

A CASP-8 Mutation Recognized by Cytolytic T Lymphocytes on a Human Head and Neck Carcinoma

By Susanna Mandruzzato,* Francis Brasseur,* Guy Andry,†
Thierry Boon,* and Pierre van der Bruggen*

From the *Ludwig Institute for Cancer Research, Brussels Branch, and Unité de Génétique Cellulaire, Université Catholique de Louvain, B-1200 Brussels, Belgium; and the †Institut Jules Bordet, Service de Chirurgie, Université Libre de Bruxelles, B-1000 Brussels, Belgium

Summary

Of the antigens recognized on human tumors by autologous cytolytic T lymphocytes, all those defined thus far have been identified on melanoma or renal cell carcinoma. We report here the identification of an antigen recognized by autologous cytolytic T lymphocytes on a human squamous cell carcinoma of the oral cavity. The antigen is encoded by a mutated form of the *CASP-8* gene. This gene, also named *FLICE* or *MACH*, codes for protease caspase-8, which is required for induction of apoptosis through the Fas receptor and tumor necrosis factor receptor-1. The mutation, which was found in the tumor cells but not in the normal cells of the patient, modifies the stop codon and adds an Alu repeat to the coding region, thereby lengthening the protein by 88 amino acids. The ability of the altered protein to trigger apoptosis appears to be reduced relative to the normal caspase-8. The antigenic peptide is a nonamer presented by HLA-B*3503. The five last amino acids are encoded by the extension of the reading frame caused by the mutation. This, together with previous observations of CDK4 and β -catenin mutations, suggests that a significant fraction of the point mutations generating a tumor antigen also play a role in the tumoral transformation or progression.

Several tumor antigens recognized by autologous cytolytic T lymphocytes (CTL) have been identified, both on mouse and on human tumors. The initial work carried out with mouse mastocytoma P815 revealed that antigens recognized on tumors by CTL can arise either from point mutations occurring in genes that are expressed ubiquitously or from the transcriptional activation of genes not expressed in normal tissues (1–3). Subsequent work provided two interesting examples of mouse tumor antigens resulting from point mutations (4, 5).

A number of human tumor antigens recognized by autologous CTL also belong to these two categories. Some of them are encoded by genes active in a significant proportion of tumors of various histological types but silent in normal tissues except in testis (6, 7). Others are encoded by mutated genes (8–11). In the case of melanomas, autologous CTL can also be generated against differentiation antigens present on both melanoma cells and normal melanocytes (12, 13). All these antigens were originally identified on melanoma and renal cell carcinoma; tumors that are considered sensitive to an immune attack.

Head and neck cancer has a worldwide incidence of ~400,000 new cases per year. The most important risk factors are tobacco smoking and alcohol consumption. Head

and neck squamous cell carcinoma (SCCHN)¹ tumors are generally infiltrated by T lymphocytes (14, 15). Cytolytic T cell lines with a specificity for the autologous tumor were obtained from peripheral blood lymphocytes of a patient and from tumor-infiltrating lymphocytes in several other patients (16–18).

We have made a systematic effort to obtain tumor cell cultures from biopsy specimens of head and neck carcinomas. Permanent cell lines were established from 14 tumors, representing a 19% success rate. Using five of these cell lines, we performed mixed lymphocyte–tumor cell cultures by using irradiated tumor cells and autologous blood lymphocytes. With four patients, we failed to obtain T lymphocytes that lysed the autologous tumor cells. However, from the blood lymphocytes of patient BB49 we were able to isolate a CTL clone that lysed the autologous tumor cells. We report here the identification of the antigen recognized by this CTL clone.

¹Abbreviations used in this paper: FADD, Fas-associated death domain protein; FasL, Fas ligand; HS, human serum; MLTC, mixed lymphocyte–tumor cell culture; SCCHN, head and neck squamous cell carcinoma; TRADD, TNFR1-associated death domain protein.

Materials and Methods

Cell Lines. Squamous cell carcinoma cell line BB49-SCCHN was adapted to culture from a tumor relapse resected from the floor of the oral cavity of patient BB49, a 70-yr-old Caucasian woman in stage IV (T₄N₀M₀), who died as a result of tumor progression 4 mo later. The tumor cell line was transfected by the calcium phosphate precipitation method with expression vector pEFBOS (received from S. Nagata, Osaka Bioscience Inst., Osaka, Japan), containing the coding sequence for human costimulatory molecule B7-1 (CD80) and in which the gene conferring puromycin resistance was inserted by J.-C. Renauld (Ludwig Inst., Brussels, Belgium; 19). Transfectants were selected with 0.5 µg/ml of puromycin and cloned by limiting dilution. Clonal subline BB49-SCCHN.B7 was selected on the basis of a high expression of B7-1, as determined by cytofluorimetric analysis with mAb BB1 (Becton Dickinson, Mountain View, CA).

Culture media were supplemented with 10% FCS, and with l-arginine (116 mg/liter), l-asparagine (36 mg/liter), l-glutamine (216 mg/liter), streptomycin (0.1 mg/ml) and penicillin (200 U/ml). COS-7 and 293-EBNA cells were grown in DMEM. Cell lines BB49-EBV, K562, and LG-2-EBV were grown in Iscove's medium. Cell lines BB49-SCCHN and BB49-SCCHN.B7 were grown in Iscove's medium supplemented with ACL-4 (20). All the cultures were maintained at 37°C and in 8% CO₂.

