sized in plant tissues and how the ratio of chl a to chl b is regulated during development of the chloroplast.

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Inhibition of Lymphocyte Proliferation by a Synthetic Peptide Homologous to Retroviral Envelope Proteins

Abstract. *The retroviral transmembrane envelope protein p15E is immunosuppressive in that it inhibits immune responses of lymphocytes, monocytes, and macrophages. A region of p15E has been conserved among murine and feline retroviruses; a homologous region is also found in the transmembrane envelope proteins of the human retroviruses HTLV-I and HTLV-II and in a putative envelope protein encoded by an endogenous C-type human retroviral DNA. A peptide (CKS-17) was synthesized to correspond to this region of homology and was examined for its effects on lymphocyte proliferation. CKS-17 inhibited the proliferation of an interleukin-2–dependent murine cytotoxic T-cell line as well as alloantigen-stimulated proliferation of murine and human lymphocytes. Four other peptides, representing different regions of virus proteins, were inactive. These results suggest that the immunosuppressive portion of retroviral transmembrane envelope proteins may reside, at least in part, in a conserved sequence represented by the CKS-17 peptide.*

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Immunosuppression frequently accompanies retroviral infections and often precedes the development of neoplasms in animals infected by oncogenic retroviruses (*1*). Although the mechanisms are not yet known, there is increasing evidence that the envelope protein p15E participates in the pathogenesis of retroviral-induced immunosuppression (*2*). P15E is a hydrophobic transmembrane protein approximately 19,000 daltons in size that is cleaved from an envelope precursor polypeptide of approximately 80,000 to 90,000 daltons (*3*). Lymphocyte blastogenic responses to mitogens and alloantigens are inhibited by isolated p15E of feline leukemia virus (FeLV), and the transformation of human lymphocytes by concanavalin A is blocked by FeLV p15E (*4, 5*). Inhibition of proliferation of a murine cytotoxic T-lymphocyte (CTL) cell line by a p15E-like protein from FeLV has also been reported (*6*). Furthermore, murine leukemia virus (MuLV) p15E inhibits macrophage accumulation at inflammatory foci in mice (*7*). Proteins with similar anti-inflammatory properties that are antigenically and physicochemically related to p15E have been identified in virus-free murine tumor cell lines and primary murine tumors (*8*). Human cancerous effusions contain p15E-related proteins that inhibit the responses of human monocytes to chemotactic stimuli (*9*), and p15E-related proteins have been identified in human malignant cells and in the plasma of leukemic patients (*10, 11*). It has therefore been postulated that certain tumor cells, not exogenously infected with retroviruses, may produce immunosuppressive products related to p15E and thereby evade immune surveillance (*2*).

Human T-cell leukemia/lymphoma virus (HTLV) is the designation for a family of lymphotrophic, exogenous retroviruses (*12*). Immunosuppression often accompanies HTLV-associated lymphocyte malignancies, and coculture of normal human lymphocytes with ultraviolet-irradiated HTLV-I inhibits their blastogenic responses to mitogens (*13*). The transmembrane envelope protein of HTLV-I and HTLV-II, gp21E, shares significant amino acid sequence homolo-

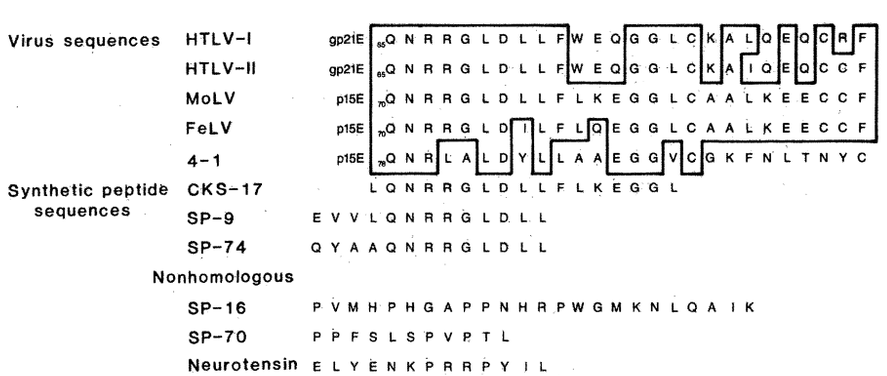

Fig. 1. Amino acid sequences (*19*) for the conserved region of retrovirus transmembrane envelope proteins gp21E and p15E and partially homologous or nonhomologous synthetic peptides. MoLV, Moloney murine leukemia virus. The number at the lower left of each sequence represents the residue at which the sequence begins.

