Although tumor suppression and growth suppression functions of *RB* are often taken to be equivalent, these activities have been dissociated in DU145 cells because their growth rate in culture was not significantly altered by *RB* replacement. Tumorigenicity is thus far the only consistent parameter for measuring the biological function of *RB*. Naturally occurring, mutated RB proteins shown to be inactive in tumor suppression include those of DU145 and J82, and a 95-kD protein in Saos-2 osteosarcoma cells that is truncated at its COOH-terminal region encoded by exons 21 to 27 (*25*). We have also characterized a mutated RB protein in a small-cell lung carcinoma that lacks the region encoded by exon 16 (*15*). These results suggest that the tumor suppression function of RB can be disturbed by deletion of any of several regions. Loss of exon 21 or 16 is also correlated with severely decreased or absent RB phosphorylation and with lack of binding to SV40 T antigen (*14, 15, 26*). Several studies have suggested that different phosphorylated forms of RB may have different functional properties (*27*). Analysis of other natural or experimental mutants may further elucidate mechanisms of tumor suppression by the *RB* gene product.

Note added in proof: We have recently identified an internal deletion of *RB* in a primary prostate carcinoma specimen.

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20. PCR amplification and direct sequencing of DU145 DNA has unexpectedly revealed a point mutation within exon 21 at nucleotide 2281. A single base substitution converts a lysine codon (AAG) to an in-frame stop codon (TAG), so this substitution cannot be a neutral sequence polymorphism. A coexisting normal base at this position was not observed. This preliminary result was duplicated in two separate amplification and sequencing experiments. This mutation does not explain the absence of exon 21 in *RB* mRNA by known splicing mechanisms.
21. J82 also differed from DU145 at a polymorphic Bam HI restriction site within *RB* [R. Bookstein *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2210 (1988)], ruling out genetic identity of these two cell lines.
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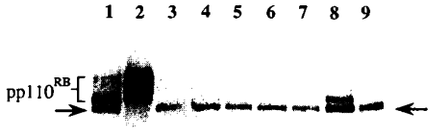

Fig. 4. Tumors formed from Rb-infected DU145 cells lacked expression of the exogenous *RB*. Five tumors (lanes 3 to 7) formed in right flanks of nude mice were individually pulverized in mortars on dry ice and lysed with RIPA buffer. In addition, 2.5×10^6 confluent cultured B5 cells (lane 1), DU145/Rb cells (lane 8), DU145/Lux cells (lane 9), and control Molt-4 cells (lane 2) were lysed with PLC buffer (50 m*M* tris, *p*H 7.4, 250 m*M* NaCl, 0.1% NP-40, 5 m*M* EDTA, 50 m*M* NaF, 2 μ*M* aprotinin, 50 μ*M* leupeptin, and 1 m*M* PMSF). Lysates were immunoprecipitated with anti-fRB, and immunoprecipitates were separated in 7.5% SDS-PAGE and transferred to Immobilon-P membrane (Millipore, Bedford, Massachusetts) by standard techniques (*29*). The membrane was first incubated with blocking buffer, then incubated with monoclonal antibody PMG3-245 followed by alkaline phosphatase–conjugated goat antibody to mouse immunoglobulin G, as described (*16*). Endogenous RB protein (arrows) in lanes 1 and 3 to 9 served as a control for sufficient DU145 cellular protein and proper immunoprecipitation. Expression of exogenous RB is evident in cultured DU145/Rb or B5 cells and is undetectable in tumors; pp110^{RB}, normal RB protein.

Antigen Presentation Requires Transport of MHC Class I Molecules from the Endoplasmic Reticulum

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The role of exocytosis of major histocompatibility complex (MHC) class I molecules in the presentation of antigens to mouse cytotoxic T lymphocytes (CTLs) was examined by use of a recombinant vaccinia virus that expresses the E19 glycoprotein from adenovirus. E19 blocked the presentation of vaccinia and influenza virus proteins to CTLs in a MHC class I allele–specific manner identical to its inhibition of MHC class I transport from the endoplasmic reticulum. This finding indicates that (i) the relevant parameter for antigen presentation is the rate of MHC class I molecule exocytosis, not the level of class I cell surface expression, and (ii) association of class I molecules with antigen is likely to occur within the endoplasmic reticulum.