Stimulation of CTL. Blood mononuclear cells (PBMC) from patient BB49 were isolated by density-gradient centrifugation on lymphoprep (Nycomed Pharma AS, Oslo, Norway) and cryopreserved at -80°C in Iscove's medium supplemented with 10% human serum (HS) and 10% DMSO. We have verified by DNA fingerprinting that the BB49-SCCHN line was of the same genetic origin as the PBMC (21).

Autologous mixed lymphocyte-tumor cell culture (MLTC) was performed as follows: 2 × 10⁶ PBMC were stimulated with 10⁵ irradiated (100 Gray from a cesium source) BB49-SCCHN.B7 cells in 2 ml of Iscove's medium supplemented with 10% HS and with asparagine, glutamine, and arginine as indicated above (hereafter referred to as complete Iscove's medium). 25 U/ml of IL-2 was added at day 3. On days 7 and 15, the responder lymphocytes were restimulated with 10⁵ irradiated BB49-SCCHN.B7 cells, in the presence of 25 U/ml of IL-2. On day 21, the responder cells were separated into CD8⁺ and CD8⁻. In brief, the cells were labeled with anti-human CD8 mAb coupled to magnetic microbeads (Miltenyi Biotec GmbH, Germany). CD8⁺ cells were separated by passage through a Mini-MACS separation column. 3 × 10⁵ CD8⁺ lymphocytes were then restimulated with 5 × 10⁴ irradiated BB49-SCCHN.B7 cells and 10⁶ irradiated (30 Gray) CD8⁻ cells, in the presence of 25 U/ml of IL-2. On day 28, the responder cells were cloned by limiting dilution. Each microwell contained 6 × 10³ irradiated tumor cells, 2 × 10⁴ irradiated LG-2-EBV cells, and 7,500 irradiated CD8⁻ cells as feeder cells, in 100 µl of complete Iscove's medium supplemented with 50 U/ml of IL-2 and 5 U/ml of IL-4. The CTL clones were maintained by passaging weekly 5 × 10⁵ CTL with 10⁵ irradiated BB49-SCCHN.B7 cells pretreated for 72 h with 100 U/ml of IFN-γ, in 2 ml of complete Iscove's medium containing 50 U/ml of IL-2, 0.8 × 10⁶ irradiated LG-2-EBV cells and 1.5 × 10⁶ allogeneic irradiated PBMC.

Lysis Test and TNF Assay. The BB49-SCCHN target cells used in the ⁵¹Cr release assay were pretreated for 72 h with 100 U/ml of IFN-γ and the test was performed as previously described (22). Target cells were also tested for their ability to stimulate the production of TNF by the CTL. In brief, 1,500 CTL

were added in flat-bottomed microwells containing 10⁴ target cells, in a total volume of 100 µl of Iscove's complete medium containing 25 U/ml of IL-2. After 4 or 24 h, the supernatant was collected and its TNF content was determined by testing its cytotoxic effect on cells of WEHI-164 clone 13 (23) in a MTT colorimetric assay (24, 25). Inhibition with mAbs W6/32 (anti-HLA class I), B1.23.2 (anti-HLA-B,C), BB7-2 (anti-HLA-A2), or 2B6 (anti-HLA-DR) was performed by addition of a 1/20 dilution of ascites during the experiment.

Construction and Transfection of the cDNA Library. Poly(A)⁺ RNA was extracted from BB49-SCCHN cells using the FasTrack mRNA extraction kit (Invitrogen Corporation, San Diego, CA). mRNA was converted to cDNA with the Superscript Choice System (GIBCO BRL, Gaithersburg, MD) using an oligo-dT primer containing a NotI site at its 5' end. cDNA were then ligated to BstXI adaptors and digested with NotI. After size fractionation, the cDNA were unidirectionally cloned into the BstXI and NotI sites of plasmid pcDNAI/Amp (Invitrogen BV, Leek, The Netherlands). Recombinant plasmids were electroporated into *Escherichia coli* DH5α F'IQ and the transfectants were selected with 50 µg/ml of ampicillin. The recombinant library was divided into pools of about 100 cDNA clones. Each pool of bacteria was amplified to saturation and plasmid DNA was extracted using the QIAprep 8 plasmid kit (Qiagen GmbH, Hipden, Germany).

Transfection experiments were performed by the DEAE-dextran-chloroquine method (26, 27). In brief, 1.5 × 10⁴ COS-7 cells were transfected with about 100 ng of plasmid DNA of a pool of the cDNA library and 50 ng of plasmid pcDNA3 containing an HLA-B*3501 cDNA obtained from the RNA of patient LB1047.

Isolation of a cDNA Coding for the Autologous HLA-B35 Molecule. Using RT-PCR, we amplified the HLA-B*3503 coding sequence from RNA of EBV-transformed B cells of patient BB49. Primers 5'-CGG GAT CCG CCG AGA TGC GGG TCA C-3' and 5'-ACT GCC CGA ATT CTC TCA GTC CCT CAC AAG GCA GCT GTC-3', which are able to bind to most HLA-B sequences, were used as sense and antisense primers. The PCR was carried out for 35 cycles of 1 min at 94°C, 5 s at 59°C, and 5 min at 75°C, using the Pfu DNA polymerase (Stratagene GmbH, Heidelberg, Germany). The PCR product was then cloned into expression vector pcDNA3 (Invitrogen BV) and the sequence was verified.

