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**Béatrice Gaugler · Nathalie Brouwenstijn
Valérie Vantomme · Jean-Pierre Szikora
Corry W. Van der Spek · Jean-Jacques Patard
Thierry Boon · Peter Schrier · Benoît J. Van den Eynde**

# A new gene coding for an antigen recognized by autologous cytolytic T lymphocytes on a human renal carcinoma

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**Abstract** Previous reports have described antigens that are recognized on human melanoma cells by autologous cytolytic T lymphocytes (CTL). The genes coding for a number of these antigens have been identified. Here we report the cloning of a gene that codes for an antigen recognized by autologous CTL on a human renal carcinoma cell line. This antigen is presented by *HLA-B7* and is encoded by a new gene that we have named *RAGE1*. No expression of *RAGE1* was found in normal tissues other than retina. RAGE1 expression was found in only one of 57 renal cell carcinoma samples, and also in some sarcomas, infiltrating bladder carcinomas, and melanomas. This represents the first identification of an antigen recognized by autologous CTL on a renal tumor.

## Introduction

A large number of cytolytic T lymphocytes (CTL) clones directed against autologous melanoma have been obtained from either peripheral blood lymphocytes (PBL) or lymphocytes infiltrating the tumor (TIL; Boon et al. 1994). In a number of instances, the antigens recognized by these CTL have been characterized. They can be classified into several groups. The first group comprises antigens encoded by genes such as *MAGE1, MAGE3, BAGE,* and *GAGE,* which are expressed in a number of tumors but are completely silent in normal tissues except testis (Boël et al. 1995; Gaugler et al. 1994; Van den Eynde et al. 1995; van der Bruggen et al. 1991). The antigens of the second group are differentiation antigens expressed in melanoma and normal melanocytes. They derive from tyrosinase, Melan-A$^{MART-1}$, gp100$^{pme117}$ and gp75$^{TRP1}$ (Bakker et al. 1994; Brichard et al. 1993; Coulie et al. 1994; Cox et al. 1994; Wang et al. 1995). The third class comprises antigens produced by point mutations in genes like *MUM1* or *CDK4,* which are expressed ubiquitously (Coulie et al. 1995; Wölfel et al. 1995).

Like melanoma, renal cell carcinoma (RCC) is a tumor type that appears to be sensitive to immune attack. Primary kidney tumors are usually infiltrated by numerous T lymphocytes displaying cytolytic activity against the tumor cells, and metastatic lesions were found to regress spontaneously in up to 7% of the cases (Balch et al. 1990; Belldegrun et al. 1988; Oliver et al. 1989). Some long-term tumor responses obtained with interleukin-2 in patients with metastatic RCC may also result from the elicitation of T-cell responses (Belldegrun et al. 1993; Palmer et al. 1992; Rosenberg 1992; Rosenberg et al. 1994; Weiss et al. 1992). This suggests that kidney tumors express antigens that can be recognized by T lymphocytes potentially capable of rejecting the tumor cells. However, none of the tumor antigens thus far defined on melanoma cells is expressed on renal carcinoma. Genes *MAGE1, MAGE3, BAGE,* and *GAGE* are expressed in several tumor types, but not in renal cell carcinoma.

Recently we isolated a number of CTL clones directed against autologous RCC line LE9211-RCC, and we obtained evidence that these clones do not recognize normal kidney cells (Brouwenstijn et al., 1996). Here we report the identification of the gene coding for the antigen recognized by one of these CTL clones.

B. Gaugler · V. Vantomme · J.-P. Szikora · J.-J. Patard · T. Boon
B. J. Van den Eynde (✉)
Ludwig Institute for Cancer Research, Brussels Branch,
74 avenue Hippocrate, UCL 7459, B1200 Brussels, Belgium and
Cellular Genetics Unit, Université Catholique de Louvain,
B1200 Brussels, Belgium

N. Brouwenstijn · C. W. Van der Spek · P. Schrier
Dept. of Clinical Oncology, Academic Hospital Leiden,
P. O. Box 9600, 2300 RC Leiden, The Netherlands

## Materials and methods

*Cell lines*

Renal cell carcinoma cell line LE9211-RCC was derived from the primary tumor of patient LE9211 and was cultured in Dulbecco's modified eagle medium supplemented with 10% FCS, L-glutamine

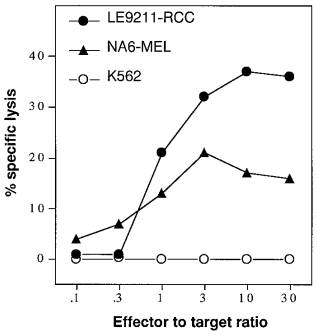



**Fig. 1** Specific lytic activity of CTL clone 263/17. LE9211-RCC is the autologous renal carcinoma cell line, and NA6-MEL is an allogeneic HLA-B7$^+$ melanoma line

(216 mg/ml), L-arginine (116 mg/ml), L-asparagine (36 mg/ml), 200 units/ml penicillin, and 100 µg/ml streptomycin. CTL clone 263/17 was derived from PBL of patient LE9211 by in vitro stimulation with irradiated LE9211-RCC cells and was maintained in culture as described (Hérin et al. 1987). CTL clone 361A/17 was obtained similarly from the same experiment and was used in one experiment in place of CTL 263/17 which failed to grow indefinitely. Both CTL recognize the same antigen. LB23-SAR is a sarcoma cell line derived from an *HLA-B7* patient. It was transfected by the calcium-phosphate precipitate method with the *RAGE1* cDNA cloned into plasmid pEFBOS [kindly provided by Shigekazu Nagata, Osaka, Japan (Mizushima and Nagata 1990)] into which a puromycin resistance gene had been introduced (constructed by J. C. Renaud in the laboratory).