CYTOTOXIC T LYMPHOCYTES PLAY A critical role in host immunity to intracellular pathogens and cancer. CTLs recognize antigens only in association with class I molecules of the MHC (*1*). Class I molecules consist of an invariant β_2-microglobulin molecule light chain (molecular mass, 12 kD) noncovalently complexed to a 44-kD type I integral membrane glycoprotein heavy chain. Light and heavy chains associate in the endoplasmic reticulum (ER) within minutes of their assembly. Class I molecules are then transported to the plasma membrane via the cis, medial, and trans cisternae of the Golgi complex. An important question about the processing and presentation of antigens to CTLs is where and when class I molecules associate with foreign antigens.

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Table 1. E19 inhibits K^d- and L^d-restricted recognition of Vac antigens. Cells were infected for 3 hours at 37°C with 20 plaque-forming units (PFU) of E19-Vac or a Vac recombinant expressing the respiratory syncytial virus fusion protein (RSV-F-Vac) (*22*). After being labeled for 1 hour with $Na^{51}CrO_4$, cells were incubated for 4 hours at 37°C with effector cells at the effector to target ratios indicated, and the amount of ^{51}Cr released into the medium was determined by gamma radiation counting. Effector cells were splenocytes from mice immunized with Vac and restimulated in vitro for 6 days with autologous Vac-infected splenocytes. Lysis of uninfected target cells ranged from 3 to 17% and was subtracted from all values. All experimental values represent the average of triplicates; all data points had a standard error of the mean of less than 4% specific release. This experiment was performed four times with similar results.

Virus	Target cells	H-2	Percent specific ^{51}Cr release mediated by anti-Vac CTLs derived from			
			BALB/c mice ($H\text{-}2^d$)		CBA/J mice ($H\text{-}2^k$)	
			28:1	9:1	22:1	7:1
E19-Vac	L929-K^d	$K^dK^kD^k$	32	16	55	48
RSV-F-Vac			71	47	67	56
E19-Vac	L929-L^d	$K^kD^kL^d$	24	19	24	11
RSV-F-Vac			64	41	33	15
E19-Vac	L929-D^d	$K^kD^kD^d$	14	9	26	13
RSV-F-Vac			16	10	36	19

Table 2. E19 inhibits presentation of influenza virus antigens. P815 cells were co-infected for 3 hours at 37°C with 20 PFU of E19-Vac or RSV-F-Vac and one of the following Vac recombinants expressing the influenza virus genes encoding hemagglutinin (H1-Vac), nonstructural 1 (NS1-Vac), nucleoprotein (NP-Vac), or basic polymerase 2 (PB2-Vac) (*23*). After being labeled for 1 hour at 37°C with $Na^{51}CrO_4$, cells were incubated for 4 hours at 37°C with anti-influenza effector cells (*24*), and the amount of ^{51}Cr released into the medium was determined by gamma radiation counting. Lysis of uninfected target cells ranged from 2 to 18% and was subtracted from all values. Experimental values represent the average of triplicates; all values had a standard error of the mean of less than 4% specific release. Data shown are from two separate experiments; lysis by effector population (ii) is from one experiment, and the rest are from a second experiment. In parallel with the second experiment, cells co-infected with Vac recombinants for 6 hours were analyzed by use of indirect immunofluorescence MAbs specific for influenza virus proteins (*25*). Cells were fixed and permeabilized with ethanol before assay to detect both intracellular and cell surface–associated viral antigens.

P815 targets	Restriction element	Effector populations	Percent specific ^{51}Cr release		Immunofluorescence	
					Antibody specificity	Percent positive (mean intensity)
H1-Vac + RSV-F-Vac	K^d	(i)	54	38	HA	89 (120)
H1-Vac + E19-Vac		(i)	0	1	HA	92 (88)
NP-Vac + RSV-F-Vac	K^d	(ii)	59	32	NP	94 (25)
NP-Vac + E19-Vac		(ii)	4	2	NP	94 (16)
PB2-Vac + RSV-F-Vac	D^d	(iii)	75	52	PB2	84 (14)
PB2-Vac + E19-Vac		(iii)	58	44	PB2	89 (9)
NS1-Vac + RSV-F-Vac	L^d	(iv)	65	44	NS1	88 (54)
NS1-Vac + E19-Vac		(iv)	30	20	NS1	94 (65)

Results of studies with brefeldin A, an inhibitor of exocytosis of secretory and integral membrane proteins (including class I molecules), were consistent with the idea that antigens associate with nascent class I molecules prior to the transport of class I molecules from the Golgi complex (*2, 3*). This interpretation is, however, limited by the possibility that brefeldin A blocks other processes necessary for antigen processing or presentation. To determine more definitively the role of class I exocytosis in the presentation of protein antigens processed from the cytosol, and to localize the site of antigen association with class I molecules, we examined the effect of the 19-kD adenovirus 5 E3 glycoprotein (E19) on antigen presentation.