DNA Sequence Analysis. The double-stranded plasmid template was sequenced on the sense and antisense strands by the dideoxy-chain termination method (ThermoSequenaseTM cycle sequencing kit; Amersham, Buckinghamshire, UK). The computer search for sequence homology was performed using the program that is available at blast@ncbi.nlm.nih.gov. Sequence alignments were performed with Geneworks[®] software (Intelligenetics, Mountain View, CA).

Production of Progressive Deletions in cDNA 668. To generate progressive deletions from the 3' end of cDNA 668, and thereby obtain a large number of truncated cDNA clones, we used the Erase-a-base System (Promega, Madison, WI) as described (28, 29).

Antigenic Peptides and CTL Assay. Peptides were synthesized on solid phase using F-moc for transient NH₂-terminal protection as described (30) and were characterized using mass spectrometry. All peptides were >80% pure, as indicated by analytical HPLC. Lyophilized peptides were dissolved at 20 mg/ml in DMSO, diluted at 2 mg/ml in 10 mM acetic acid, and stored at

-20°C. Peptides were tested in chromium release assays where 1,000 ⁵¹Cr-labeled target cells were incubated for 30 min at room temperature in 96-well microplates with various concentrations of peptide, before adding an equal volume containing 10,000 CTL 121 and 50,000 unlabeled K562. The indicated concentrations of peptide represent the final concentrations during the incubation of the target cells with the CTL. The assay was terminated after 4 h of incubation at 37°C.

Site-directed Mutagenesis. In vitro site-directed mutagenesis was performed on cDNA 668 cloned into expression vector pcDNA1/Amp with the Chameleon™ Double-stranded, Site-directed Mutagenesis Kit (Stratagene GmbH, Germany) using the selection primer 5'-CTT GTC TTC CCT TCT GAT TGA TGG TGC TAT TTT G-3'. The coding sequence of mutant cDNA clone 668-wt was verified.

Cell Death Assays. In each microwell, 50,000 293-EBNA cells were transiently transfected with 1 µl of lipofectAMINE (GIBCO BRL), 40 ng of pcDNA3-β-galactosidase, and 120 ng of the pcDNA1/Amp constructs containing either cDNA clone 668 or 668-wt in a final volume of 50 µl of OPTIMEM (GIBCO BRL). The experiment was performed in triplicate in two different microplates. After 18 h, cells were fixed in one plate and stained with X-gal (31). The data shown are the percentage of blue apoptotic cells among the 300 blue cells that were counted. In the other plate, the extent of cell death was counted using the Cell Death Detection Plus™ ELISA kit (Boehringer Mannheim; Indianapolis, IN). 20 µl of the supernatant of the cell culture (one tenth of total volume) was analyzed. Nucleosomes that are present in the supernatant of the transfected cells are purified by a histone-specific antibody and detected photometrically with an anti-DNA antibody coupled to peroxidase.

Results

A CTL Clone Against a Head and Neck Carcinoma. Cell line BB49-SCCHN (referred to below as CHN) was derived from a large squamous cell carcinoma resected from the oral cavity of patient BB49. After irradiation, these cells were used to stimulate either blood mononuclear cells or

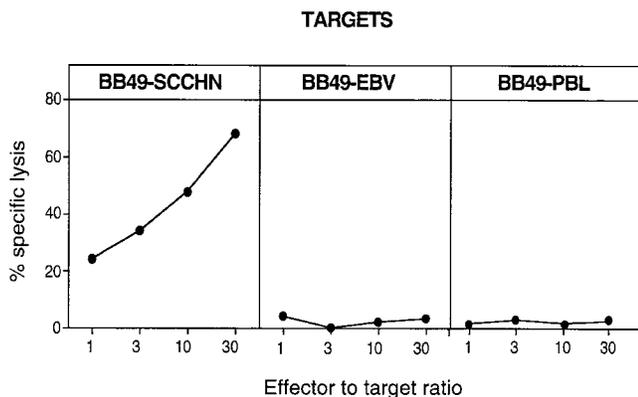


Figure 1. Specific lysis of the autologous tumor cells by CTL 121. Squamous cell carcinoma line of patient BB49 (BB49-SCCHN), autologous EBV-transformed B cell line (BB49-EBV), and autologous PHA-treated blood lymphocytes (BB49-PBL) were used as ⁵¹Cr-labeled target cells in the presence of a 50-fold excess of unlabeled K562. Chromium release was measured after 4 h.

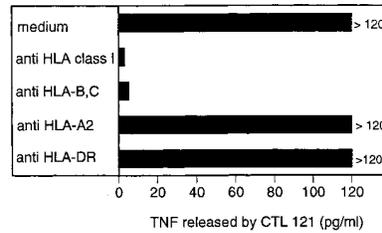


Figure 2. Recognition of BB49-SCCHN by CTL 121 is inhibited by anti-HLA-B,C mAb. 1,500 CTL were incubated with 20,000 BB49-SCCHN in microwells for 4 h without mAb (medium) or in the presence of either mAb W6/32 (anti-HLA-class I), B1.23.2 (anti-HLA-B,C), BB7-2 (anti-HLA-A2), or 2B6 (anti-HLA-DR). TNF production was estimated by testing the toxicity of the supernatant for TNF-sensitive WEHI-164.13 cells.

purified CD8⁺ T cells collected from patient BB49, but no lytic responder T cells were produced. To improve their stimulatory capacity, CHN cells were transfected with a cDNA encoding costimulatory molecule B7-1. The transfectants were cloned by limiting dilution, and clonal line CHN.B7 was selected for further work on the basis of its high expression of B7-1.