*Assay for cytolytic activity*

Lytic activity of CTL clones was tested as previously described (Boon et al. 1980). Target cells were preincubated with 100 units/ml IFNγ 48 h before the assay. Chromium release was measured after 4 h.

*Transfection of the cDNA library into COS-7 cells*

The cDNA library was prepared as described (Van den Eynde et al. 1995). Transfection experiments were performed by the DEAE-dextran-chloroquine method (Brichard et al. 1993; Coulie et al. 1994; Seed and Aruffo 1987). Briefly, 1.5 $10^4$ COS-7 cells were transfected with about 100 ng of plasmid DNA from a bacteria pool of the cDNA library and with 60 ng of plasmid pcD-SRα containing the *HLA-B7* cDNA. The cDNA encoding *HLA-B7* was obtained by reverse transcription-polymerase chain reaction (RT-PCR) from RNA sarcoma line LB23-SAR using the method described by Ennis and co-workers (1990) and was cloned into plasmid pcD-SRα (a gift from Kevin Moore, DNAX, Palo Alto, CA). Transfected COS-7 cells were tested for their ability to stimulate the production of TNF by the CTL clone as described (Traversari et al. 1992).

*DNA sequence analysis*

DNA sequencing was performed by the dideoxy-chain termination method (T7 Sequencing Kit; Pharmacia Uppsala, Sweden and ΔTaq™–Cycle-Sequencing Kit; US Biochemicals, Cleveland, OH) using specific priming with synthetic oligonucleotides. The computer search for sequence similarity was done with the Blast program at NCBI. Sequence alignments were performed with Geneworks (Intelligenetics, Mountain View, CA).

*Screening of the cDNA library*

Colonies from the cDNA library of LE9211-RCC cells were grown on hybridization transfer membranes (GeneScreen Plus®; Dupont, Boston MA) and were hybridized with a $^{32}$P-labeled PCR product (nucleotide 26–219 of *RAGE1*).

*Identification by RACE PCR of the 5′ end of the RAGE cDNA*

Amplification of the 5′ end was performed on poly-A$^+$ RNA using the 5′ Amplifinder™ RACE Kit (Clontech, Palo Alto, CA). The primer used for cDNA synthesis was 5′-GGTGTGCCGATGACATCG-3′ (BG16, anti-sense nucleotide, position 202–219 of *RAGE1*). PCR amplification was performed with the sense Amplifinder anchor primer and the anti-sense primer 5′-CCAGGAAAGAGGGGCTGC-3′ (BG17, anti-sense, nucleotides 150–167 of *RAGE1*). Amplified products were cloned into vector pCR-Script™ SK(+) (pCR-Script™ SK(+) Cloning Kit; Stratagene, La Jolla, CA) and sequenced.

*PCR assay for the expression of RAGE genes*

Total RNA was extracted by the guanidine-isothiocyanate procedure as described (Davis et al. 1986). Reverse transcription was performed as described elsewhere (Van den Eynde et al. 1995). For PCR amplification, 1/20 of the cDNA reaction was supplemented with 5 µl of 10 × thermostable DNA polymerase buffer (Finnzymes OY, Espoo, Finland), 1 µl each of 10 mM dNTP, 2 µl each of 20 µM primers solutions, 1 unit of Dynazyme (Finnzymes OY) and water to a final volume of 50 µl. The PCR primers for the amplification of all *RAGE* genes were 5′-GTGTCTCCTTCGTCTCTACTA-3′ (TB4, sense, nucleotides 26–44 of *RAGE1*) and anti-sense primer 5′-GGTGTGCCGATGACATCG-3′ (BG16, anti-sense, nucleotides 202–219 of *RAGE1*). For the specific amplification of *RAGE1*, sense primer TB4 was used with anti-sense primer 5′-GAGGTATTCCTGATCCTG-3′ (BG25, anti-sense, nucleotides 248–265 of *RAGE1*). After a first denaturation step of 5 min at 94 °C, PCR was run with 33 cycles of 1 min at 94 °C, 2 min at 60 °C (56 °C when primer BG25 was used), and 3 min at 72 °C. A final extension step of 15 min at 72 °C was done. Aliquots of 10 µl of the PCR products were size-separated on agarose gels. RNA integrity was checked by reverse transcription and amplification of the β-actin mRNA.

## Results

CTL clone 263/17 was isolated from a responder lymphocyte population obtained by stimulating peripheral blood lymphocytes of patient LE9211 with irradiated cells of the autologous renal cell carcinoma (RCC) line. This CTL clone lyses autologous renal carcinoma cells but not NK-sensitive target cells K562 (Fig. 1; Brouwenstijn et al. 1996). The antigen recognized by CTL clone 263/17 was called LE9211-A. This CTL clone released TNF when stimulated with LE9211-RCC tumor cells (Fig. 3). The *HLA* type of patient LE9211 is *A3*, *B7*, *B35*, *Cw7*, and *Cw4*. An antibody directed against *HLA-B7* inhibited TNF secretion by clone 263/17 (Brouwenstijn et al. 1996). In agreement with the conclusion that the antigen is presented by *HLA-B7*, we found that *HLA-B7*-positive melanoma line NA6-MEL was also lysed by CTL 263/17 (Fig. 1).

