-Dickinson FACS 440.

Table 1. Net Growth per Biweekly Passage

Passage Number ^a	Cell Number × 10 ⁵ /ml				
	A.E7	A.E7-M2-D3	A.E7-M2-2	A.E7-M2-3	A.E7-M2-11
15	10.5	4.3	2.5	1.6	2.5
16	11.2	2.8	ND	ND	5.4
17	12.0	4.0	3.5	2.0	2.5
18	18.0	6.3	6.7	1.6	4.1
19	9.2	3.0	3.3	6.4	2.9

^a Passage number 1 was the first passage postmutagenesis.
ND, not determined.

normal size mRNA were found in the parent and L-CA⁻ cells (Figure 3B). SDS-PAGE of immunoprecipitated L-CA from parent A.E7 cells, labeled overnight with [³⁵S]methionine, revealed two molecular weight species of 180,000 and 160,000 M_r. However, each of the L-CA⁻ mutant clones contained only the 160,000 M_r form, albeit in lesser amounts. These data indicate that neither the transcription nor the translation of L-CA is impaired, but the defect is in the maturation and surface expression of the glycoprotein.

To determine whether or not the defect was specific for L-CA, the expression of the cell surface molecules LFA-1,

Thy-1, Pgp-1, and CD4 were examined by flow cytometry (Figure 4). Unlike surface L-CA, there was no detectable difference in the expression of these glycoproteins between the A.E7 parent and the L-CA⁻ T cell clones. The combined immunoprecipitation and flow cytometry data strongly suggest that the defect in the L-CA⁻ clones is specific to the L-CA glycoprotein.

Analysis of the Proliferative Capacity of L-CA T Cell Clones

The L-CA⁻ clones were visibly different from the parent line (data not shown). While a few scattered cells were

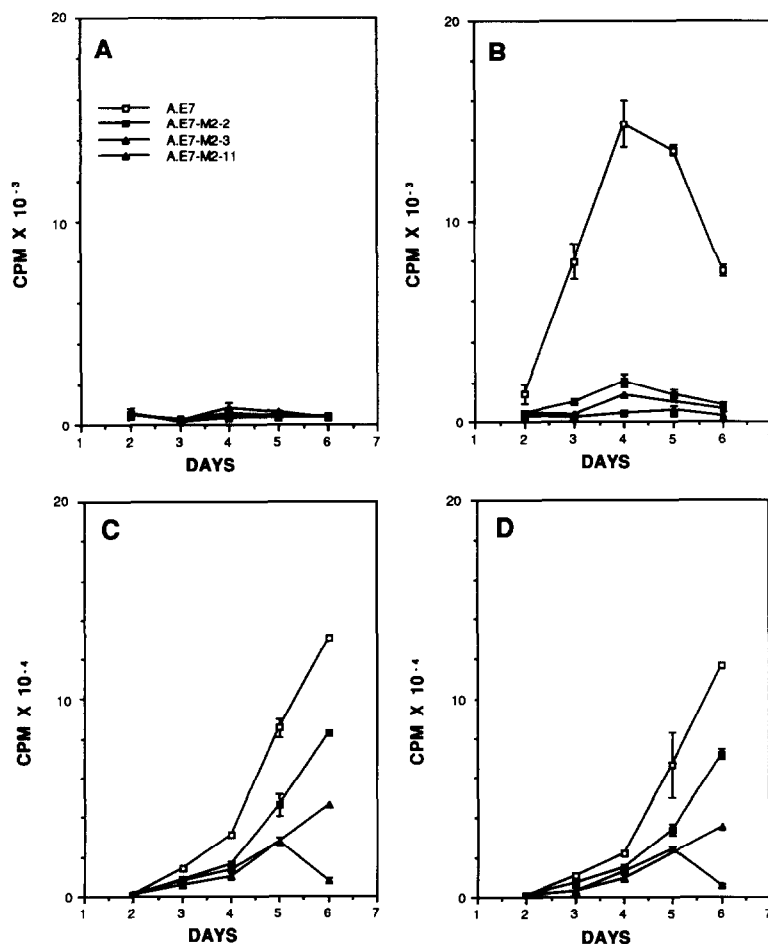


Figure 5. Proliferation Assay of A.E7 and L-CA⁻ T Cell Clones

T cells incubated with: (A) irradiated syngeneic spleen cells; (B) irradiated spleen cells and pigeon cytochrome c; (C) irradiated spleen cells and 5% phorbol stimulated EL-4 cell supernatant as an IL-2 source; and (D) irradiated spleen cells, cytochrome c, and 5% EL-4 supernatant. [³H]thymidine was added to the cultures 24 hr prior to harvesting. The cells were: A.E7 (open squares); A.E7-M2-2 (closed squares); A.E7-M2-3 (open triangles); and A.E7-M2-11 (closed triangles). Error bars represent standard deviation of triplicate cultures.