Irradiated cells were used to stimulate autologous blood mononuclear cells. After restimulation with the CHN.B7 cells on days 7 and 15, the responder population was found on day 21 to exert low lytic activity on the CHN cells. The CD8⁺ T cells, which represented less than 5% of the responder population, were sorted and restimulated with CHN.B7. 1 wk later, the responder population efficiently lysed the CHN cells. It was cloned by limiting dilution, and the stable CD8⁺ CTL clone 121 was obtained. The CTL lysed the autologous tumor cells but did not lyse an autologous EBV-transformed B cell line (BB49-EBV) or autologous PHA-treated blood lymphocytes (Fig. 1).

Patient BB49 was typed HLA-A2, -A11, -B35, -B62, -Cw01, -Cw04. CTL clone 121 secreted TNF upon stimulation with the autologous tumor cells, and TNF release was inhibited by a mAb recognizing HLA-B and HLA-C molecules. This indicates that the antigen recognized by CTL 121 is presented by HLA-B35, -B62, -Cw01, or -Cw04 (Fig. 2).

A cDNA Coding for the Antigen Recognized by CTL 121. To identify the gene coding for the antigen, we used a genetic approach involving the transfection of a cDNA library into simian cell line COS-7, together with the sequence coding for the HLA presenting molecule (26, 27). In this method, the cDNA library is prepared in an expression vector that contains an origin of replication of SV40, resulting in considerable episomal multiplication of the transfected plasmids in COS-7 cells and therefore in high expression of transfected genes. The transfected COS-7 cells that have integrated the sequence coding for the antigen are then identified on the basis of their ability to stimulate the production of TNF by the CTL.

Not knowing which of the B35, B62, Cw01, and Cw04 was the HLA presenting molecule, we started the screening

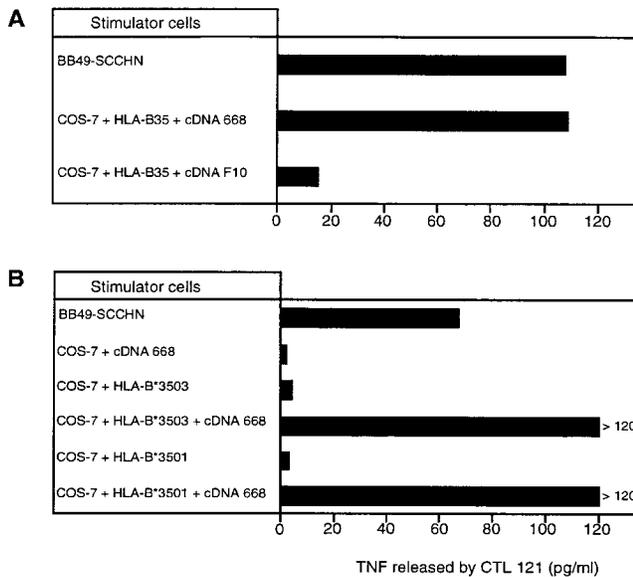


Figure 3. Stimulation of CTL 121 by COS-7 cells transiently cotransfected with cDNA clone 668 and with a sequence encoding an HLA-B35 molecule. 1,500 CTL were added into microwells containing 10,000 BB49-SCCHN or 20,000 transfected COS-7 cells. The production of TNF was measured after 24 h of coculture by testing the toxicity of the supernatants for the TNF-sensitive WEHI-164.13 cells. (A) COS-7 cells were cotransfected with cDNA 668 and a cDNA coding for HLA-B35 obtained from the RNA of patient LB1047. The autologous BB49-SCCHN cells were used as a positive control for stimulation. COS-7 cells, cotransfected with HLA-B35 and nonrelevant cDNA clone F10, were used as a negative control. (B) COS-7 cells were cotransfected with cDNA 668 and either a cDNA coding for the autologous HLA-B*3503 molecule or with a cDNA coding for the allogeneic HLA-B*3501 molecule. Stimulator cells included the autologous BB49-SCCHN cells as a positive control and COS-7 cells transfected with either cDNA 668, the HLA-B*3503 cDNA, or the HLA-B*3501 cDNA alone as negative controls.

of the library with a cDNA encoding HLA-B35 obtained from the RNA of patient LB1047. The cDNA library was prepared with RNA from the CHN cells into expression vector pcDNA1/Amp. The library was divided into pools of 100 recombinant clones and DNA from each pool was cotransfected into microcultures of COS-7 cells with the HLA-B35 coding sequence. 48 h later, the transfected microcultures were screened for the expression of the antigen by adding CTL 121 in the microwells and by measuring TNF production after 24 h of coculture. Of the 800 cDNA pools that were tested, four proved positive when cotransfected with HLA-B35. By subcloning the bacteria of the first positive pool, and by repeating the transfection and screening procedures outlined above with individual plasmids, we obtained several clones that transferred the expression of the antigen. The result obtained with representative cDNA clone 668 is shown in Fig. 3 A.

Sixteen alleles of the HLA-B35 gene have been described (32). Therefore, we set out to confirm these results by cotransfecting COS-7 cells with cDNA clone 668 and the sequence coding for the autologous HLA-B35 molecule. Using primers specific for most HLA-B sequences, a

RT-PCR product was obtained from the RNA of BB49-EBV and cloned into expression vector pcDNA3. Sequencing indicated that the autologous allele was B*3503, whereas the B35 allele that we had used for the transfection of the cDNA library was B*3501. When COS-7 cells were transfected with cDNA 668 together with the autologous HLA-B*3503 cDNA, the transfectants also stimulated the production of TNF by CTL 121 (Fig. 3 B).