### *A cDNA encoding antigen LE9211-A*

To identify the gene coding for antigen LE9211-A, we prepared a cDNA library in plasmid pcDNAI/Amp with RNA from LE9211-RCC cells. We divided the library into 1400 pools of approximately 100 bacteria, and we cotransfected DNA from each pool into COS-7 cells together with the *HLA-B7* cDNA. Forty-eight h later we screened the transfectants for expression of the antigen by adding CTL

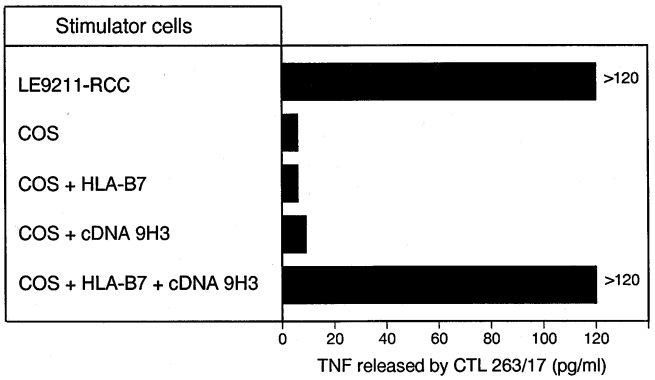



**Fig. 2** Stimulation of CTL 263/17 by COS-7 cells transfected with the *HLA-B7* cDNA and with cDNA 9H3. Control stimulator cells included autologous LE9211-RCC cells and COS-7 cells transfected either with the *HLA-B7* cDNA alone or with cDNA 9H3 alone. The production of TNF was measured after 18 h of culture with the stimulator cells, by testing toxicity of the supernatants for TNF-sensitive cells WEHI-164.13

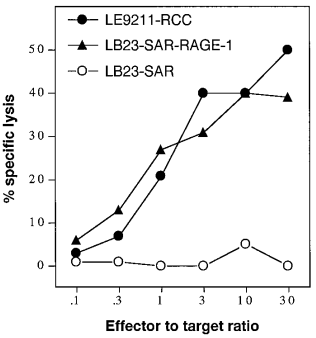



**Fig. 3** Lysis of stable transfectants obtained with the *RAGE1* cDNA. LB23-SAR is an HLA-B7$^+$ sarcoma line. It was transfected with the *RAGE1* cDNA (LB23-SAR-RAGE1). The lysis test was performed with CTL clone 361A/17, which recognizes the same antigen as CTL clone 263/17

263/17 to the microcultures, and by measuring TNF production after 18 h. One of the cDNA pools produced a transfected culture that strongly stimulated TNF release by CTL 263/17. By subcloning the bacteria of this positive pool and by transfecting DNA from individual colonies as above, we isolated cDNA clone 9H3 which transferred the expression of the antigen (Fig. 2). When this clone was transfected into the HLA-B7(+) sarcoma line LB23-SAR, it produced stable transfectants that stimulated TNF release (data not shown) and were lysed by the CTL (Fig. 3).

The 9H3 cDNA was 1118 base pairs (bp) long, and its sequence showed no significant similarity with any gene reported in data banks. The new gene was named *RAGE1*, for Renal tumor AntiGEn. The *RAGE1* sequence contains several sizeable open reading frames (ORF) in the three different phases (Fig. 4). The first ORF (positions 173–271) is probably not translated because its AUG initiator codon is not surrounded by the consensus motif required for efficient initiation of translation (Kozak 1991). The second ORF (positions 204–326) is likely to be translated because it has a purine in position −3 of the initiator codon. It is 123 bp long and encodes a putative protein of 40 amino acids (Fig. 5). Three additional sizeable ORFs are found downstream (Fig. 4), two of which have strong initiator codons.

When cDNA 9H3 was used to hybridize a northern blot prepared with RNA from LE9211-RCC, it revealed diffuse bands corresponding to mRNAs of sizes between 1.6 and 1.9 kb (data not shown). We concluded that there were several mRNA species related to *RAGE*, and that clone 9H3 was incomplete. Using primers specific for the 5′ end of

