Mutations in the Gene Encoding Cystatin B in Progressive Myoclonus Epilepsy (EPM1)

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Progressive myoclonus epilepsy of the Unverricht-Lundborg type (EPM1) is an autosomal recessive inherited form of epilepsy, previously linked to human chromosome 21q22.3. The gene encoding cystatin B was shown to be localized to this region, and levels of messenger RNA encoded by this gene were found to be decreased in cells from affected individuals. Two mutations, a 3′ splice site mutation and a stop codon mutation, were identified in the gene encoding cystatin B in EPM1 patients but were not present in unaffected individuals. These results provide evidence that mutations in the gene encoding cystatin B are responsible for the primary defect in patients with EPM1.

Epilepsy is a heterogeneous disorder that affects 3% of the world's population (*1*). Although the etiology of most epilepsies is unknown, genetic factors play an important role in the disease. Genetic linkage has been established for several inherited epilepsies, and in one case a mutation in a specific gene has been identified (*2*). Progressive myoclonus epilepsy refers to a heterogeneous group of severe inherited epilepsies characterized by myoclonic seizures, generalized epilepsy, and progressive neurological deterioration, including dementia and ataxia (*3*). One of the five recognized members of this group is progressive myoclonus epilepsy of the Unverricht-Lundborg type (EPM1) (*4*). This form of epilepsy is inherited as an autosomal recessive disease with severe stimulus-sensitive myoclonus and tonic-clonic seizures beginning between ages 6 and 15 and a variable rate of progression between and within families (*5*). Seizures tend to diminish at 25 to 30 years of age, although mild dementia generally develops late in the course of the disease. Unlike other progressive myoclonus epilepsies, inclusion bodies or storage material are not observed in EPM1 and diagnosis is usually based on clinical history, typical electroencephalographic abnormalities, and exclusion of the other four subtypes (Lafora's disease, myoclonus epilepsy and ragged red fibers syndrome, neuronal ceroid lipofuscinosis, and sialidosis).

Linkage analysis initially localized the gene responsible for EPM1 to a region of 2 million base pairs on human chromosome 21 between the DNA markers CBS and CD18 (*6*). Founder effects and bottlenecks in the history of the Finnish population allowed us to use linkage disequilibrium and recombination breakpoint mapping with Finnish EPM1 patients to refine the location of the gene to a region between markers D21S2040 and D21S1259 (Fig. 1) (*7*). This region is entirely encompassed in a 750–kilobase pair (kb) bacterial clone contig we generated by sequence-tagged site–content mapping and walking. We used a detailed restriction map of the contig to determine that the distance between the DNA markers defining the boundaries of EPM1 is about 175 kb (*8*). We used this combination of genetic and physical mapping information, as well as the clone reagents, to perform a systematic search for the gene encoding EPM1.

For part of this positional cloning effort, we used direct complementary DNA (cDNA) selection to isolate segments of expressed DNA from the 175-kb region (*9*). Several cDNAs identified with bacterial artificial chromosome (BAC) clone 52C10 with the use of this method encode a previously described protein, cystatin B, a cysteine protease inhibitor (Fig. 1) (*10*). Hybridization and polymerase chain reaction (PCR) amplification experiments indicated that these cDNA segments were derived from a 9-kb Eco RI restriction fragment in the BAC clone and the overlapping cosmid clones that were used to build the contig (Fig. 1) (*8*). These results indicated that the gene encoding cystatin B, which had previously not been mapped to a human chromosome, lies in this segment of human chromosome 21. Further analysis with other restriction enzymes and hybridizations demonstrated that the gene is oriented 5′ to 3′ in the telomere-to-centromere direction.

We confirmed previous reports that the gene encoding cystatin B is widely expressed by demonstrating that a probe made from the cDNA clone detects an mRNA approximately 0.8 kb in length in all tissues examined (Fig. 2A). These results suggested that measurement of mRNA levels in lymphoblastoid cell lines could be used as an initial screen for alterations in the cystatin B gene in affected individuals (*11*). On Northern (RNA) blots, lymphoblastoid cells from affected individuals from a Finnish family, an American family (Fig. 2B), and two other families (*12*) had reduced levels of cystatin B mRNA compared to those in an unaffected, noncarrier individual (Fig. 2B) and carrier parents of EPM1 patients (Fig. 2B). These results suggest that the gene encoding cystatin B from these affected individuals is mutated in a manner that results in decreased amounts of mature mRNA and that these mutations play a primary role in EPM1.