Bacteria of the three other positive pools were also subcloned and individual plasmid DNA were screened using the transfection procedure described above. In each of these three pools, we identified several clones that stimulated the production of TNF by CTL 121 when transfected into COS-7 cells together with the HLA-B*3501 cDNA. However, when transfected together with the autologous HLA-B*3503 cDNA, these clones were unable to stimulate CTL 121 (data not shown), indicating that the corresponding genes do not code for the antigen recognized on the autologous HLA-B*3503 tumor.

Identification of the Antigenic Peptide. cDNA 668 is 2647 bp long. To identify the region encoding the peptide presented to CTL 121, exonuclease III was used to generate progressive deletions from the 3' end of the cDNA. A large number of truncated cDNA clones were cotransfected into COS-7 cells together with the HLA-B*3503 cDNA to test their ability to code for the antigen. Sequence comparison of the longest negative cDNA clone (nucleotides 1–1,446) with the shortest positive clone (nucleotides 1–1,600) indicated that the COOH-terminus of the antigenic peptide had to be encoded between nucleotides 1,446 and 1,600. In this region of the putative protein, we searched for peptides bearing the HLA-B35 binding motif, i.e., Pro in position 2, and Tyr or hydrophobic residues at the COOH terminus of the peptide (33, 34). Two peptides corresponding to this motif were identified, namely nonapeptide FPSD-SWCYF (amino acids 476–484) and octapeptide FPSD-SWCY (amino acids 476–483). They were synthesized and

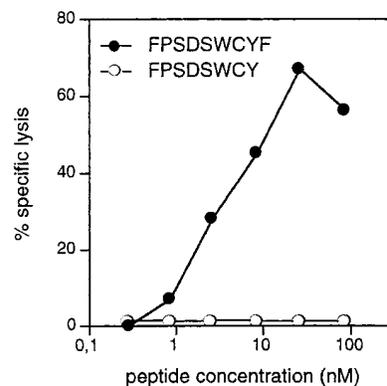


Figure 4. Lysis by CTL 121 of autologous EBV-transformed B cell line (BB49-EBV) incubated with synthetic peptide FPSDSWCYF. 1,000 chromium-labeled BB49-EBV cells were incubated at room temperature for 30 min with the indicated peptides at various concentrations, before adding an equal volume containing 10,000 CTL 121 and 50,000 unlabeled K562. Chromium release was measured after 4 h.

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1   M D F S R N L Y D I G E Q L D S E D L A S L K F L S L D Y I P Q R
70  ATGGACTTCA GCAGAAATCT TTATGATATT GGGGAACAAC TGGACAGTGA AGATCTGGCC TCCTCAAGT TCCTGAGCCT GGACTACATT CCGCAAAGGA

34  K Q E P I K D A L M L F Q R L Q E K R M L E E S N L S F L K E L L F
170 AGCAAGAAC CATCAAGGAT GCCTTGATGT TATTCAGAG ACTCCAGGAA AAGAGAAATG TGGAGGAAAG CAATCTGTCC TTCCTGAAGG AGCTGCTCTT

68  R I N R L D L L I T Y L N T R K E E M E R E L Q T P G R A O I S A
270 CCGAATTAAT AGACTGGATT TGCTGATTAC CTACCTAARAC ACTAGAAGG AGGAGATGGA AAGGGAACCT CAGACACCAG GCAGGGCTCA AATTTCTGCC

101 Y R V M L Y Q I S E E V S R S E L R S F K F L L Q E E I S K C K L
370 TACAGGCTCA TGCTCTATCA GATTCAGAAA GAAGTGAACA GATCAGAATT GAGTCTTTTT AAGTTTCTTT TGCAAGAGGA AATCTCCAAA TGCAAACCTG

134 D D D M N L L D I F I E M E K R V I L G E G K L D I L K R V C A Q I
470 ATGATGACAT GAACCTGCTG GATATTTTCA TAGAGATGGA GAAGAGGGTC ATCTCTGGAG AAGAAAGTGT GGACATCTGT AAAAGAGTCT GTGCCCAAAT

168 N K S L L K I I N D Y E E F S K E R S S L E G S P D E F S N G E
570 CACCAAGAGC CTGCTGAAGA TAATCAACGA CTATGAAGAA TTCAGCAAG AGAGAAGCAG CAGCTTGAA GGAAGTCTGT ATGAATTTTC AAATGGGGAG

201 E L C G V M T I S D S P R E Q D S E S Q T L D K V Y Q M K S K P R
670 GAGTCTGTG GGGTAATGAC AATCTGGGAC TCTCCAAGAG AACAGGATAG TGAATACAG ACTTTGGACA AAGTTTACCA AATGAAAAGC AAACCTCGGG

234 G Y C L I I N N H N F A K A R E K L H S I R D R N G T H L D A
770 GATCTGCTC ATCTCAATC AATCACAATT TTGCAAAAGC ACGGGAGAAA VTGCKAAGC TAGGCAGTCT TAGGGAGAGC AATGGAACAC ACTTGGATGC

268 G A L T T T F E E L H F E I K P H D D C T V E O I V E I L K I Y Q
870 AGGGGCTTTG ACCACGACTT TGAAGAGCT TCNTTTTGAG ATCAAGCCCC ACGATGACTG CACAGTAGAG CAATCTATG AGATTTTGA AATCTACCAA

301 L M D H S N M D C F I C C I L S H G D K G G I I Y G T D G Q E A P I
970 CTCATGACC ACAGTAACAT GGACTGCTTC ATCTGTGTA TCCTCTCCA TGGAGACAAG GGCATCATCT ATGGCAGTGA TGGACAGGAG GCCCCCATCT C(FLICE)