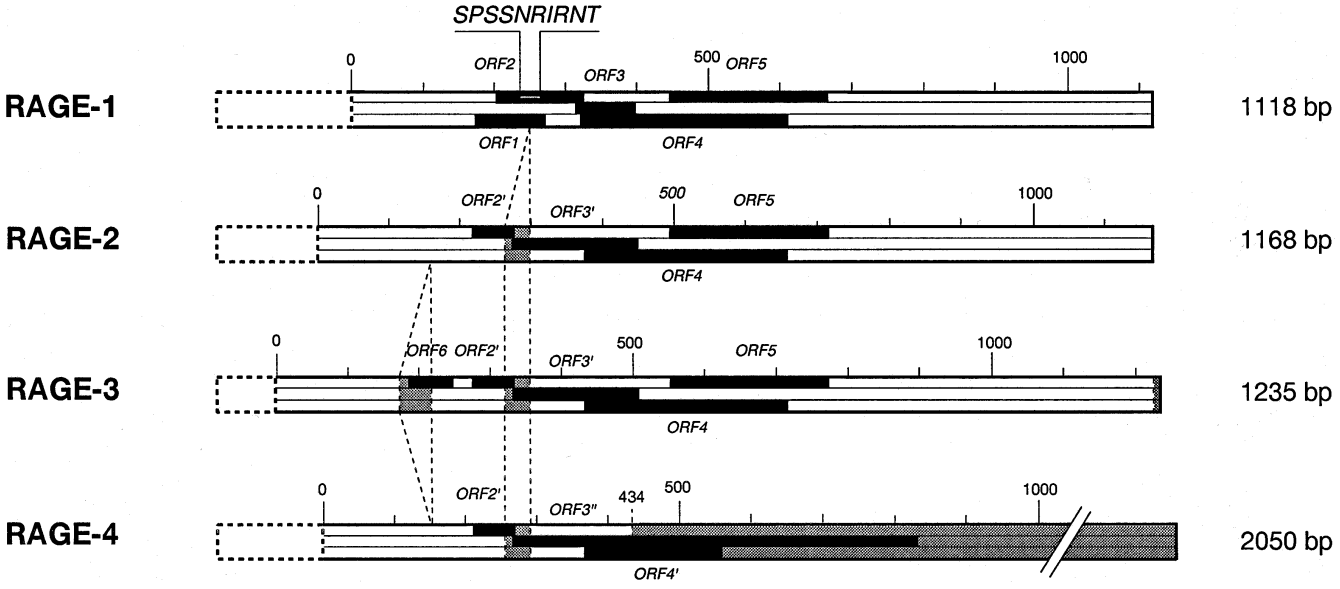
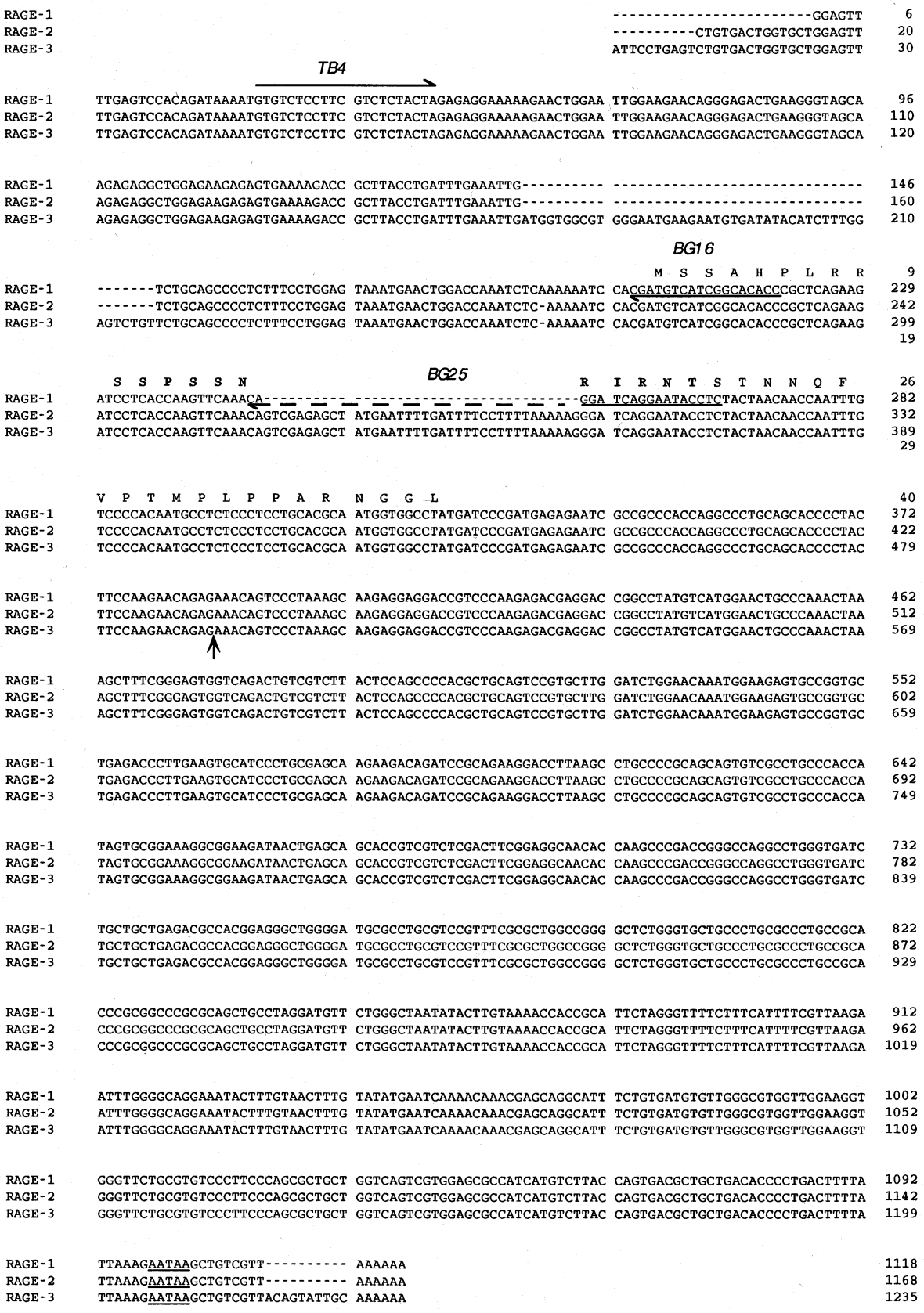



**Fig. 4** Schematic representation of the *RAGE1, 2, 3,* and *4* cDNAs. *Closed black boxes* indicate the different ORFs in each of the three reading frames. *Shaded areas* in the *RAGE2, 3,* and *4* cDNAs represent sequences unrelated to the *RAGE1* sequence, including two insertions. The 5′ terminal sequence obtained by PCR is indicated by *dashed boxes*. The 3′ end of this PCR sequence is identical to the overlapping 5′ end sequences of the *RAGE2, 3,* and *4* cDNAs. The antigenic peptide encoded by *RAGE1* is indicated. Positions of the ORFs are as follows:
*RAGE1:* ORF1 (173–271); ORF2 (204–326); ORF3 (313–399); ORF4 (323–610); ORF5 (444–665)
*RAGE2:* ORF2′ (217–276); ORF3′ (273–449)
*RAGE3:* ORF6 (185–247)
*RAGE4:* ORF3″ (269–832); ORF4′ (369–557)