E19 is an ER resident protein that specifically binds class I molecules and prevents their transport from the ER (*4*). Difficulties in synchronously and rapidly attaining high levels of E19 expression in antigen presenting cells suitable for detailed analysis of CTL recognition allowed the effect of E19 on antigen presentation to be examined only to the extent of documenting that CTL recognition of E19-expressing cells is diminished in parallel with a decrease in cell surface class I expression (*5, 6*). To circumvent these problems we constructed a vaccinia virus (Vac) recombinant containing the E19 gene (the recombinant is termed E19-Vac) that synthesizes E19 at high levels early in the Vac infectious cycle (*7*).

In preliminary experiments, we established that after infection with E19-Vac, two mouse cell lines, P815 and L929, produced E19 that reacted with antiserum to E19 (some of the results obtained with P815 cells are shown in Fig. 1). E19 from both cell lines migrated with the expected mobility in SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Authentic E19 contains two N-linked oligosaccharides in high-mannose forms sensitive to the action of endo-β-*N*-acetylglucosaminidase H (endo H) (*8*). Both E19-associated oligosaccharides remained sensitive to endo H for at least 4 hours at 37°C after their synthesis in E19-Vac–infected P815 or L929 cells, a result that is consistent with E19 being located in the ER of these cells.

We initially examined the effect of E19 on the recognition of Vac-infected P815 ($H\text{-}2^d$) and L929 ($H\text{-}2^k$) cells by Vac-specific CTLs derived from C3H.OH ($H\text{-}2K^dD^k$) mice or A/J ($H\text{-}2K^kD^dL^d$) mice. Both C3H.OH- and A/J-derived CTLs recognized E19-Vac–infected L929 cells and control Vac–infected cells at equivalent levels. In contrast, recognition of E19-Vac–infected P815 cells by either CTL population was greatly decreased. This experiment suggested that E19 can inhibit the presentation of Vac antigens and that the magnitude of the effect is far greater with K^d- and D^dL^d-restricted antigens than with K^k- and D^k-restricted antigens. To test this idea, we examined the recognition of L929 cells expressing transfected $H\text{-}2^d$ genes [termed L929-K^d (*9*), L929-D^d, and L929-L^d (*10*)] by Vac-specific CTLs derived from CBA ($H\text{-}2^k$) or BALB/c ($H\text{-}2^d$) mice (Table 1). Again, we observed little difference between the ability of E19-Vac and a control Vac recombinant to sensitize any of the cell lines for recognition by $H\text{-}2^k$–restricted Vac-specific CTLs. Furthermore, we found no difference in the recognition of E19-Vac– and of control Vac–infected L929-D^d cells by $H\text{-}2^d$–restricted CTLs. In contrast, recognition of E19-Vac–infected L929-K^d or L929-L^d cells was greatly decreased relative to recognition of control Vac–infected cells.

A trivial explanation for the deficit in L^d- and K^d-restricted presentation by E19-Vac–infected cells is a deficiency in the synthesis of viral proteins recognized by L^d- and K^d-restricted CTLs. To eliminate this possibility we examined the effect of coinfecting P815 cells with E19-Vac and Vac recombinants containing influenza virus genes encoding hemagglutinin (HA), nucleoprotein (NP), basic polymerase 2 (PB2), or nonstructural 1 (NS1) (Table 2). When $H\text{-}2^d$–restricted influenza-specific CTLs are used, HA and NP are recognized in association with K^d; PB2 is recognized in association with D^d, and NS1 with L^d (*11*). Cytofluoro-

graphic analysis of fixed and permeabilized cells indirectly stained with monoclonal antibodies (MAbs) specific for each of the viral components revealed that cells coinfected with E19-Vac and Vac recombinants encoding influenza virus gene products expressed influenza virus proteins in amounts similar to those of cells coinfected with a control Vac recombinant and Vac recombinants encoding influenza virus gene products. Despite this, cells coinfected with E19-Vac did not detectably present the K^d-restricted proteins HA and NP and presented the L^d-restricted NS1 protein at reduced levels. Presentation of the D^d-restricted PB2 protein was only slightly diminished by E19-Vac coinfection.