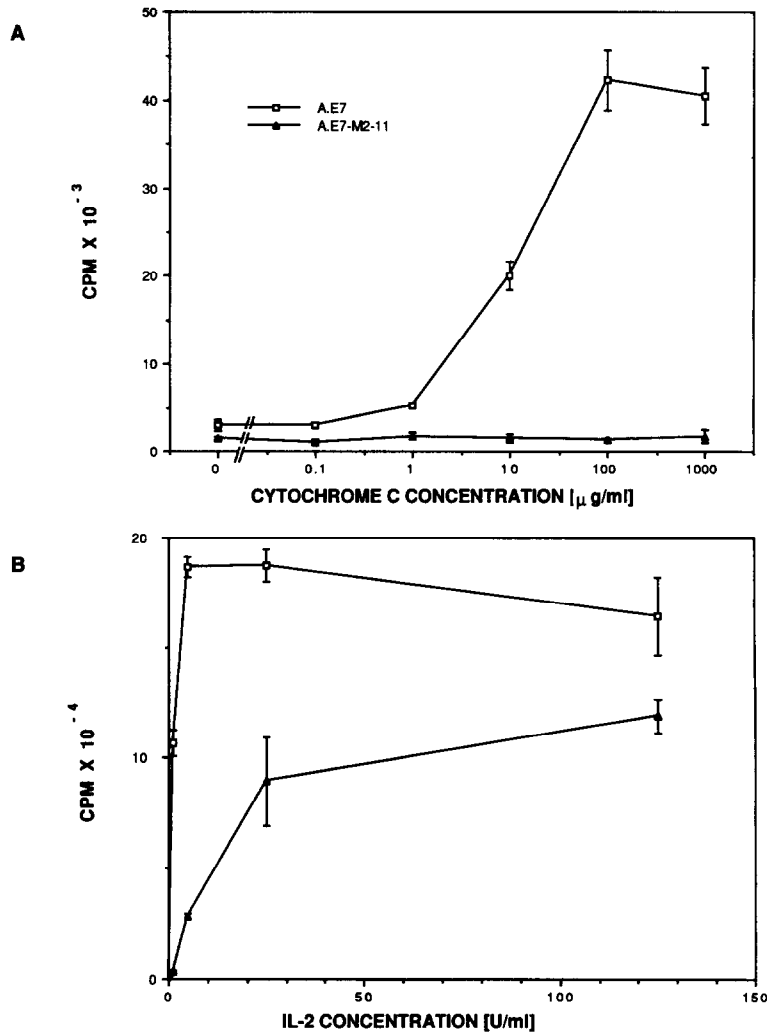


Figure 6. Dose Response Assay for Antigen and IL-2

(A) Proliferation was measured by [3 H]thymidine incorporation at 72 hr. A.E7 and A.E7-M2-11 were stimulated with serial dilutions of pigeon cytochrome c and irradiated syngenic spleen cells.

(B) Proliferation of A.E7 and A.E7-M2-11 96 hr poststimulation with recombinant IL-2. The cells were: A.E7 (open squares); and A.E7-M2-11 (closed triangles). Error bars indicate standard deviation of triplicate cultures.

similar in shape to the parent line, most cells were larger and spherical. In contrast, the parent line contained between 20%–50% cells with amoeboid morphology. The L-CA⁻ clone cultures also contained more cellular debris and a higher frequency of dead cells. This is reflected in total cell growth. Table 1 displays the number of cells obtained at the end of a series of biweekly passages. Consistently, the mutant cell lines gave approximately 4-fold fewer cells.