RAGE-1 ----------GGAGTT 6
RAGE-2 ----------CTGTGACTGGTGCTGGAGTT 20
RAGE-3 ATTCCTGAGTCTGTGACTGGTGCTGGAGTT 30
TB4
RAGE-1 TTGAGTCCACAGATAAAATGTGTCTCCTTC GTCTCTACTAGAGAGGAAAAAGAACTGGAA TTGGAAGAACAGGGAGACTGAAGGGTAGCA 96
RAGE-2 TTGAGTCCACAGATAAAATGTGTCTCCTTC GTCTCTACTAGAGAGGAAAAAGAACTGGAA TTGGAAGAACAGGGAGACTGAAGGGTAGCA 110
RAGE-3 TTGAGTCCACAGATAAAATGTGTCTCCTTC GTCTCTACTAGAGAGGAAAAAGAACTGGAA TTGGAAGAACAGGGAGACTGAAGGGTAGCA 120
RAGE-1 AGAGAGGCTGGAGAAGAGAGTGAAAAGACC GCTTACCTGATTTGAAATTG---------- ---------- 146
RAGE-2 AGAGAGGCTGGAGAAGAGAGTGAAAAGACC GCTTACCTGATTTGAAATTG---------- ---------- 160
RAGE-3 AGAGAGGCTGGAGAAGAGAGTGAAAAGACC GCTTACCTGATTTGAAATTGATGGTGGCGT GGGAATGAAGAATGTGATATACATCTTTGG 210
BG16
M S S A H P L R R 9
RAGE-1 -------TCTGCAGCCCCTCTTTCCTGGAG TAAATGAACTGGACCAAATCTCAAAAAATC CACGATGTCATCGGCACACCCGCTCAGAAG 229
RAGE-2 -------TCTGCAGCCCCTCTTTCCTGGAG TAAATGAACTGGACCAAATCTC-AAAAATC CACGATGTCATCGGCACACCCGCTCAGAAG 242
RAGE-3 AGTCTGTTCTGCAGCCCCTCTTTCCTGGAG TAAATGAACTGGACCAAATCTC-AAAAATC CACGATGTCATCGGCACACCCGCTCAGAAG 299
19
S S P S S N BG25 R I R N T S T N N Q F 26
RAGE-1 ATCCTCACCAAGTTCAAACA---------- ---------- ----------GGA TCAGGAATACCTCTACTAACAACCAATTTG 282
RAGE-2 ATCCTCACCAAGTTCAAACAGTCGAGAGCT ATGAATTTTGATTTTCCTTTTAAAAAGGGA TCAGGAATACCTCTACTAACAACCAATTTG 332
RAGE-3 ATCCTCACCAAGTTCAAACAGTCGAGAGCT ATGAATTTTGATTTTCCTTTTAAAAAGGGA TCAGGAATACCTCTACTAACAACCAATTTG 389
29
V P T M P L P P A R N G G L 40
RAGE-1 TCCCCACAATGCCTCTCCCTCCTGCACGCA ATGGTGGCCTATGATCCCGATGAGAGAATC GCCGCCCACCAGGCCCTGCAGCACCCCTAC 372
RAGE-2 TCCCCACAATGCCTCTCCCTCCTGCACGCA ATGGTGGCCTATGATCCCGATGAGAGAATC GCCGCCCACCAGGCCCTGCAGCACCCCTAC 422
RAGE-3 TCCCCACAATGCCTCTCCCTCCTGCACGCA ATGGTGGCCTATGATCCCGATGAGAGAATC GCCGCCCACCAGGCCCTGCAGCACCCCTAC 479
RAGE-1 TTCCAAGAACAGAGAAACAGTCCCTAAAGC AAGAGGAGGACCGTCCCAAGAGACGAGGAC CGGCCTATGTCATGGAACTGCCCAAACTAA 462
RAGE-2 TTCCAAGAACAGAGAAACAGTCCCTAAAGC AAGAGGAGGACCGTCCCAAGAGACGAGGAC CGGCCTATGTCATGGAACTGCCCAAACTAA 512
RAGE-3 TTCCAAGAACAGAGAAACAGTCCCTAAAGC AAGAGGAGGACCGTCCCAAGAGACGAGGAC CGGCCTATGTCATGGAACTGCCCAAACTAA 569
RAGE-1 AGCTTTCGGGAGTGGTCAGACTGTCGTCTT ACTCCAGCCCCACGCTGCAGTCCGTGCTTG GATCTGGAACAAATGGAAGAGTGCCGGTGC 552
RAGE-2 AGCTTTCGGGAGTGGTCAGACTGTCGTCTT ACTCCAGCCCCACGCTGCAGTCCGTGCTTG GATCTGGAACAAATGGAAGAGTGCCGGTGC 602
RAGE-3 AGCTTTCGGGAGTGGTCAGACTGTCGTCTT ACTCCAGCCCCACGCTGCAGTCCGTGCTTG GATCTGGAACAAATGGAAGAGTGCCGGTGC 659
RAGE-1 TGAGACCCTTGAAGTGCATCCCTGCGAGCA AGAAGACAGATCCGCAGAAGGACCTTAAGC CTGCCCCGCAGCAGTGTCGCCTGCCCACCA 642
RAGE-2 TGAGACCCTTGAAGTGCATCCCTGCGAGCA AGAAGACAGATCCGCAGAAGGACCTTAAGC CTGCCCCGCAGCAGTGTCGCCTGCCCACCA 692
RAGE-3 TGAGACCCTTGAAGTGCATCCCTGCGAGCA AGAAGACAGATCCGCAGAAGGACCTTAAGC CTGCCCCGCAGCAGTGTCGCCTGCCCACCA 749
RAGE-1 TAGTGCGGAAAGGCGGAAGATAACTGAGCA GCACCGTCGTCTCGACTTCGGAGGCAACAC CAAGCCCGACCGGGCCAGGCCTGGGTGATC 732
RAGE-2 TAGTGCGGAAAGGCGGAAGATAACTGAGCA GCACCGTCGTCTCGACTTCGGAGGCAACAC CAAGCCCGACCGGGCCAGGCCTGGGTGATC 782
RAGE-3 TAGTGCGGAAAGGCGGAAGATAACTGAGCA GCACCGTCGTCTCGACTTCGGAGGCAACAC CAAGCCCGACCGGGCCAGGCCTGGGTGATC 839
RAGE-1 TGCTGCTGAGACGCCACGGAGGGCTGGGGA TGCGCCTGCGTCCGTTTCGCGCTGGCCGGG GCTCTGGGTGCTGCCCTGCGCCCTGCCGCA 822
RAGE-2 TGCTGCTGAGACGCCACGGAGGGCTGGGGA TGCGCCTGCGTCCGTTTCGCGCTGGCCGGG GCTCTGGGTGCTGCCCTGCGCCCTGCCGCA 872
RAGE-3 TGCTGCTGAGACGCCACGGAGGGCTGGGGA TGCGCCTGCGTCCGTTTCGCGCTGGCCGGG GCTCTGGGTGCTGCCCTGCGCCCTGCCGCA 929
RAGE-1 CCCGCGGCCCGCGCAGCTGCCTAGGATGTT CTGGGCTAATATACTTGTAAAACCACCGCA TTCTAGGGTTTTCTTTCATTTTCGTTAAGA 912
RAGE-2 CCCGCGGCCCGCGCAGCTGCCTAGGATGTT CTGGGCTAATATACTTGTAAAACCACCGCA TTCTAGGGTTTTCTTTCATTTTCGTTAAGA 962
RAGE-3 CCCGCGGCCCGCGCAGCTGCCTAGGATGTT CTGGGCTAATATACTTGTAAAACCACCGCA TTCTAGGGTTTTCTTTCATTTTCGTTAAGA 1019
RAGE-1 ATTTGGGGCAGGAAATACTTTGTAACTTTG TATATGAATCAAAACAAACGAGCAGGCATT TCTGTGATGTGTTGGGCGTGGTTGGAAGGT 1002
RAGE-2 ATTTGGGGCAGGAAATACTTTGTAACTTTG TATATGAATCAAAACAAACGAGCAGGCATT TCTGTGATGTGTTGGGCGTGGTTGGAAGGT 1052
RAGE-3 ATTTGGGGCAGGAAATACTTTGTAACTTTG TATATGAATCAAAACAAACGAGCAGGCATT TCTGTGATGTGTTGGGCGTGGTTGGAAGGT 1109
RAGE-1 GGGTTCTGCGTGTCCCTTCCCAGCGCTGCT GGTCAGTCGTGGAGCGCCATCATGTCTTAC CAGTGACGCTGCTGACACCCCTGACTTTTA 1092
RAGE-2 GGGTTCTGCGTGTCCCTTCCCAGCGCTGCT GGTCAGTCGTGGAGCGCCATCATGTCTTAC CAGTGACGCTGCTGACACCCCTGACTTTTA 1142
RAGE-3 GGGTTCTGCGTGTCCCTTCCCAGCGCTGCT GGTCAGTCGTGGAGCGCCATCATGTCTTAC CAGTGACGCTGCTGACACCCCTGACTTTTA 1199
RAGE-1 TTAAAGAATAAGCTGTCGTT---------- AAAAAA 1118
RAGE-2 TTAAAGAATAAGCTGTCGTT---------- AAAAAA 1168
RAGE-3 TTAAAGAATAAGCTGTCGTTACAGTATTGC AAAAAA 1235