334 Y E L T S O F T G L K C P S L A G K P K V F F I Q A C Q G D N Y Q K
1070 ACTACTGAC ATCTGACTG ACTGCTTTGA KCTGCCCTC CTTGCTGGA AAACCCAAAG TGTTTTTAT TCAGGCTTCT CAGGGGGATA ACTACCAGAA

368 G I P V E T D S E E Q P Y L E M D L S S P O T R Y I P D E A D F L
1170 AGTATACCT GTTGAGACTG ATTCAGAGGA GCAACCCAT TTAGAAAATG ATTTATCATC ACCTCAAACG AGATATATCC CGGATGAGGC TGACTTCTG

401 L G M A T V N N C V S Y R N P A E G T W Y I O S L C Q S L R E R C
1270 CTGGGATGG CCACTGTGAA TAACTGTGTT TCCTACCGAA ACCCTGCAGA GGGAACTTGG TACATCCAGT CACTTTGCCA GAGCCTGAGA GAGCGATGTC G(FLICE)

434 P R G D D I L T T I L T E V N Y E V S N K D D K K N M G K Q M P O P T
1370 CTCGAGCGGA TGATATTCCT ACCATCCCTGA CTGAAGTGA CTATGAAGTA AGCAACAAG ATGACAAGAA AAACATGGGG AAACAGATGC CTCAGCCTAC

468 F T L R K K L V F P S D S W C Y F V C F V L F C F F E T E S R S V
1470 TTTCACACTA AGAAAAAATC TTGCTTCCC TTCTGATCA TGGTGTATT TTGTTTGTG TGTTTGTTT TGTTTTTTG AGACAGAACT TCGCTCTGTC TGA(MACHα1)
1570 TGA(FLICE)

501 A O A G V O W R D L G S P O A P P P G F T P F S C L S L P S S W D
1570 CCCAGGCTG GAGTGCAGT GCGTGATCTC GGCTCACCGC AAGCTCCGCC TCCCGGGTTC ACGCCATCTC CTFGCTCAG CCTCCCGAGT AGCTGGGACT

534 Y R G P P P H L A N F L K I F L V E T G F H C V S O G G L D L L T S
1670 ACAGGGCCCC GCCACCACAC CTGGCTAATT TTTTAAARAT ATTTTLAGTA GAGACAGGGT TTCACTGTGT TAGCCAGGGT GGTCTTGATC TCCTGACCTC

1770 GTGA

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Figure 5. Sequence of the open reading frame and of the putative protein encoded by cDNA 668. The nucleotides are numbered starting from nucleotide 70 of the cDNA 668. The translated Alu sequences are underlined. The sequence corresponding to the peptide recognized by CTL 121 is boxed. For *MACHα1* and *FLICE*, only the stop codon TGA and the differences in nucleotides are indicated. There are two single nucleotide differences with *FLICE* (This sequence data is available from EMBL/GenBank/DBJ under accession number U58143), giving rise to two different amino acids, and a single conservative difference with *MACHα1* (This sequence data is available from EMBL/GenBank/DBJ under accession number X98172). The 5'-untranslated region of cDNA 668 is identical to that of *MACHα2*. Therefore, compared with the cDNA sequences of *CASP-8* already described, cDNA 668 is representative of a new type of transcript.

tested for recognition. Only nonapeptide FPSDSWCYF sensitized BB49-EBV to lysis by CTL 121. Half-maximal lysis was obtained with only 3 nM of peptide (Fig. 4).

It is worthy to note that, in addition to CTL 121, we have derived from another blood sample of patient BB49 a second CTL clone recognizing the same antigenic peptide. These two clones express the same TCRBV14S1 and the sequence of the joining region is identical (data not shown).

The Antigenic Peptide Is Encoded by a Mutated *CASP-8* Gene. Recently, two groups reported the identification of a new gene named *FLICE* or *MACH* (35, 36). This new gene was more recently renamed *CASP-8* and the encoded protein, caspase-8 (37). Caspase-8 is required for induction of apoptosis through the Fas and TNFR1 receptors. cDNA clone 668 is almost identical to the coding sequence of *FLICE* or the $\alpha 1$ splice variant of *MACH*. In the region encoding the antigenic peptide, we found a cytosine (C) at position 1508 in cDNA 668 instead of the guanine (G) present in both *FLICE* and *MACHα1* (Fig. 5). This led us to consider the possibility that a tumor-specific point mutation in this gene might be responsible for the expression of the antigen recognized by CTL 121. To compare the *CASP-8* sequences in tumor cells and in normal cells of patient BB49, a DNA fragment corresponding to nucleotides 683–1,530 in cDNA 668 was amplified by PCR from genomic DNA of tumor cell line CHN, autologous PHA-

treated blood lymphocytes, or BB49-EBV. Direct sequencing of the PCR products revealed that tumor DNA contained both a G and a C at position 1508, whereas DNA from both types of blood cells contained only a G (Fig. 6