To examine the growth parameters between the parent and mutant cells, we compared the proliferative response to antigen and IL-2 (Figure 5). As expected, both the parent and the L-CA⁻ clones failed to respond when only the spleen filler cells were present (Figure 5A). Remarkably, when the specific antigen pigeon cytochrome c was added to the cultures, all the L-CA⁻ clones failed to respond appropriately (Figure 5B). This was not merely a result of a shift in the antigen dose response of the mutants (Figure 6A). The L-CA⁻ clones failed to respond even to doses as high as 1 mg/ml. In comparison, both the parent and the L-CA⁻ clones proliferate in response to IL-2, although the response is weaker for the L-CA⁻ clones (Fig-

ure 5C). In five separate experiments, the response of the L-CA⁻ clones to antigen was on average 9% of the response of the parent, while the response to IL-2 was 77%. The IL-2 source for the experiments shown in Figure 5 was from phorbol-stimulated EL-4 cells, however, similar results were obtained if recombinant IL-2 was used (Figure 6B). In a dose response assay, the L-CA⁻ clones did respond to IL-2, but the response was not as great as that of the A.E7 parent. Examination of the p55 chain of the IL-2 receptor by flow cytometry indicated that the mutant not only expressed this component but that the L-CA⁻ clone increased the surface expression to a greater degree than that of the parent after stimulation with antigen and IL-2 (Table 2). This indicates that the diminished growth was not due to the lack of the IL-2 receptor in the L-CA⁻ clones. When antigen and IL-2 were added simultaneously to the cultures, the proliferative response of the parent and the mutant clones was always less than when only IL-2 was present (Figure 5D). Since the L-CA⁻ clones fail to respond to antigen, our ability to grow these cells in vitro is presumably a result of the addition of an exogenous IL-2 source.

Table 2. IL-2 Receptor (p55) Expression

	Day Poststimulation ^b	Mean Channel Fluorescence ^a	
		Negative Control	IL-2 Receptor (p55)
Experiment 1			
A.E7	Day 15	35.89	85.18
A.E7-M2-D3		39.66	62.75
Experiment 2			
A.E7	Day 13	46.30	97.93
A.E7-M2-D3		34.06	80.77
A.E7	Day 5	38.90	91.17
A.E7-M2-D3		39.02	121.13

^a Fluorescence was measured by flow cytometry using a Becton–Dickinson FACS 440. Fluorescent intensity was measured on a four-log scale and was divided into 255 channels.

^b Cells were stimulated with antigen and IL-2 on day 1. For each experiment, IL-2 receptor was measured on the last day prior to stimulation (day 15 and day 13 of the previous stimulation for experiments 1 and 2, respectively) and then either day 6 or day 5 poststimulation.

To determine whether the L-CA⁻ cells expressed the αβ T cell antigen receptor–CD3 complex, the cells were analyzed by flow cytometry using a recently described monoclonal antibody to a framework determinant on the αβ T cell receptor (Figure 7) (Kubo et al., 1989). There was no detectable difference between the parent and mutant cells, indicating that lack of proliferation to antigen was not due to the failure to express the αβ T cell antigen receptor–CD3 complex.

Since the L-CA⁻ clones expressed CD3, it was of interest to determine whether or not they would proliferate in response to an anti-CD3 antibody. As shown in Figure 8, while the parent responded to doses as low as 20 ng/ml, the A.E7-M2-2 clone failed to respond to any dose. We interpret these data to mean that signaling through the antigen receptor–CD3 complexes is impaired in the L-CA⁻ clones.

Analysis of an L-CA⁺ Revertant

Confirmation that the diminished antigen-induced proliferative response of the L-CA⁻ cells was a result of the failure to express surface L-CA was obtained by the analysis of an L-CA⁺ revertant. In one of the L-CA⁻ clones, A.E7-M2-11, a spontaneous revertant arose, A.E7-M2-11R, which was detected by the change in morphology and increased cell growth rate. Cells were isolated and examined by flow cytometry and confirmed to be L-CA⁺ (Figure

9A). The L-CA⁺ revertant cells, when analyzed for proliferation, regained their ability to divide in response to antigen and anti-CD3 (Figures 9B and 9C). This, therefore, indicates that the L-CA⁻ mutant, A.E7-M2-11, has a functional T cell receptor–CD3 complex but requires the L-CA-glycoprotein for the induction of the proliferative response. The revertant was identified at the fifteenth passage post-cloning and the A.E7-M2-11 clone was still entirely L-CA⁻ at the twelfth passage (Figures 1B and 2). Therefore, since the L-CA⁻ cells have a selective growth advantage over the L-CA⁺ cells, the appearance of the L-CA⁺ revertant could not be due to a contamination during cloning. It should also be noted that since the L-CA⁺ cells do have a selective growth advantage, it is not surprising that revertants are identified.