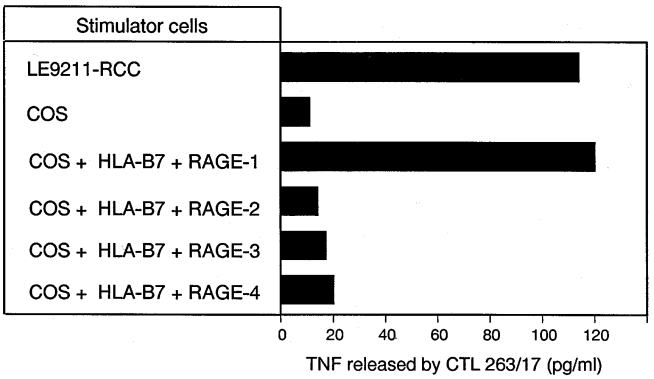



**Fig. 6** Transfection of the *RAGE1, 2, 3*, and *4* cDNAs into COS cells. The *RAGE* cDNAs were transfected into COS-7 cells together with the *HLA-B7* cDNA. CTL 263/17 was added after 24 h and the production of TNF was measured 18 h later. Autologous LE9211-RCC cells were used as control stimulator cells

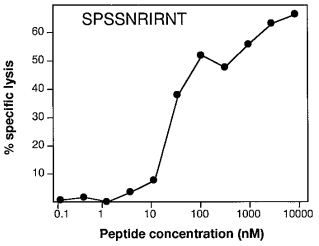
cDNA 9H3, we performed anchored-PCR amplification of cDNA from LE9211-RCC (Frohman et al. 1988), and this extended the sequence by 183 bp at the 5′ end (Fig. 4). No additional ORF was generated by the addition of this sequence, which contained termination codons in each reading frame, making it unlikely that an ORF had been overlooked because of missing nucleotides at the 5′ end. The 5′ untranslated (UT) sequence contained a stretch of 107 bp (positions –128 to –22) showing 86% identity with the 5′UT region of the cytochrome b5 cDNA (Li et al. 1995). A related sequence is also present on the anti-sense strand of two human endogenous defective retroviruses, probably due to recombination with human genomic sequences (Hirose et al. 1993; Mager and Freeman 1987).