gy with p15E of MuLV and FeLV and with the transmembrane proteins of bovine leukemia virus (BLV) and Rous sarcoma virus (RSV) (*14*). There is also homology between the nucleotide sequence of a full-length endogenous C-type human retroviral DNA (clone 4-1) and DNA sequences from MuLV, FeLV, HTLV-I, and HTLV-II in the same region (*15*). Considering the potential role of retroviral envelope proteins in the pathogenesis of immunosuppression, we hypothesized that the homologous sequence might mediate such activity. We now report that a peptide (CKS-17) synthesized to correspond to a portion of this region of homology inhibits the proliferation of an interleukin-2 (IL-2)–dependent murine CTL (CTLL-2) cell line and the alloantigen-stimulated proliferation of both murine and human lymphocytes in mixed leukocyte cultures.

The sequence of CKS-17 and the homologous sequences in human, murine, and feline retroviruses are shown in Fig. 1. Additional virus-related peptides that are partially homologous (SP-9 and SP-74) or nonhomologous (SP-16 and SP-70) to CKS-17 were synthesized; the neuropeptide neurotensin was obtained commercially (Vega Biochemicals). All peptides were synthesized by means of Merrifield solid-phase synthesis techniques and purified with ion-exchange and reversed-phase high-performance liquid chromatography (HPLC); their identities were verified by HPLC and amino acid analysis (*16*). Peptides were coupled to the carrier protein bovine serum albumin (BSA) by the following method. Peptide (1.0 m*M*) and BSA (0.030 m*M*) were mixed in 0.1*M* 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl for approximately 10 hours at 22°C and *p*H 5.0. After the addition of an equal volume of 1.0*M* glycine, the mixture was rotated overnight at 4°C and then extensively dialyzed against Hanks balanced salt solution (HBSS) that had been buffered to *p*H 7.2 with 10 m*M* Hepes. Coupling, which occurred with an efficiency of approximately 30 percent as measured with radiolabeled peptide, resulted in conjugated material at a final peptide concentration of approximately 0.15 m*M* and a final BSA concentration of 0.015 m*M*. BSA alone was processed in an identical manner and used as an additional control.

Addition of the heptadecapeptide CKS-17 resulted in a dose-dependent inhibition of growth of the CTLL-2 cell line (Tables 1 and 2). Addition of various concentrations of the other coupled peptides or of the BSA control did not significantly inhibit incorporation of [^{3}H]thymidine into CTLL-2 cells. The inhibition of [^{3}H]thymidine incorporation was not the result of competition with or breakdown of the radiolabeled nucleotide but represented true inhibition of cellular proliferation. When CTLL-2 cells were cultured in the presence of IL-2 plus the BSA control or media alone, cell numbers increased by 180 percent during the 24-hour assay. In contrast, addition of CKS-17 at a dilution of 1:20 limited the increase in cell number to 105 percent. Furthermore, inhibition by CKS-17 was not the result of any rapid toxic effect as (i) CTLL-2 cells that had been incubated with the peptide for 3 hours were still viable (>95 percent) as determined by exclusion of trypan blue and (ii) maximal inhibition by the peptide required at least 16 hours of cell exposure. The inhibitory action of CKS-17 upon the CTLL-2 cells was not affected by increasing the IL-2 concentrations in the cultures by up to 20-fold, suggesting that the peptide was not directly competing with the IL-2.

CKS-17 also inhibited the proliferation of murine splenocytes in a two-way mixed leukocyte reaction (MLR) culture (Fig. 2A). In three experiments the CKS-17 peptide inhibited growth at dilutions of 1:20, 1:40, and 1:80 by 94 ± 2, 74 ± 4, and 48 ± 9 percent (mean ± standard error of the mean), respectively, while the BSA control had little or no effect at the same concentrations. CKS-17 at similar concentrations had no statistically significant effect on [^{3}H]thymidine incorporation by BALB/c 3T3 or NIH 3T3 fibroblast cells cultured for