Discussion

The L-CA⁻ T cell clones proliferated very poorly in response to antigen and had a diminished proliferative capacity in response to IL-2. The data strongly suggest that the failure of the L-CA⁻ clones to respond to signaling was owing to the lack of the L-CA glycoprotein. The L-CA⁻ cells expressed the αβ T cell antigen receptor–CD3 complex, CD4, LFA-1, Thy-1, Pgp-1, and the IL-2 receptor in amounts equivalent to the parent clone. Furthermore, the only distinguishable difference of total

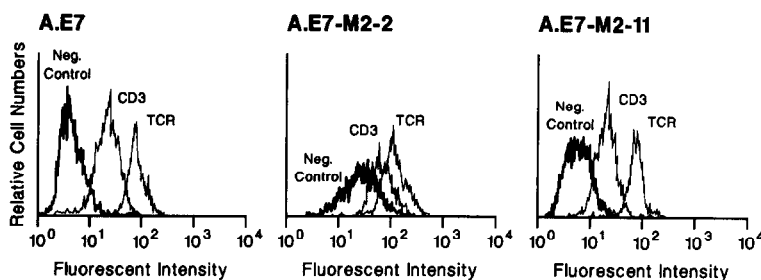


Figure 7. Flow Cytometry Analysis of the αβ T Cell Receptor and CD3 on A.E7 and L-CA⁻ T Cells

The antibodies were purified and labeled directly with fluorescein and analyzed on Becton–Dickinson FACScan. The negative control was unstained cells.

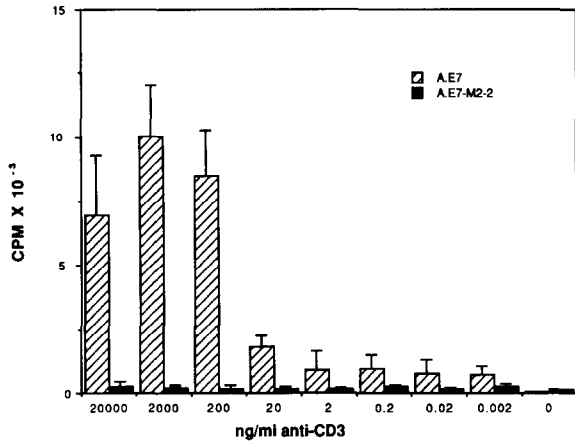


Figure 8. Proliferative Response of A.E7 and A.E7-M2-2 to Anti-CD3 Cells were added to wells precoated with the antibody, and [³H]thymidine was added 24 hr prior to harvesting. A.E7 is represented by hatched bars and A.E7-M2-2 by the closed bars. Error bars indicate standard deviation of triplicate cultures.

surface-labeled lysates between the parent and mutant cells was a single high molecular weight protein, which is most likely L-CA. This indicates that the defect in the L-CA⁻ cells was specific for the L-CA glycoprotein. Further evidence that the proliferative defect in the mutant cells was due to the failure to express properly the L-CA glycoprotein was obtained by the analysis of a L-CA⁺ revertant. These cells proliferated in response to antigen, cross-linked CD3, and IL-2 in a manner similar to the parent clone. It is highly unlikely that a double revertant would be obtained simultaneously for two separate mutations and therefore, the most straightforward interpretation is that the increase in proliferation is due to the reexpression of surface L-CA glycoprotein. The expression of the T cell receptor-CD3 complex and CD4 indicates that the recognition of antigen should not have been impaired in L-CA⁻ cells. It is significant that the L-CA⁻ cells express LFA-1, Pgp-1, and Thy-1 as well. LFA-1 and Pgp-1 are thought to be involved in cell adhesion advents (Springer et al., 1987; Goldstein et al., 1989; Stamenkovic et al., 1989). Antibod-