These findings led us to sequence the

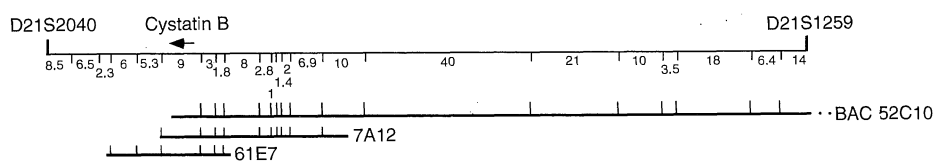

Fig. 1. Physical mapping information used in the positional cloning of the progressive myoclonus epilepsy (EPM1) gene. The top line shows an Eco RI restriction map of the 175-kb region on chromosome 21q22.3 flanked by DNA markers D21S2040 and D21S1259 (oriented from left to right in the centromeric to telomeric direction) that was shown by linkage disequilibrium and recombination breakpoint mapping to contain the gene encoding EPM1. Vertical tick marks on the expanded region indicate Eco RI restriction sites, with the numbers below indicating the sizes of the restriction fragments determined by Stone *et al.* (*8*). A bacterial artificial chromosome clone (BAC 52C10), which was used as the genomic source for direct cDNA selection, is shown below the Eco RI map, and two cosmid clones (7A12 and 61E7) that contain the gene encoding cystatin B are also depicted. The leftward arrow indicates the location and transcriptional orientation, from 5′ to 3′, of the gene encoding cystatin B, which is completely encompassed in a 9-kb Eco RI restriction fragment, on the chromosome.

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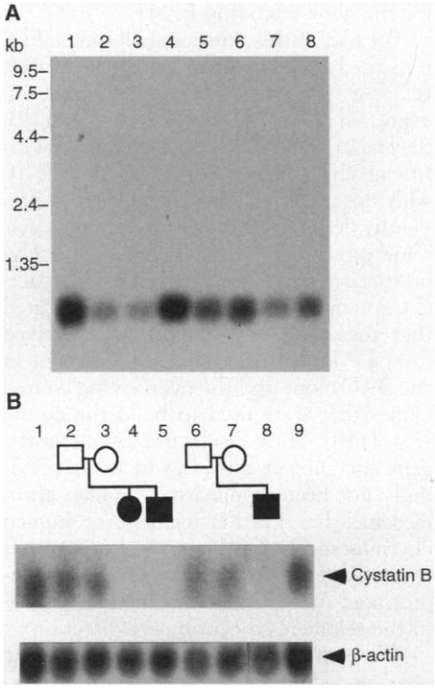

Fig. 2. Messenger RNA analysis of the gene encoding cystatin B in affected and unaffected individuals. (**A**) A 500-bp cystatin B cDNA probe was hybridized to RNA blots. Each lane contained 2 μg of polyadenylated mRNA from eight human tissues (Clontech, Palo Alto, California), including 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; and 8, pancreas. The size of the cystatin B mRNA is less than 1 kb, which is consistent with the 642 bp of the original full-length cDNA sequence described in (*13*). (**B**) The same probe was hybridized to RNA blots containing 20 μg of total RNA from lymphoblastoid cell lines (upper panel); 1 and 9 are from unaffected noncarrier controls; 2 through 5 are from a Finnish family with EPM1, including 2, carrier father; 3, carrier mother; 4, affected child; and 5, affected child; and 6 through 8 are from an American family, including 6, carrier father; 7, carrier mother; and 8, affected child. A human β-actin probe was hybridized to the same Northern blot to assess the approximate quantity of RNA loaded per lane (lower panel).