### *Other cDNA clones related to RAGE*

To obtain other cDNA clones related to *RAGE1*, 120 000 clones of the LE9211-RCC cDNA library were hybridized with the *RAGE1* cDNA. We obtained seven other cDNA clones carrying three new homologous sequences which we called *RAGE2, 3*, and *4*. We also screened a genomic library of LE9211-RCC and we isolated a cosmid containing a sequence that appeared to correspond to part of the *RAGE2* cDNA.

**Fig. 5** Alignment of the *RAGE1, 2*, and *3* cDNA sequences. The amino acid sequence of the protein encoded by *RAGE1* is shown *above* the nucleotide sequence. The sequence of the antigenic peptide is indicated in *bold*. Primers *TB4, BG16*, and *BG25* used for the analysis of *RAGE* expression by PCR are indicated by *horizontal arrows*. A *vertical arrow* indicates the position at which the *RAGE4* cDNA sequence diverges from the other *RAGE* sequences. The polyadenylation signal is *underlined*. Comparison of the *RAGE3* cDNA sequence with the genomic sequence indicated that the 10 additional bases at the 3′ end of *RAGE3* result from the addition of the poly-A tail 10 bases downstream as in the other cDNAs. The nucleotide sequences of the four *RAGE* cDNAs are available from EMBL/GenBank/DDBJ under accession numbers U46191, U46192, U46193, and U46194

**Fig. 7** Lysis by CTL 263/17 of HLA-B7$^+$ cells pulsed with *RAGE1*-encoded peptide SPSSNRIRNT. Chromium-labelled HLA-B7$^+$EBV-transformed lymphoblastoid cells (LB23-EBV) were pulsed 30 min with the indicated peptide at various concentrations before addition of CTL 263/17 at an effector/target ratio of 10. Similar results were obtained with CTL clone 361A/17




A schematic alignment of the four cDNAs is shown on Figure 4. The three new cDNA sequences all have an insertion of 37 bp at position 249 of *RAGE1*. For the *RAGE2* cDNA, comparison with the cosmid sequence indicated that this insertion corresponds to the beginning of an exon. Therefore its absence in the *RAGE1* cDNA might result from the use of an alternative downstream acceptor site. In addition, *RAGE2, 3*, and *4* differ from RAGE1 in lacking a nucleotide at position 192 of *RAGE1* (Fig. 5). These changes significantly modify the limits of the ORFs of *RAGE2, 3*, and *4* that are homologous to ORF 2 and 3 of *RAGE1*. In addition, *RAGE3* has another insertion of 47 bp at the 5′ end. Except for these differences, the *RAGE1, 2*, and *3* sequences are identical.

*RAGE4* is about 800 bases longer than the other *RAGE* cDNAs. Its 5′ sequence is identical to that of *RAGE2*, but from position 434 to the poly-A tail the *RAGE4* sequence differs totally from the other *RAGE* cDNAs. This unrelated sequence was also found at the 3′ end of the *RAGE* gene which had been isolated from the cosmid library. Therefore, the *RAGE4* cDNA appears to result from differential splicing of the *RAGE2* gene. On the basis of the present data, we cannot exclude that the *RAGE3* cDNA also derives from the same gene by alternative splicing.

To determine whether the *RAGE-2, 3*, and *4* cDNAs also encode antigen LE9211-A, we transfected them into COS-7 cells together with the *HLA-B7* cDNA. They did not confer recognition by CTL 263/17 (Fig. 6). As the 37 bp insertion present in *RAGE2, 3*, and *4* caused premature termination of the second ORF of *RAGE1* (Fig. 4), this result suggested that the antigenic peptide recognized by CTL 263/17 derived from the C-terminal part of the protein encoded by this ORF. In agreement with this prediction, we found that decameric peptide SPSSNRIRNT efficiently sensitized HLA-B7$^+$ Epstein-Barr virus-B cells to lysis by CTL 263/17 (Fig. 7).

### *Expression of RAGE*

We analyzed the expression of the *RAGE* messages in various tissues by RT-PCR using primers common to the four *RAGE* cDNAs (Table 1). We found no expression in kidney and in the other normal tissues, with the exception of retina. A number of tumors of various histological types were positive.