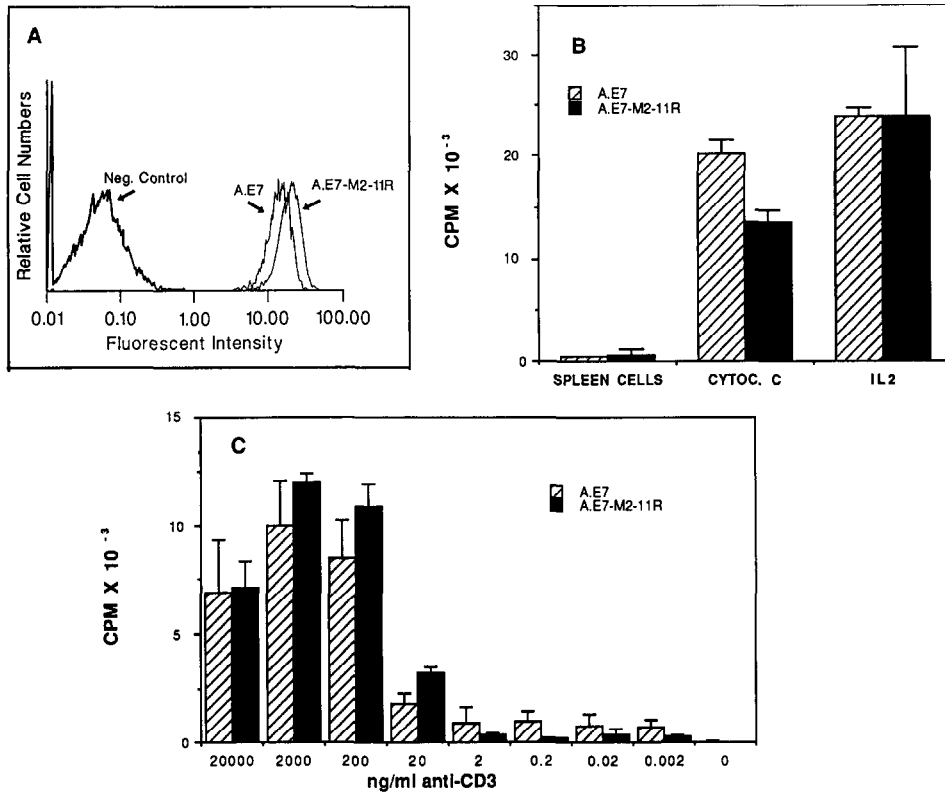


Figure 9. Analysis of the Revertant A.E7-M2-11R

(A) Flow cytometry of cells stained with anti-L-CA I3/2.3. The negative control was cells stained only with secondary antibody. Cells were analyzed on a Becton-Dickinson FACS 440.

(B) Proliferation assay of A.E7 and A.E7-M2-11R in response to irradiated spleen cells and either pigeon cytochrome c or IL-2. Phorbol-stimulated EL-4 cell supernatant was used as a source of IL-2. Cells were incubated for 48 hr before the addition of [³H]thymidine and harvested at 72 hr. Hatched bars represent A.E7 and closed bars represent A.E7-M2-11R. Error bars indicate standard deviation of triplicate cultures.

(C) Proliferation assay in response to anti-CD3. Cells were added to wells precoated with anti-CD3. After 48 hr [³H]thymidine was added, and cells were harvested at 72 hr. Hatched bars represent A.E7 and closed bars represent A.E7-M2-11R. Error bars indicate standard deviation of triplicate cultures.

TYROSINE KINASES		*	POSITION
<i>c-src</i>		LEDYFTSTEPQYQPGENL	527
<i>lck</i>		-D-F--A--G---QP	509
<i>c-fgr</i>		-----A-----DQT	529
<i>c-yes</i>		-----A-----	543
TYROSINE PHOSPHATASES			
h L-CA		ARFGMVSTFEQYQFLYDV	1199
m L-CA		---V-CSY-----I	1187
r L-CA		-----GS-----I	1168
h LAR		Q--A--Q-ED---LC-RA	1850

Figure 10. Sequence Comparison between Tyrosine Kinases and Phosphatase Sequences Adjacent to the Regulatory Kinase Phosphorylation Site

Dashes indicate identical residues; the kinases are compared with *c-src* and the phosphatases with human L-CA. The asterisk denotes the tyrosine phosphorylation site in *c-src* and *lck*. The sequences are: *c-src* (Hunter, 1987); *lck* (Marth et al., 1988); *c-fgr* (Katamine et al., 1988); *c-yes* (Sukegawa et al., 1987); human, mouse, and rat L-CA (Thomas, 1989); and human LAR (Streuli et al., 1988). The single letter amino acid code is used.

ies to Thy-1 have been shown to cause mouse T cells to proliferate, and proliferation requires the coexpression of the T cell receptor-CD3 complex (Gunter et al., 1987). Therefore, the normal expression of many of the glycoproteins known to be involved in recognition, adherence, and signal transduction further indicates that the lack of L-CA surface expression is the cause of the proliferative defect.