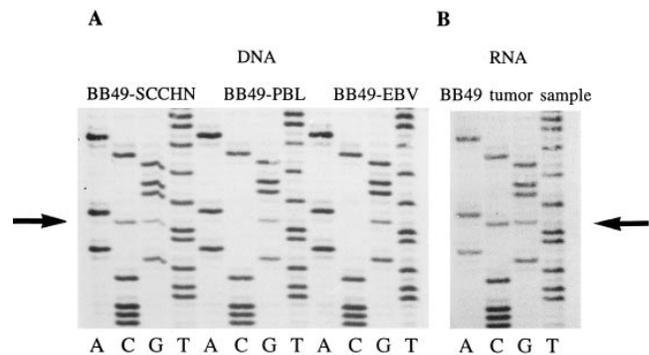


Figure 6. Autoradiography showing the sequence ladder in the region coding for the antigenic peptide in the squamous cell carcinoma line of patient BB49 (BB49-SCCHN), autologous PHA-treated blood lymphocytes (BB49-PBL), autologous EBV-transformed B cell line (BB49-EBV), and the tumor sample resected from patient BB49. (A) PCR products were obtained by amplification with specific primers on genomic DNA. (B) The RT-PCR product was obtained by amplification with specific primers on RNA extracted from the tumor mass resected from patient BB49. The PCR fragments were purified and directly sequenced. The arrow indicates the mutation G to C in one of the alleles.

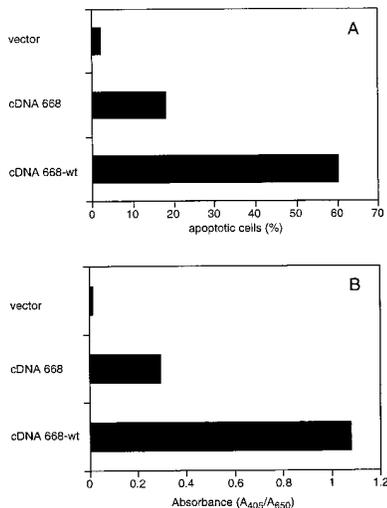


Figure 7. Triggering of apoptosis in cells transiently transfected with cDNA 668 or the equivalent wild-type sequence, cDNA 668-wt. 293-EBNA cells were transiently transfected by lipofectAMINE. Transfection marker β -galactosidase, inserted into expression vector pcDNA3, was cotransfected with expression vector pcDNA1/Amp either empty, containing cDNA 668, or containing cDNA 668-wt (wt for wild type). cDNA 668-wt was obtained by site-directed mutagenesis performed on cDNA 668, in which the TCA codon was replaced by stop codon TGA. The experiment was performed in triplicate in two different microplates. (A) After 18 h, cells were fixed in one plate and stained with X-gal. A total number of 300 blue cells were counted. The x-axis gives the percentage of blue apoptotic cells. (B) In the other plate, the extent of cell death was quantitate using an ELISA-based assay in which nucleosomes in the supernatant of the transfected cells are purified by a histone-specific antibody and detected photometrically with an anti-DNA antibody coupled to peroxidase.

A), proving that a point mutation had occurred in the tumor. The mutation was also found in the RNA of a frozen surgical sample of the tumor, excluding the possibility that it had arisen during cell culture (Fig. 6 B).

In cDNA 668, the point mutation changes the stop codon TGA into TCA, increasing the size of the encoded protein by 88 amino acids. This protein extension is almost entirely encoded by an Alu repetitive sequence. The antigenic peptide is encoded by the region surrounding the mutation. The last five amino acids are encoded by the extension of the reading frame caused by the mutation (see Fig. 5).

Reduced Triggering of Apoptosis by the Mutated Caspase-8. Apoptosis is triggered by transient transfection of *CASP-8*, in common with other genes involved in apoptosis (35, 36). This is presumably due to overexpression of the protein. To examine whether the mutation in cDNA 668 affected the ability of caspase-8 to induce apoptosis, 293-EBNA cells were transiently transfected with the β -galactosidase coding sequence, as a transfection marker, and cDNA 668. As a positive control, we used a cDNA clone obtained by site-directed mutagenesis of cDNA clone 668, clone 668-wt (wt for wild type), in which the mutated codon TCA is replaced by the wild-type stop codon TGA. 18 h after transfection, the cells were stained for β -galactosidase activity

and the morphological changes were observed among the blue transfected cells. The majority of cells cotransfected with clone 668-wt displayed a rounded and condensed shape, typical of cells undergoing apoptosis. In three different experiments, the proportion of blue apoptotic cells ranged from 60–76%. Upon transfection with cDNA 668, this percentage ranged from 14–20%. A typical experiment is shown in Fig. 7 A. Compared with the equivalent wild-type sequence, the transfection of cDNA 668 in all experiments was 3.3 to 5.6 times less efficient in triggering apoptosis. We also used an ELISA-based assay to estimate the level of nucleosomal DNA fragments in cells undergoing apoptosis. The amount of nucleosomes in the supernatant of the transfected cells paralleled the percentage of blue apoptotic cells (Fig. 7 B).

Discussion

The tumor antigen described here is encoded by a mutated *CASP-8* gene, which codes for protein caspase-8, a key component in the pathway of Fas and TNFR1-induced apoptosis (35, 36). Activation of Fas recruits FADD, the Fas-associated death domain protein, which in turn binds and activates caspase-8 (35, 36, 38, 39). Similarly, TNFR1 recruits TRADD, the TNFR1-associated death domain protein (40). TRADD subsequently acts as an adaptor protein to recruit FADD, which, in a manner that remains to be determined, binds and activates caspase-8. It is believed that the activated caspase-8 activates other caspases, thereby leading to apoptosis.