Table 1. Effect of CKS-17 on [^{3}H]thymidine incorporation by murine CTLL-2 cells. Cells (5×10^3 per well) were cultured for 24 hours at 37°C in humidified 5 percent CO_2 in 96-well tissue culture plates. Culture medium was RPMI 1640 supplemented with 1 m*M* sodium pyruvate, 2 m*M* *L*-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), 2 percent fetal bovine serum (Sterile Systems, Logan, Utah), and 1 percent partially purified human IL-2 (Electronucleonics, Silver Spring, Maryland). Cells were cultured either in medium alone or in medium with the indicated concentrations of materials to be tested. Incorporation was measured by adding 1.0 μCi [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear) per well for the final 4 hours of culture, collecting the cells with a multiple automated sample harvester onto glass-fiber filters, and determining the incorporated radioactivity by scintillation spectrophotometry. Values represent the mean ± standard error of the mean of quadruplicate samples. Cultures grown in the absence of IL-2 incorporated <200 count/min per well.

Material tested	Incorporation (count/min) at peptide dilutions		
	1:20	1:40	1:80
	Experiment 1		
CKS-17	18,511 ± 1898	33,602 ± 718	42,076 ± 1489
BSA*	40,982 ± 1815	45,765 ± 2328	51,623 ± 1178
Media alone	44,676 ± 2063		
	Experiment 2		
CKS-17	10,950 ± 1082	23,128 ± 1644	29,980 ± 1745
BSA*	25,772 ± 358	31,317 ± 1730	27,169 ± 1802
Media alone	29,002 ± 1898		
	Experiment 3		
CKS-17	17,253 ± 453	26,122 ± 398	27,484 ± 463
BSA*	31,580 ± 829	28,619 ± 829	29,149 ± 743
Media alone	28,180 ± 1116		

*BSA that underwent the same coupling conditions as used for CKS-17.

Table 2. Effects of synthetic peptides on [^{3}H]thymidine incorporation by murine CTLL-2 cells. Cells were cultured as described in Table 1 in the presence or absence of the indicated concentrations of the peptides. Incorporation of [^{3}H]thymidine by cells grown in the absence of peptides ranged between 25,000 and 50,000 count/min (cpm) for this series of experiments. The results represent the mean ± standard error of the mean where percent inhibition is [(cpm in medium alone − cpm in the presence of peptide)/cpm in medium alone] × 100.

Peptide coupled to BSA	*N**	Inhibition of [^{3}H]thymidine incorporation (%) at peptide dilutions		
		1:20	1:40	1:80
None	16	5.8 ± 1.2	6.4 ± 1.5	4.0 ± 1.3
CKS-17	24	49.6 ± 5.4	22.0 ± 4.8	9.4 ± 1.3
SP-9	2	0	0	0
SP-74	2	0	0	0
SP-16	2	6.5 ± 6.5	0	0
SP-70	2	5.0 ± 5.0	0	0
Neurotensin	4	0.5 ± 0.5	0	0

**N*, number of experiments

either 2 or 3 days. Growth of human mononuclear cells that had been stimulated in a two-way MLR was also blocked by CKS-17 (Fig. 2B). In four experiments, CKS-17 at dilutions of 1:20, 1:40, and 1:80 inhibited [^{3}H]thymidine incorporation by 92 ± 2, 64 ± 11, and 18 ± 3 percent (mean ± standard error of the mean), respectively.

Inhibition of the human MLR by CKS-17 was observed when the peptide was added on day 3 (of a 4-day culture) or day 5 (of a 6-day culture) but not when the peptide was added at the initiation (time 0) of the culture. This was in contrast to the murine MLR, where addition of the peptide at time 0 inhibited proliferation in both 3- and 5-day cultures. The reason for this has not yet been determined. However, Copelan *et al.* reported that p15E inhibition of human lymphocyte blastogenic responses to concanavalin A was reversed by addition of monocytes to the cultures (*5*). As approximately 25 percent of the human MLR culture were monocytes, these cells may either take up or degrade the CKS-17 peptide, making less available to the lymphocytes in the cultures. This possibility has also been used to explain the reversal of the effect of p15E on lymphocytes by the addition of monocytes (*5*).