The L-CA mutant clones synthesize a protein recognized by the I3/2.3 antibody, however, the protein is not transported to the cell surface (Figures 2 and 3). This suggests that the mutation causes a specific retention of L-CA at some stage of transport and may be similar to mutations described for influenza hemagglutinin protein, the E1 viral coat protein of coronavirus, and the low density lipoprotein receptor (Gething et al., 1986; Machamer and Rose, 1987; Pathak et al., 1988). Structural mutations have been described for these proteins that cause a specific retention in the biosynthetic pathway. Since it is still formally possible that the mutation in the L-CA⁻ cells effects L-CA and some other unknown component of the T cell receptor-CD3 complex, we are currently conducting experiments to transfect the complete L-CA cDNA into the L-CA⁻ cells to determine whether or not this will also correct the proliferative defect. If expression of L-CA by transfection does not correct the proliferative defect, then this would indicate that some other component of the T cell receptor complex is also effected in these cells. However, the most likely explanation for the proliferative defect described here is the failure to properly express the L-CA glycoprotein.

Lymphocyte Division and Tyrosine Phosphorylation

These data yield two observations that first appear to be surprising. L-CA is expressed by all leukocytes, so it was not anticipated that a negative L-CA phenotype would dramatically effect specific antigen signaling through the T cell receptor-CD3 complex. However, this is reasonable if one assumes that T cells have evolved from primitive cell types that at one time were not antigen-specific. It is possible that T cells have adapted antigen-specific triggering for cell division utilizing molecules that are generally used in leukocyte cell division. This is supported by

the observation that the L-CA tyrosine phosphatase domain is highly conserved throughout invertebrate and vertebrate evolution, indicating that this domain is extremely important in some aspect of cell physiology, such as cell cycling (R. J. Matthews and M. L. Thomas, unpublished data).

The lack of L-CA on the cell surface resulted in the failure of the cells to divide in response to antigen. Since this, presumably, is due to the failure of the proper signals being transduced to the tyrosine phosphatase domain, it is logical to conclude that antigen-induced cell proliferation requires the dephosphorylation of a tyrosine residue. Many growth factor receptors have a tyrosine kinase domain. It is thought, therefore, that tyrosine phosphorylation is also required for the induction into cell cycle. These two observations may at first appear to be incongruous. However, the *src* gene family of tyrosine kinases is regulated by the phosphorylation of a tyrosine residue near the carboxyl terminus (Hunter, 1987). Dephosphorylation at this site causes an increase in tyrosine kinase activity (Cooper and King, 1986; Cartwright et al., 1987; Amrein and Sefton, 1988; Marth et al., 1988). It is possible, therefore, that L-CA functions by regulating tyrosine kinase activity. Dephosphorylation of unknown kinase would cause an increase in the tyrosine kinase activity, thus initializing a cascade of events that results in cell cycling. It is interesting, therefore, that Ostergaard et al. (1989) have indicated that *lck*, a member of the *src* gene family, is a substrate for L-CA. They have observed that position 505 in *lck*, the regulatory tyrosine site, is not phosphorylated in L-CA⁺ lymphomas but is phosphorylated in L-CA⁻ lymphoma mutants. The *lck* tyrosine kinase has been identified in complexes with CD4 and CD8 and is a candidate for signaling through the T cell antigen receptor (Rudd et al., 1988; Veillette et al., 1988). It is possible that a failure to activate *lck* by dephosphorylation accounts for the results obtained here. The sequence around position 505 in *lck* is highly conserved in all members of the *src* gene family (Figure 10). It is interesting to note, therefore, that a similar sequence is to be found near the carboxyl terminus of the second L-CA subdomain (Figure 10).

L-CA Activation

Since the interactions for the external domain of L-CA may be important in activating the tyrosine phosphatase domains, a question arises regarding the ligand for the external domain. Evidence exists that the carbohydrate structures are an important functional moiety of the L-CA glycoprotein (reviewed in Thomas, 1989). Briefly stated, the external domain of L-CA is heavily glycosylated, bearing many N-linked and O-linked carbohydrates, and is a major surface glycoprotein on lymphocytes, comprising approximately 10% of the cell surface. Therefore, L-CA bears many of the carbohydrates of these cells. Furthermore, there are differences in L-CA carbohydrate structures between lymphocyte lineages, and these structures can change upon activation (Brown and Williams, 1982; Cook et al., 1987). More compelling though, is the observation that the difference between family members is due to differential splicing of three exons that encode O-linked

carbohydrate sites. Since the expression of these carbohydrate sites is controlled in a precise developmental and activational manner, this strongly suggests that the carbohydrates are of functional importance. It has also been shown that the carbohydrate groups from L-CA will block NK cell binding to targets (Gilbert et al., 1988). The carbohydrate residues on L-CA may interact with lectins on cell surfaces, and this cell-cell interaction is necessary for induction into cell cycling. While the data strongly suggest that carbohydrate groups on L-CA are functionally important, this does not eliminate the possibility that L-CA could be activated by other interactions (for example, interactions with a soluble ligand or movement in the cell membrane).