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                                                    5' Upstream
acccagcctgcggcgagtggtggccaggctccccgcccgcgccccgcccgcgccccgcccgcgcgtcccttctt

gcggggccaccgcgaccccgcaggggactccgaagccaaagtgcctcctccccgcccttggttccgcCGCGCGTC
                                                    5' UTR
ACGTGACCCCAGCGCCTACTTGGGCTGAGGAGCCGCCGCGTCCCCTCGCCGAGTCCCCTCGCCAGATTCCCTCCGTC

GCCGCCAAG ATG ATG TGC GGG GCG CCC TCC GCC ACG CAG CCG GCC ACC GCC GAG ACC CAG C
          M   M   C   G   A   P   S   A   T   Q   P   A   T   A   E   T   Q
                                                                    Intron 1
AC ATC GCC GAC CAG gtgggtgggccgcggggacggggccggcccggagtcctgccttagc...1364 bp...
H   I   A   D   Q
                                  c
cactgagacatcctcattctgtcccttctgtctag GTG AGG TCC CAG CTT GAA GAG AAA GAA AAC AAG
                                      V   R   S   Q   L   E   E   K   E   N   K

AAG TTC CCT GTG TTT AAG GCC GTG TCA TTC AAG AGC CAG GTG GTC GCG GGG ACA AAC TAC
 K   F   P   V   F   K   A   V   S   F   K   S   Q   V   V   A   G   T   N   Y
                                                    Intron 2
TTC ATC AAG gtagagtgtgggcctcaggagggcctg...266 bp...atcagaggcttcgctcactccgctctc
 F   I   K
                                                  T
ttcccag GTG CAC GTC GGC GAC GAG GAC TTC GTA CAC CTG CGA GTG TTC CAA TCT CTC CCT
        V   H   V   G   D   E   D   F   V   H   L   R   V   F   Q   S   L   P

CAT GAA AAC AAG CCC TTG ACC TTA TCT AAC TAC CAG ACC AAC AAA GCC AAG CAT GAT GAG
 H   E   N   K   P   L   T   L   S   N   Y   Q   T   N   K   A   K   H   D   E
                                                    3' UTR
CTG ACC TAT TTC TGATCCTGACTTTGGACAAGGCCCTTCAGCCAGAAGACTGACAAAGTCATCCTCCGTCTACC
 L   T   Y   F   *

AGAGCGTGCACTTGTGATCCTAAAATAAGCTTCATCTCCGCTGTGCCCTTGGGGTGGAAGGGGCAGGATTCTGCAGC

TGCTTTTGCATTTCTCTTCCTAAATTTCATTGTGTTGATTTCTTTCCTTCCCAATAGGTGATCTTAATTACTTTCAG
                                                    3' Downstream
AATATTTTCAAAATAGATATATTTTTAAAATCCTTACagattgcctcctttgcttttagacttttttcttgctgcta

accaccccgggcaggtccttcccctccaggcaggagggcggagagagtc
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Fig. 3. Genomic sequence of the gene encoding human cystatin B. Uppercase letters for nucleotides indicate the portions of the gene present in the mature mRNA transcript, and lowercase letters designate the 5′ flanking region, the two introns, and the 3′ flanking region. Amino acids in the cystatin B protein are designated below the nucleotide sequence (*13*). Underlined bases designate potential Sp1 binding sites in the 5′ flanking region. Only portions of the two introns, which are 1445 and 325 bp in length, are shown; 5′ and 3′ untranslated regions are indicated by 5′ UTR and 3′ UTR, respectively. The two mutations we identified in this study are designated by boxes, where the mutant sequence is shown above the wild-type sequence. The 2500-bp sequence of the gene encoding cystatin B was determined as follows. We first determined that the entire gene is present on a 9-kb Eco RI fragment in several genomic clones from a cosmid and BAC contig of the region. Oligonucleotide primers based on the cDNA sequence were used to determine the sequences of the exon-intron junctions, and additional primers were generated on the basis of this information to determine the complete sequence of the gene, which has been deposited in GenBank (accession number U46692).

gene encoding cystatin B from affected individuals. Because only cDNA and not genomic sequence information was available, we first determined the entire nucleotide sequence of the human gene from an unaffected chromosome (Fig. 3). This sequencing revealed that the gene is 2500 base pairs (bp) in length and contains three small exons encoding the 98–amino acid protein, whose mature mRNA and amino acid sequence were previously known (*13*). We used this information to amplify by PCR the gene encoding cystatin B and to determine its sequence from an affected individual from each of the four EPM1 families in our study (*14*). Our previous haplotype analysis suggested that three or four independent EPM1 mutations are present in these families (*6*, *7*). Sequence comparison identified two different mutations in the gene encoding cystatin B in these individuals. One is a G-to-C transversion at the last nucleotide of intron 1, altering the sequence of the 3′ splice site AG dinucleotide that appears in this position in almost all introns (Figs. 3 and 4A) (*15*). The second mutation, which was found in alleles of the cystatin B gene from two of the four families, changes CGA to TGA, generating a translation stop codon at amino acid position 68 (Figs. 3 and 4B).