In view of the E19-mediated inhibition of NP presentation to K^d-restricted CTLs, it was important to examine presentation of a synthetic peptide recognized by NP-specific CTLs. Target cells were coincubated with CTLs for 4 hours at 37°C in the presence of a peptide corresponding to NP residues 147 to 158, lacking arginine at position 156 (*12*). E19-Vac– and control Vac–infected cells were lysed at identical levels, with peptide at concentrations ranging from limiting to saturating. Since exogenous peptides can associate with class I molecules at the cell surface (*2, 13*), this finding suggested that E19 does not inhibit antigen presentation by decreasing the quantities of cell surface class I molecules. Two additional findings bolster this conclusion. First, the effect of E19 on presentation of Vac antigens to L^d-restricted CTLs was apparent even when target cells were infected for only 2 hours before addition of CTLs. Second, infection of P815 cells with E19 for 5 hours did not decrease the levels of cell surface class I molecules relative to cells infected with a control Vac recombinant, as detected by cytofluorography after indirect immunofluorescence with Mabs specific for K^d, D^d, and L^d.

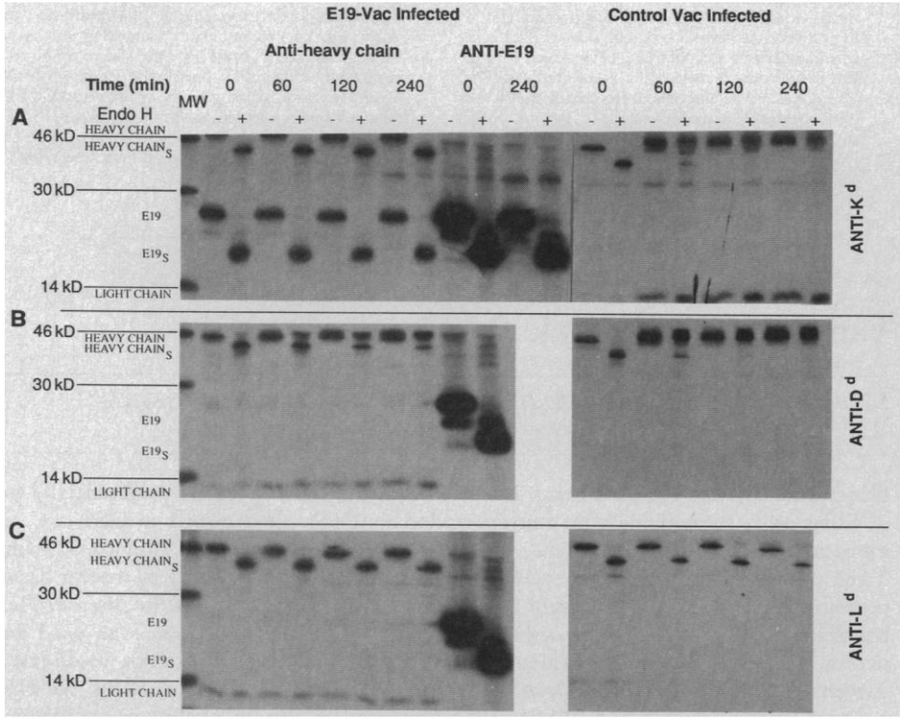

Fig. 1. E19-Vac differentially affects the transport of K^d, D^d, and L^d from the ER. P815 cells were infected with E19-Vac or RSV-F-Vac for 2 hours at 37°C. After incubation in methionine-deficient medium for 30 min at 37°C, cells were labeled for 10 min with [^{35}S]methionine and incubated with unlabeled methionine for the indicated times at 37°C. Labeled cells were extracted and immunoprecipitated with protein-A–Sepharose beads that had been loaded with either class I heavy chain–specific MAbs or with a polyclonal rabbit antiserum to a synthetic peptide consisting of the 15 COOH-terminal amino acids of E19. Half of the immunoprecipitates were digested with endo H. All procedures utilized were essentially as described previously (*18*). Immunoprecipitates were analyzed on 12% polyacrylamide gels by use of the buffer system of Laemmli (*19*). Shown are fluorographs produced by exposing preflashed Kodak XAR-5 film to dried gels at −80°C. The following class I heavy chain MAbs were used for immunoprecipitation: (**A**) anti-K^d, SF1.1.1 (ATCC HB-159); (**B**) anti-D^d, 34-5-8S (ATCC HB-102) (*20*); and (**C**) anti-L^d, 28-14-8S (ATCC HB-27) (*21*). These data represent a number of experiments; precipitation with the E19-specific antisera is shown for each experiment to demonstrate that differences in class I heavy chain transport are not due to variations in levels of E19 expression. Faster migrating, deglycosylated forms of class I heavy chains and E19 resulting from endo H digestion are designated with the subscript S. Lanes marked MW contain ^{14}C-methylated ovalbumin (46 kD), carbonic anhydrase (30 kD), and lysozyme (14 kD).