Experimental Procedures

Mutagenesis

The A.E7 is a T_H1 CD4⁺ clone, I-E^K restricted, and specific for pigeon cytochrome c. It was obtained from Dr. Casey Weaver (Matis et al., 1983). The cells were passaged by culturing 5 × 10⁵ cells/ml in RPMI 1640 containing 50 μM 2-mercaptoethanol and 10% fetal calf serum (FCS; Hyclone) with 2.5 × 10⁶ CBA/J spleen cells/ml (irradiated with 3000 rads) and 100 μg/ml pigeon cytochrome c (Sigma). After 2 days cells were expanded 5-fold in media containing 5% filtered supernatant obtained from EL-4 cells stimulated for 24 hr with 10 ng/ml phorbol myristate acetate (the EL-4 supernatant contains between 100–500 U/ml IL-2). The A.E7 cells were passaged at 2 week intervals.

A.E7 cells were taken at day 6 of passage and cellular debris removed by centrifugation through Ficoll gradients (Ficoll-Paque, Pharmacia). A total of 5 × 10⁷ cells was resuspended at 10⁷ cells/ml in complete media with 7.5 μg/ml N-methyl-N'-nitro-N-nitrosoguanidine (Sigma) for 45 min at 37°C, 5% CO₂. After washing twice with Hanks' balanced salt solution (HBSS) containing 2% FCS, cells were resuspended in 25 ml of complete media containing 5% EL-4 supernatant and incubated at 37°C, 5% CO₂. After 6 days, cellular debris was removed by centrifugation through Ficoll gradients and the cells were treated at 10⁷ cells/ml with a 1:50 dilution of I3/2.3 ascites (Trowbridge, 1978) for 45 min on ice, followed by treatment with 1:50 dilution rabbit anti-rat immunoglobulin (United States Biochemical) for 45 min on ice. Cells were lysed by treating with 1:12 dilution rabbit complement (Cedarlane, low-tox-M) for 45 min at 37°C, washed once with HBSS, 2% FCS, and retreated with the I3/2.3 rabbit anti-rat immunoglobulin and rabbit complement as above, except that the incubation times were 20 min, 20 min, and 30 min, respectively. Cells were resuspended in 1 ml of complete media with 1 μg/ml ConA. The next day, media were removed and cells were passaged as normal. ConA was added to the cultures a day prior to passage for the first three passages. After the fourth passage, cells were selected again with one cycle of antibody and complement as above. Cells were sorted and cloned after the tenth passage using a Becton-Dickinson FACS 440 staining with a second antibody of fluoresceinated goat anti-rat immunoglobulin and cloning by limiting dilution using 0.3 cells/well.

Flow Cytometry

Cells, 10⁶, were washed once with PBS (10 mM Na phosphate [pH 7.4], 150 mM NaCl) containing 0.02% BSA and 2 mM NaN₃ (B-PBS), resuspended in 200 μl of monoclonal antibody tissue culture supernatant and incubated for 30 min at room temperature. The monoclonal antibodies used were: anti-L-CA, I3/2.3 (Trowbridge, 1978), 30F11.1 (Seaman et al., 1981), M1/9.3.4, HL.2 (Springer et al., 1978), 104-2 (anti-Ly-5.2; Shen, 1981), anti-α-e1- T cell antigen receptor, H57-157 (Kubo et al., 1989), anti-CD3, 145-2C11 (Leo et al., 1987), anti-CD4, GK1.5 (Daly et al., 1983), anti-IL-2 receptor p55, 7D4 (Malek et al., 1983), anti-LFA-1, FD411.8 (Sarmiento et al., 1982), anti-Thy-1, HO-22-1 (Marshak-Rothstein et al., 1979), and anti-Pgp-1, IM78.1 (Trowbridge et al., 1982). Cells were washed twice in B-PBS, resuspended in 200 μl of a 1:30 dilution of fluoresceinated goat anti-rat immunoglobulin (cross-reactive with mouse immunoglobulin), and incubated for 30 min at room temperature. Antibodies that were neither rat nor mouse were

purified and coupled directly with fluorescein. After washing twice with B-PBS, cells were resuspended in 300 μl of B-PBS containing 25 μg/ml propidium iodide and filtered through nytex. Cells were analyzed using a Becton-Dickinson FACS 440.