The 3′ splice site mutation destroys a recognition site for the restriction enzyme Bfa I, which allowed us to develop a simple test to screen alleles in large numbers of unaffected individuals (Fig. 4C). We found no mutant alleles after screening 190 chromosomes for this change in 95 unrelated, unaffected individuals. Similarly, we used direct sequencing of PCR products to screen 70 alleles for the stop codon mutation from unaffected control individuals and found no mutant alleles in this sample (*16*).

Despite identifying these two mutations in affected chromosomes from three of the four families, we were unable to detect any sequence differences in the gene encoding cystatin B from the remaining one or two alleles that we had available for our study. However, these alleles are accounted for by the common Finnish haplotype alone or by that haplotype and one other mutation. This sequence comparison included the 100 bp flanking the transcription start site, the entire coding region, several hundred base pairs of the introns at each junction, and 100 bp of the 3′ flanking region. In spite of the fact that the Finnish ancestral mutation has not yet been identified, our Northern blot experiments (Fig. 2) showed that the expression of the gene from this allele, as well as from all other alleles we tested, is defective.

Cystatin B is a small protein that is a member of a superfamily of cysteine protease inhibitors (*10*). It is a tightly binding revers-

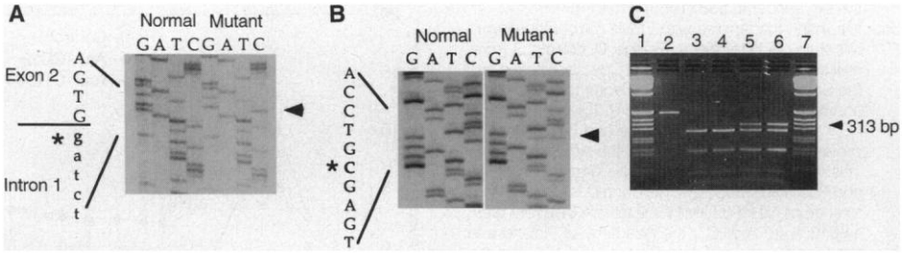

Fig. 4. DNA sequence analysis of the gene encoding cystatin B in EPM1 patients. (**A**) A portion of an autoradiogram showing the DNA sequence of cloned PCR products from an unaffected chromosome (left) and an affected chromosome from an American family (right), both of which we had transferred into somatic cell hybrids to allow single alleles to be separately analyzed. Amplification primers were designed on the basis of the cystatin B genomic sequence to produce 803-bp products (primers F11 and R1), which were cloned into a plasmid vector. Ten independent clones were sequenced with primer F11, all of which produced the mutant sequence shown on the right. This transversion mutation changes the last nucleotide of the 3′ splice acceptor of intron 1 from a G to a C in the affected chromosome, as noted by the arrowhead and asterisk. (**B**) A portion of an autoradiogram showing the nucleotide sequence determined directly from amplified PCR products from an unaffected chromosome (left) and from an affected chromosome from a Finnish family (right). The PCR products were excised from agarose gels and purified by Gene-Clean (Bio-101) before sequencing. The asterisk and arrowhead indicate the C-to-T transition mutation in this patient that results in the formation of a stop codon in the cystatin B coding sequence. Because the affected individual is heterozygous for the stop codon mutation, two bands are seen at this position in the autoradiogram. (**C**) A restriction enzyme screen for the 3′ splice site mutation. This mutation destroys a site for the restriction enzyme Bfa I. To screen large numbers of genomic DNA samples, we amplified 100 ng of genomic DNA with 20 pmol of primers F11 and R10 under the same conditions we used to amplify the 3′ segment of the gene encoding cystatin B for sequencing (*14*). The 474-bp product was digested with 10 units of Bfa I for 3 hours, and the fragments were resolved by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining. PCR products from an unaffected chromosome result in DNA fragments 260, 140, 53, and 21 bp in length, whereas PCR products from individuals heterozygous for this mutation result in the generation of an additional fragment 313 bp in length that is a result of the loss of a Bfa I site separating the 260-bp and 53-bp fragments. Lanes 1 and 7, DNA size markers (1-kb ladder; BRL); lane 2, undigested 474-bp PCR product; lane 3, Bfa I–digested PCR product from an unaffected individual; lane 4, Bfa I–digested PCR product from the father of an EPM1 patient; lane 5, Bfa I–digested PCR product from the mother of the same EPM1 patient; and lane 6, Bfa I–digested PCR product from an EPM1 patient, the child of the parents analyzed in lanes 4 and 5. The mother carries the allele of the gene encoding cystatin B that contains the 3′ splice mutation, whereas the father carries a different mutant allele.