Additional experiments demonstrated that E19 inhibition of antigen presentation correlated precisely with its inhibition of transport of class I molecules from the ER. P815 or L929 cells infected for 2 hours with E19-Vac or a recombinant Vac control were labeled for 10 min with [^{35}S]methionine and then incubated for up to 4 hours at 37°C with unlabeled methionine. Detergent extracts from cells were immunoprecipitated with MAbs specific for class I heavy chains or with antiserum to E19, and half the immunoprecipitates were digested with endo H to monitor the delivery of radiolabeled molecules to the Golgi complex (*14*). D^k and K^k heavy chains acquired endo H resistance with identical kinetics in E19-Vac– and control recombinant Vac–infected cells. E19 was not coprecipitated with D^k or K^k molecules from E19-Vac–infected L929 cells.

In contrast, immunoprecipitation of K^d resulted in clear co-precipitation of approximately equimolar amounts of E19 (Fig. 1A). β_2-Microglobulin was not present in the immunoprecipitates (*15*). K^d heavy chains did not detectably acquire endo H resistance during the 4-hour treatment with unlabeled methionine. The lack of K^d transport can be specifically attributed to the presence of E19, since K^d heavy chains synthesized by cells infected with a control Vac recombinant acquired endo H resistance with a half-time of less than 60 min (*16*).

E19 was present in much smaller quantities in L^d immunoprecipitates and was barely detected in D^d immunoprecipitates, both of which contained β_2-microglobulin in amounts similar to that of control precipitates. The effect of E19 on D^d was to delay, rather than completely block its transport from the ER (Fig. 1B). This finding provides a ready explanation for the slight effect of E19 on CTL recognition of PB2 (Table 2). Only a small portion of L^d acquired endo H resistance in cells infected with the control Vac recombinant. In E19-Vac–infected cells, no L^d molecules detectably acquired endo H resistance, a result consistent with E19 inhibition of L^d-restricted antigen presentation to CTLs (Fig. 1C).

Our findings demonstrate that (i) E19 can inhibit the presentation of protein antigens processed from the cytosol without decreasing the levels of class I expression at the cell surface; (ii) this inhibition correlates with a selective allele-specific blockade of class I transport; and (iii) presentation of the NP(147–158) lacking Arg^{156} is not affected by E19. These data complement and extend earlier findings obtained with brefeldin A (*2, 3*) and conclusively establish that presen-

tation of protein antigens processed from the cytosol requires the transport of newly synthesized class I molecules from the ER, whereas presentation of exogenous synthetic peptides does not.

The dependence of protein antigen presentation on the transport of class I molecules from the ER indicates that antigen association occurs either within the ER or in a post-ER exocytic compartment that is rapidly emptied of nascent class I molecules after exocytosis at the ER is blocked. A report that exogenous peptides induce assembly of class I heavy and light chains and transport from the ER in a mutant cell line deficient in these functions (*17*) favors the first possibility. Additional studies are needed to firmly establish the site of antigen association with class I molecules and to assess the contributions of other cellular gene products to the process.