Immunoprecipitation

For surface labeling, cells were purified by centrifugation through Ficoll gradients, and 10⁷ cells were grown overnight at 2 × 10⁶ cells/ml with 5% EL-4 supernatant. The next day cells were washed once with media, once with PBS without calcium or magnesium, and resuspended in 1 ml of PBS with 50 mM glucose, 0.5 mg/ml glucose oxidase, and 200 μl of 2.5 μg/ml lactoperoxidase added. The reaction was initiated by the addition of 20 μl of 5 mCi/ml Na¹²⁵I (ICN Pharmaceuticals) and incubated for 45 min at room temperature; the reaction terminated by the addition of 9 ml of ice-cold RPMI 1640 containing 10 mM NaN₃ and 2 mM L-glutamine.

Cells were biosynthetically labeled by washing 5 × 10⁶ cells twice with methionine-free RPMI 1640, incubating at 37°C for 45 min with methionine-free RPMI containing 200 mM L-glutamine, adding [³⁵S]-methionine to 150 mCi/ml (Amersham), and culturing overnight at 8 × 10⁵ cells/ml.

Cells were washed three times with RPMI 1640 containing 0.02% NaN₃, resuspended in 0.5 ml PBS containing 1% Triton X-100, 5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml leupeptin and incubated for 15 min on ice. The nuclei were pelleted, and 250 μl portions of the supernatant were brought to 1 ml with PBS containing 1% Triton X-100, 1% SDS, 0.5% deoxycholate, 0.5% bovine serum albumin, 0.5% human serum albumin, 0.02% NaN₃, 5 mM iodoacetamide, 1 mM PMSF, 2100 KIU/ml aprotinin, and 10 μg/ml leupeptin. Either 10 μl of ascites or 100 μl of tissue culture supernatant of a monoclonal antibody was added and incubated overnight at 4°C. The next day, 50 μl of goat anti-rat immunoglobulin coupled to Sepharose CL 4B was added, mixed for 2 hr at 4°C, pelleted, and washed three times with the above buffer and once with PBS. Samples were dissolved in 50 μl of reducing Laemmli sample buffer and analyzed by SDS-PAGE and autoradiography.

Northern Blot Analysis

Total RNA was isolated from 7 × 10⁷ cells by the guanidine isothiocyanate procedure as described previously (Thomas et al., 1985). Five micrograms was electrophoresed on a 1% agarose-formaldehyde denaturing gel (Maniatis et al., 1982) and transferred to a Zetaprobe filter (BioRad) by capillary blotting using 20 mM NaHPO₄ (pH 6.6). The filter was hybridized with a full-length L-CA cDNA (Thomas et al., 1987) (nick-translated to a specific activity of 2 × 10⁸ CPM/μg with [³²P]dCTP [New England Nuclear]) overnight at 42°C and washed with 0.2× SSC (Maniatis et al., 1982) at 55°C.

Antigen- and IL-2-Induced Proliferation Assays

A total of 2 × 10⁴ A.E7 T cells was mixed with 10⁶ irradiated CBA/J spleen cells with 100 μg/ml cytochrome c and/or 5% phorbol myristate acetate-stimulated EL-4 supernatant as an IL-2 source. Twenty-four hours prior to harvesting, 20 μl of [³H]thymidine at 20 μCi/ml was added. Each point was assayed in triplicate. For the antigen dose response assays, proliferation was measured at day 3 poststimulation and for recombinant IL-2 at day 4 poststimulation. The recombinant IL-2 was generously provided by Dr. Robert Schreiber, Washington University.

CD3-Stimulated Proliferation

Wells of microtiter plates were coated by incubating serial dilutions of purified anti-CD3 (Leo et al., 1987) in PBS for 5 hr at room temperature. Wells were washed three times with PBS and 2 × 10⁴ cells were added to each well in 200 μl of RPMI 1640 with 10% FCS. After 48 hr, 20 μl of [³H]thymidine at 20 μCi/ml was added to each culture. Cells were harvested at 72 hr.

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