ible inhibitor of cathepsins L, H, and B, is found in all tissues, and is thought to inactivate proteases that leak out of the lysosome (*10, 13, 17*). Despite ubiquitous expression of this protein, it is not understood why mutation of the gene encoding cystatin B causes the symptoms of EPM1, an apparent tissue-specific phenotype. Another member of this family of protease inhibitors, cystatin C, has been shown to be responsible for hereditary cerebral amyloid angiopathy (*18*). This dominantly inherited disease is characterized by the deposition of cystatin C–rich amyloid fibrils in affected brain arteries. However, EPM1 is inherited in a recessive manner and is likely the result of decreased amounts of cystatin B, suggesting different mechanisms for the two diseases.

The genes responsible for Lafora's disease and juvenile myoclonus epilepsy, which have symptoms similar to EPM1, have been localized to specific chromosomal regions by meiotic linkage analysis (*2*). The identification of cystatin B defects in EPM1 suggests that other members of the cystatin superfamily or their substrates might be defective in these related epilepsies. Even in chronic and severe cases, patients with EPM1 show marked improvement when treated with the anti-epileptic drug sodium valproate (*19*). However, phenytoin, another drug that is effective against some other forms of epilepsy, does not improve the condition of EPM1 patients, often shows toxic effects, and, in some cases, is fatal. The identification of mutant genes encoding cystatin B in patients with EPM1 may help to understand the differential response to these two drugs. Furthermore, this knowledge provides a biochemical pathway and molecular target for the treatment of EPM1 and perhaps other forms of epilepsy.

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11. Total RNA and DNA were extracted from cultured cells with the TRIZOL reagent (BRL, Gaithersburg, MD). Northern blot hybridization was in 5× SSPE, 10× Denhardt's solution, 100 μg/ml of salmon sperm DNA, 50% formamide, and 2% SDS at 42°C for 18 hours. Filters were washed with 2× saline sodium citrate (SSC) at room temperature for 30 min and in 0.1× SSC at 60°C for 30 min.
12. L. A. Pennacchio *et al.*, data not shown.
13. The GenBank number for the genomic sequence determined here is U46692. Abbreviations for the amino acids residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; and asterisk, stop codon [A. Ritonja, W. Machleidt, A. J. Barrett, *Biochem. Biophys. Res. Commun.* **131,** 1187 (1985)]. The GenBank number for the cDNA sequence, deposited by K. S. Bhat, is L03558.
14. The gene encoding cystatin B was amplified by PCR in two overlapping segments from genomic DNA of affected individuals from four families. These included a 5′ segment with oligonucleotide primers pF2 and 51814R1 and a 3′ segment with primers F11 and R1 (pF2: 5′-CTCCGACTGCCCCT-TCCCTAT-3′; 51814R1: 5′-GAGACACAGGGAAA-GTTGCCATCT-3′; F11: 5′-CCACCGTACCCAGCT-GGAACTGT-3′; and R1: 5′-CGGAGGATGACTTT-GTCAGTCTTC-3′). These primers were used for PCR amplification and for sequencing. The following primers were also used for sequencing: F3: 5′-TAAG-GCCGTGTCATTCAAGAGCCA-3′; F5: 5′-CGCCG-AGACCCAGCACATC-3′; and R10: 5′-TCTTAGCT-CCCCAGAAGCCCTAGT-3′. The PCR assay for the 5′ segment of the gene included 10% dimethyl sulfoxide and 50% deaza–deoxyguanosine triphosphate and the following cycling conditions: initial incubation at 95°C for 5 min followed by 30 cycles of 30 s at 95°C, 30 s at 65°C, and 2 min at 72°C, with a final incubation for 10 min at 72°C. Conditions for amplifying the 3′ segment of the gene included an initial incubation at 94°C for 5 min followed by 30 cycles for 30 s at 94°C, 30 s at 60°C, and 2 min at 72°C, with a final incubation for 10 min at 72°C. PCR products were purified with a Centricon-100 concentrator (Amicon, Beverly, MA) and sequenced directly with cycle sequencing with SequiTherm DNA polymerase (Epicentre, Madison, WI) or cloned into a plasmid vector. Cloned products were manually sequenced with Sequenase (U.S. Biochemicals, Cleveland, OH). The sequencing reaction products were separated on 6% polyacrylamide gels and visualized by autoradiography.
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16. The 3′ splice site mutation was screened in the general population in 95 unrelated Americans (190 chromosomes), 90% of whom were of European ances-