Significant inhibition of lymphocyte proliferation was not observed when uncoupled CKS-17 was tested, suggesting that biological activity might be dependent on a particular conformation that is conferred on the peptide molecule by coupling it to a carrier. If this is the case, we have no way of estimating the percentage of coupled CKS-17 molecules that are in the required conformation. Thus, the concentration of CKS-17 required for biological activity (approximately 7.5 μM at a 1:20 dilution) might be substantially less under conditions in which all the molecules assumed the appropriate conformation.

Previous studies have shown that retroviral p15E can inhibit feline and human lymphocyte transformation (*4, 5*). Our results show that CKS-17, a synthetic peptide corresponding to a conserved region of retroviral envelopes, inhibited the proliferative responses, not only of a murine CTL cell line but of both murine and human alloantigen-stimulated lymphocytes. No significant inhibition was seen with (i) a variety of other peptides that were from different regions of either p15E or HTLV gp21E (SP-9 and SP-74), (ii) peptides from HTLV gp46 (SP-70) or HTLV p24 (SP-16), (iii) synthetic neurotensin, or (iv) BSA that had been processed in an identical manner as BSA-coupled CKS-17. The lack of activity with SP-9 and SP-74, which are partially homologous to CKS-17, suggests that the additional or different amino acids contained in CKS-17 may be necessary for biological activity.

Based on our observation of a p15E-like protein in human cancerous effusions and in mitogen-transformed human lymphocytes (*9, 10*), we suggested that normal human cells might contain a gene coding for a p15E-related protein (*2*). The partial homology of CKS-17 with a portion of the envelope protein encoded by an endogenous human retrovirus DNA sequence (*15*) (Fig. 1, sequence 4-1) lends some support to this concept. We hypothesized that under certain conditions, such as neoplastic transformation, the gene might become activated and, by releasing an immunosuppressive substance, enhance the virulence of tumors that express it. Furthermore, Jacquemin *et al.* reported that there are elevated plasma concentrations of a p15E-related glycoprotein in patients with leukemia (*11*). Lymphocytes from patients with acquired immune deficiency syndrome (AIDS) produce an immunosuppressive protein (*17*). The transmembrane envelope region of HTLV-III, the virus which is the etiological agent of AIDS (*18*), has limited (approximately 35 percent) homology to the CKS-17 sequence. Whether this protein plays any role in immunosuppression associated with AIDS remains to be determined.

The present studies suggest that the biologically relevant portion of retroviral envelope proteins may reside in a relatively homologous region represented, at least in part, by CKS-17. Such synthetic peptides might well have diagnostic or therapeutic potential.

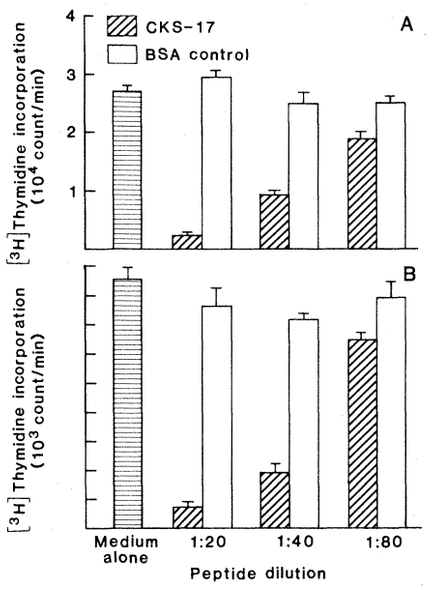

Fig. 2. Effects of CKS-17 on murine (A) or human (B) two-way mixed leukocyte culture reactions. Murine cultures contained 10^5 BALB/c 3T3 and NIH/Swiss splenocytes per well and were cultured for 3 days as described in Table 1, except that the culture medium contained 50 μM 2-mercaptoethanol and lacked IL-2. Cultures were labeled for the final 4 hours with 1.0 μCi of [^{3}H]thymidine per well and were collected on glass-fiber filters; incorporated radioactivity was determined as described in Table 1. Results are the mean ± standard error of the mean of quadruplicate samples. Human mononuclear cells, isolated from the blood of two healthy individuals by Ficoll-Hypaque density gradient centrifugation, were cultured for 6 days under the same conditions as murine cells, with the exception that CKS-17 or the BSA control were present for only the final 24 hours of culture and 2-mercaptoethanol was excluded from the media.

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19. The following abbreviations were used for amino acids: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine.

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