Caspases are proteases that bear a cysteine in their active site and cleave after an aspartic acid (37). They are synthesized as inactive proenzymes that are cleaved at specific aspartate residues to form active subunits. In the case of caspase-1, the NH₂-terminal prodomain is removed and the other part of the protein is cleaved into a p20 and a p10 subunit. These subunits form heterodimers and their crystal structure has revealed that the mature, active form of caspase-1 is a tetramer composed of two heterodimers (41). Caspase-8 can be cleaved in vitro by granzyme B into an active form containing p20 and p10 subunits (35).

The tumor cells of patient BB49 express both a normal and a mutated allele of *CASP-8*. The mutated allele codes for a protein with 88 extra residues at the COOH-terminus. Therefore, instead of producing a p10 subunit with 105 amino acids, this altered protein ought to produce a p19 subunit of 193 amino acids. Assuming that caspase-8 adopts a conformation similar to the p20/p10 tetramer of caspase-1, the mutation may decrease the activity of the enzyme because the active tetramer cannot be assembled with the abnormal p19 subunits. Moreover, the p19 subunit may impede the folding of the tetramers containing subunits of the normal caspase-8, thereby resulting in a partially dominant effect.

We have tried to obtain evidence that the *CASP-8* mutation effectively impairs the signaling leading to apoptosis after Fas ligation. When incubated with an anti-Fas antibody, two EBV-transformed B cell lines and the Jurkat cell

line died, as expected. In contrast, the CHN cells did not die, as expected if the mutation impairs apoptosis (data not shown). However, this evidence is not conclusive, because the CHN cells express a lower level of Fas than the EBV-B cells and the Jurkat cells, and we observed that another cell line, expressing the same low level of Fas but not the *CASP-8* mutation, did not undergo apoptosis upon treatment with an anti-Fas antibody.

More conclusive evidence for the impairment of the function of the altered caspase-8 was obtained when the mutated *CASP-8* gene was transiently transfected in 293-EBNA cells: its ability to trigger apoptosis was reduced relative to the normal caspase-8 but not abolished. This reduction may be more profound in the tumor cells of patient BB49 than in the transient transfectants. Indeed, in the latter, the reduced effectiveness of the altered protein may be partially compensated by a significant overexpression.

Impaired triggering of apoptosis induced by Fas ligand (FasL) or TNF may provide tumor cells with increased resistance to killing by the immune cells. Clearly, the fact that CHN tumor cells were lysed by CTL 121 indicates that they were not completely protected against an immune attack. This is not surprising, because CTL are known to mediate cytotoxicity not only via Fas but also via a perforin/granzyme-based pathway (42–44). The latter mechanism may not require signaling through caspase-8, because granzyme B is capable of activating other caspases by itself (45, 46). On the other hand, cytolytic T cells are not the only cells that express FasL. Neutrophils, which express both membrane-bound and soluble FasL, have been shown to infiltrate tumors and they may kill tumor cells through Fas (47).

The possibility cannot be excluded that other unknown signals leading to apoptosis are also transduced through caspase-8 and that a partial or complete failure to transmit these signals may have offered a selective advantage to the tumor cells of patient BB49. In this regard, another death domain-containing receptor, named DR3, has recently been identified (48). DR3 is related to TNFR1 and Fas, its intracellular signal is transmitted through caspase-8, and its ligand is still unknown.

Using PCR, we screened 150 tumor samples for the presence of a mutation in the stop codon of *CASP-8*. None was found, suggesting at first that this mutation does

not have a very important selective value for tumors. However, it is likely that the mutation in the stop codon is not the only one to be deleterious to the activity of caspase-8. Mutations in other critical parts of the protein, such as the different cleavage sites, the catalytic site, or the domain that interacts with FADD, may be observed in other tumors.

Several tumor antigens recognized on melanoma by autologous CTL also result from point mutations, such as those found in *MUM-1*, cyclin-dependent kinase 4 (CDK4), and β -catenin (8, 10, 11). The CDK4 and β -catenin mutations have been found in different patients and are clearly both antigenic and oncogenic (10, 49). The CDK4 mutation prevents the protein from binding to its inhibitor, p16. This appears to alter the regulation of the cell cycle, favoring uncontrolled growth of the tumor cells. The β -catenin mutation results in stabilization of the protein, with high levels of free β -catenin driving formation of complexes with transcription factors such as Lef-1. Constitutive β -catenin–Lef-1 complexes may result in persistent transactivation of as yet unidentified target genes, thereby stimulating cell proliferation or inhibiting apoptosis (50). Our results suggest that the *CASP-8* mutation may also be oncogenic. It seems likely that other antigens resulting from mutations will be identified with CTL. Some of these mutations may be oncogenic. If the coding genes are unknown, it may well be worth studying their function.

The point mutations of *MUM-1*, CDK4, and β -catenin change one amino acid and this single substitution generates the antigenic peptide. *CASP-8* is interesting in this respect because the last five amino acids of the antigenic peptide recognized by CTL clone 121 are encoded by an extension of the reading frame. The altered caspase-8 comprises 88 extra amino acids that could contain several epitopes presented to other CTL by various HLA molecules.

After the identification of the first tumor antigens recognized by CTL on melanoma cells, concern was expressed that tumors of other histological types may be less likely to express such antigens. The results obtained with renal carcinoma (7, 9) and those reported here for a head and neck carcinoma suggest, however, that there is no fundamental difference in this respect between melanoma and other tumors.

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Address correspondence to Pierre van der Bruggen, 74 avenue Hippocrate, B-1200 Bruxelles, Belgium. Phone: 32-2-764-74-31; FAX: 32-2-762-94-05; E-mail: vanderbruggen@licr.ucl.ac.be

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