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7. For construction of the E19-Vac recombinant, a 5424-bp DNA fragment encoding the Ad5 E3 region (provided by R. Ricciardi, Wistar Institute, Philadelphia, PA) was digested with Sac I and gel-purified to yield a 2214-bp fragment containing the E19 gene. The fragment was ligated into the Sac I polylinker site of pTZ.18R, which was digested with Hinf I and gel-purified to yield a 733-bp fragment. The Hinf I fragment was blunt-ended with the Klenow fragment of DNA polymerase I and treated with calf intestinal phosphatase before being ligated into the Sma I site of pSC11 [S. Chakrabarti, K. Brechling, B. Moss, *Mol. Cell. Biol.* **101**, 725 (1985)]. The resulting plasmid, pJC1, contains 109 bp of adenovirus DNA upstream from the E19 initiation codon and 145 bp of adenovirus DNA downstream from the E19 termination codon. CV1 cells were co-transfected with wild-type Vac and the pJC1 plasmid and subjected to selection for thymidine kinase–deficient virus. All infectious recombinant vaccinia virus stocks were grown from an isolated virus plaque in BSC cells and stored at −70°C (*16*).
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15. This differs from previous observations of E19–class I complexes (including K^d) precipitated by class I–specific antibodies from human cells (*5*). This could reflect antibody-related differences, or perhaps more likely, differences in the interaction of mouse and human β_2-microglobulin with heavy chains.
16. The ability of E19 to block the surface expression of newly synthesized K^d was confirmed with a Vac recombinant expressing K^d [B. E. H. Coupar, M. E. Andrew, D. B. Boyle, R. V. Blanden, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7879 (1986)]. Co-infection of L929 cells with K^d-Vac and a control Vac recombinant resulted in the expression of K^d on the surface of more than 90% of cells as detected by cytofluorographic analysis of viable cells stained by indirect immunofluorescence. Cells co-infected with E19-Vac and K^d-Vac did not detectably express K^d on their surfaces. K^d accumulated intracellularly in these cells, since cytofluorographic analysis of cells permeabilized by ethanol treatment revealed equivalent fluorescence between cells co-infected with K^d-Vac plus E19-Vac and cells co-infected with K^d-Vac plus a control Vac recombinant.
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24. Effector cells were obtained from in vitro virus-stimulated cultures of splenocytes from influenza virus or Vac recombinant–immunized mice as follows (effector:target ratio) (i) H1-Vac–immunized mice restimulated with A/Puerto Rico/8/34 (H1N1) (PR8) (78:1 and 9:1); (ii) PR8 immunized mice restimulated with A/Northern Territories/60/68 (H3N2) (30:1 and 3:1); (iii) PB2-Vac–immunized mice restimulated with PR8 (66:1 and 7:1); and (iv) PR8-immunized mice restimulated with PR8 (15:1 and 5:1).
25. Cells fixed and permeabilized in 70% ethanol were washed and incubated with the following MAbs: H28-E23, HA-specific; H19-S24, NP-specific; NS1-1A7, NS1-specific; 170-1C12, PB2-specific. Cells were then washed and incubated with fluorescein-labeled rabbit immunoglobulin to mouse immunoglobulin, washed, and then analyzed via cytofluorography.

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T Cells Responsive to Myelin Basic Protein in Patients with Multiple Sclerosis

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Gene mutation in vivo in human T lymphocytes appears to occur preferentially in dividing cells. Individuals with multiple sclerosis (MS) are assumed to have one or more populations of dividing T cells that are being stimulated by autoantigens. Mutant T cell clones from MS patients were isolated and tested for reactivity to myelin basic protein, an antigen that is thought to participate in the induction of the disease. The hypoxanthine guanine phosphoribosyltransferase (*hprt*) clonal assay was used to determine mutant frequency values in MS patients with chronic progressive disease. Eleven of 258 thioguanine-resistant (*hprt*$^-$) T cell clones from five of the six MS patients who were tested proliferated in response to human myelin basic protein without prior in vitro exposure to this antigen. No wild-type clones from these patients, nor any *hprt*$^-$ or wild-type clones from three healthy individuals responded to myelin basic protein. Thus, T cell clones that react with myelin basic protein can be isolated from the peripheral blood of MS patients.

ALTHOUGH THE ETIOLOGY OF MS IS unknown, an autoimmune basis for this demyelinating disorder has been proposed (*1*). The similarity of MS to experimental allergic encephalomyelitis (EAE), a relapsing disease of mice, has suggested that MS, like EAE, might be a consequence of autosensitization to myelin basic protein (MBP) (*2*). However, attempts to isolate MBP-reactive T cells from the blood, cerebrospinal fluid, or plaque tissue obtained at autopsy from MS patients has been unsuccessful without stimulation

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