try and 10% of whom were of other ethnic backgrounds. The stop codon mutation was screened in 70 Finnish EPM1 carrier parents. All 70 of these individuals contained the common ancestral haplotype around the EPM1 locus on one of their chromosomes. To distinguish mutations from polymorphisms, we considered only the nonancestral haplotype chromosome of these 70 individuals. DNA from these individuals was amplified by PCR, and the products were directly sequenced with the AmpliCycle sequencing kit (Perkin-Elmer).
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20. We thank the families with EPM1 for contributing to this study; C. Iannicola, C. Prange, D. Vollrath, J. Kere, and members of the Myers and Cox laboratories and the Stanford Human Genome Center for discussions and support; A.-L. Träskelin and R. Tolvanen for technical assistance; and R. Eldridge and B. J. Wilder for providing patient samples from the American family. This work was supported by NIH grants HD-24610 and P50 HG-00206 (to R.M.M. and D.R.C.), postdoctoral grant NIH IF32GM17502 (to J.A.W.), NIH grant NS31831 (to A.d.l.C.), the Academy of Finland and the Sigrid Juselius Foundation (to A.d.l.C. and A.-E.L.), and the Epilepsy Research Foundation of Finland (to A.-E.L.). Part of this study was done at the Folkhälsan Institute of Genetics (Helsinki).

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Enhancement of Antitumor Immunity by CTLA-4 Blockade

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One reason for the poor immunogenicity of many tumors may be that they cannot provide signals for CD28-mediated costimulation necessary to fully activate T cells. It has recently become apparent that CTLA-4, a second counterreceptor for the B7 family of costimulatory molecules, is a negative regulator of T cell activation. Here, in vivo administration of antibodies to CTLA-4 resulted in the rejection of tumors, including preestablished tumors. Furthermore, this rejection resulted in immunity to a secondary exposure to tumor cells. These results suggest that blockade of the inhibitory effects of CTLA-4 can allow for, and potentiate, effective immune responses against tumor cells.

Despite expressing antigens recognizable by a host's immune system, tumors are very poor in initiating effective immune responses. One reason for this poor immunogenicity may be that the presentation of antigen alone is insufficient to activate T cells. In addition to T cell receptor engagement of an antigenic peptide bound to major histocompatibility complex (MHC) molecules, additional costimulatory signals are necessary for T cell activation (*1*). The most important of these costimulatory signals appears to be provided by the interaction of CD28 on T cells with its primary ligands B7-1 (CD80) and B7-2 (CD86) on the surface of specialized antigen-presenting cells (APCs) (*2–4*). Expression of B7 costimulatory molecules is limited to specialized APCs. Therefore, even though most tissue-derived tumors may present antigen in the context of MHC molecules, they may fail to elicit effective immunity because of a lack of costimulatory ability. Several studies support this notion. In a variety of model systems, transfected tumor cells expressing costimulatory B7 molecules induced potent responses against both modified and unmodified tumor cells (*5–8*). It appears that tumor cells transfected with B7 are able to behave as APCs, presumably allowing direct activation of tumor-specific T cells.

Recent evidence suggests that costimulation is more complex than originally thought and involves competing stimulatory and inhibitory signaling events (*3, 9–12*). CTLA-4, a homolog of CD28, binds both B7-1 and B7-2 with affinities much greater than does CD28 (*13–16*). In vitro, antibody cross-linking of CTLA-4 has been shown to inhibit T cell proliferation and interleukin-2 production induced by antibody to CD3 (anti-CD3), whereas blockade of CTLA-4 with soluble intact or Fab fragments of antibody enhances proliferative responses (*17, 18*). Similarly, soluble intact or Fab fragments of anti–CTLA-4 greatly augment T cell responses to nominal peptide antigen or the superantigen *Staphylococcus* enterotoxin B in vivo (*19, 20*). It has also been suggested that CTLA-4 engagement can induce apoptosis in activated T cells (*21*). Finally, mice deficient in CTLA-4 exhibit severe T cell proliferative disorders (*22*). These results demonstrate that CTLA-4 is a negative regulator of T cell responses and raise the possibility that blockade of inhibitory signals delivered by CTLA-4–B7 interactions might augment T cell responses to tumor cells and enhance antitumor immunity.

We first sought to determine whether CTLA-4 blockade with nonstimulatory, bivalent antibody (*18, 20*) would accelerate rejection of B7-positive tumor cells. Previously, we showed that B7-1 expression was partially successful at inducing rejection of the transplantable murine colon carcinoma 51BLim10 (*23*). We reasoned that CTLA-4 blockade would remove inhibitory signals in the costimulatory pathway, resulting in enhanced rejection of the tumor cells. We injected groups of BALB/c mice with B7-1–transfected 51BLim10 tumor cells (B7-51BLim10) (*23*). Two groups were treated with a series of intraperitoneal injections of either anti–CTLA-4 or anti-CD28 (*18, 24*). Treatment with anti–CTLA-4 inhibited B7-51BLim10 tumor growth as compared with the anti-CD28–treated mice or the untreated controls (Fig. 1). All mice in the untreated and anti-CD28–treated groups developed small tumors that grew progressively for 5 to 10 days and then ultimately regressed in 8 of the 10 mice by about day 23 after injection. The two small tumors that did not regress remained static for more than 90 days. In contrast, three of five mice treated with anti–CTLA-4 developed very small tumors, all of which regressed completely by day 17. Although these results were encouraging and were consistent with our hypothesis, they were not very dramatic because B7-1 expression resulted in fairly rapid rejection of transfected 51BLim10 cells even in the absence of CTLA-4 blockade; however, these results confirmed that anti–CTLA-4 did not inhibit tumor rejection.

We next examined the effects of CTLA-4 blockade on the growth of V51BLim10, a vector control tumor cell line that does not express B7 (*23*). All mice either injected with 4×10^6 V51BLim10

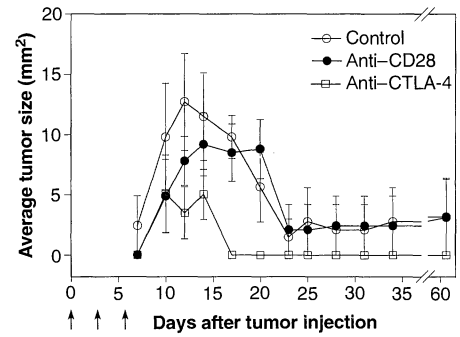

Fig. 1. Treatment with anti–CTLA-4 accelerates rejection of a B7-1–positive colon carcinoma (*23*). A volume of 100 μl of cell suspension (4×10^6 cells) was injected subcutaneously into the left flanks of groups of five female BALB/c mice. Two of the groups received three intraperitoneal injections of either anti–CTLA-4 or anti-CD28 (*18*). Injections of 100, 50, and 50 μg of antibody were given on days 0, 3, and 6, respectively, as indicated by the arrows. Control animals received no injections. Data points represent the average of the products of bisecting tumor diameters. Error bars represent standard error of the mean.

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