**Table 1** Expression of *RAGE* by normal and tumoral tissues

| Normal tissues | | Tumors | | |
|---|---|---|---|---|
| Histological type | Expression of *RAGE1–4*[a] | Histological type | Number of tumors expressing | |
| | | | *RAGE1–4*[a] | *RAGE1*[b] |
| Bone marrow | – | Tumor samples | | |
| Brain | – | Renal carcinomas | 2/57 | 1/57 |
| Breast | – | Sarcomas | 5/25 | 3/25 |
| Cerebellum | – | Bladder tumors superficial | 0/29 | 0/29 |
| Colon | – | infiltrating | 3/37 | 3/37 |
| Heart | – | Melanomas primary lesions | 2/60 | 2/60 |
| Kidney | – | metastases | 8/177 | 6/177 |
| Liver | – | Head and neck tumors | 2/50 | 1/50 |
| Lung | – | Mammary carcinomas | 3/128 | 1/128 |
| Melanocytes | – | Prostatic carcinomas | 0/22 | 0/22 |
| Muscle | – | Colorectal carcinomas | 0/48 | 0/48 |
| Ovary | – | Leukemias | 0/19 | 0/19 |
| Prostate | – | Lung carcinomas (NSCLC[d]) | 0/59 | 0/59 |
| Retina | +[c] | (SCLC[e]) | 0/5 | 0/5 |
| Skin | – | Mesotheliomas | 1/3 | 0/3 |
| Splenocytes | – | Brain tumors | 0/11 | 0/11 |
| Stomach | – | Oesophageal tumors | 0/7 | 0/7 |
| Testis | – | Ovarian tumors | 0/3 | 0/3 |
| Thymocytes | – | | | |
| Urinary bladder | – | Tumor cell lines | | |
| Uterus | – | | | |
| Wound healing | – | Renal carcinomas | 8/19 | 7/19 |
| | | Bladder tumors | 3/3 | 3/3 |
| | | Mesotheliomas | 11/19 | 8/19 |
| Fetal tissues[f] | | Head and neck tumors | 3/7 | 1/7 |
| | | Sarcomas | 2/6 | 1/6 |
| Brain | – | Melanomas | 11/78 | 7/78 |
| Liver | – | Colorectal carcinomas | 1/17 | 1/17 |
| Placenta | – | Lung carcinomas (NSCLC[d]) | 0/2 | 0/2 |
| | | (SCLC[e]) | 0/26 | 0/26 |
| | | Leukemias/Lymphomas | 0/11 | 0/11 |
| | | Brain tumors | 0/1 | 0/1 |
| | | Gastric tumors | 0/2 | 0/2 |

[a] Expression of *RAGE1–4* was tested by RT-PCR on total RNA with sense primer *TB4* and antisense primer *BG16*. These primers are located in different exons and amplify a product of 193 bases which is not observed when genomic DNA is tested

[b] Expression of *RAGE1* was tested by RT-PCR on total RNA with sense primer *TB4* and antisense primer *BG25*. These primers are specific for *RAGE1* and amplify a fragment of 239 bases. They are located in different exons. Only samples positive with primers *TB4/BG16* detecting *RAGE1–4* were tested with this set of primers

[c] The retina samples were also tested with the *RAGE1*-specific primers *TB4/BG25* and were found to be positive

[d] *NSCLC* Non-small-cell lung carcinoma

[e] *SCLC* Small-cell lung carcinoma

[f] Fetal tissues derived from fetuses older than 20 weeks

Since only *RAGE1* encodes the antigen, we retested the positive samples with a set of primers that specifically amplify *RAGE1* (Table 1). Normal retina samples proved positive again. The precise retinal cell type that expresses *RAGE1* could not be defined.

In tumors, we found expression of *RAGE1* in 37% of renal cell carcinoma lines, but in only one of 57 renal cell carcinoma samples (Table 1). This positive fresh RCC sample was from the tumor that gave rise to cell line LE9211-RCC. *RAGE1* was also expressed in some samples of sarcomas, infiltrating bladder carcinomas, and melanomas.

## Discussion

Here we report the first identification of an antigen recognized by autologous CTL on human renal cell carcinoma. This antigen is encoded by a new gene named *RAGE1*, which is expressed in a number of tumors but is silent in normal adult tissues other than retina. The structure of the *RAGE1* cDNA is unusual in that it contains several relatively small ORFs rather than a major one. It is difficult to predict which of these ORFs is functional. Our data suggest that at least ORF2 is translated since the antigenic peptide recognized by CTL 263/17 is encoded by this ORF. However, this does not necessarily mean that the ORF2 protein is the only or even the major protein encoded by the *RAGE1* cDNA. It is not impossible that another protein encoded by ORF3 or ORF5 is the major product of the *RAGE1* cDNA. The other members of the *RAGE* family may also produce tumor antigens recognized by CTL, as they are also silent in normal tissues other than retina, and appear to be expressed in a number of tumors. In particular, the *RAGE4* cDNA contains a long ORF with a favorable initiation codon. The 187 amino acid protein encoded by this ORF is likely to represent the major product of *RAGE4*. It contains several peptide consensus sequences for binding to different HLA molecules. These peptides could be used to stimulate lymphocytes from normal individuals in order to generate tumor-specific CTL by primary in vitro immunization (Celis et al. 1994; van der Bruggen et al. 1994).

The molecular characterization of tumor antigens opens the possibility of new cancer immunotherapy trials involving immunization with defined antigens. The expression of *RAGE1* in retina raises a concern about the safety of immunization procedures based on *RAGE*-derived antigens, since autoimmune adverse effects could be generated.

However most intra-ocular tissues including retina do not appear to express MHC class I molecules (Abi-Hanna et al. 1988). Therefore, it is possible that under normal conditions the antigenic peptide derived from *RAGE1* is not displayed at the surface of the retinal cells that express the gene. A similar situation has been observed for the *MAGE1* gene, whose expression in normal tissues appears to be restricted to testicular germ cells which do not express MHC class I molecules (Chomez et al. 1995; Takahashi et al. 1995). In addition, the eye is an immunologically privileged site, containing a variety of potent immunosuppressive agents protecting it from destructive inflammatory reactions (Barker and Billingham 1977; Streilein 1995). That this immune protection might suffice to prevent ocular autoimmunity after immunization against the RAGE-1 antigens is suggested by the observation of patients who were injected with TIL directed against melanoma differentiation antigens. Notwithstanding the presence of melanocytes in the retinal choroid, no ophthalmic toxicity was observed, even though tumor responses were observed in some patients and even though some patients developed autoimmune responses against cutaneous melanocytes resulting in vitiligo (Kawakami et al. 1994). If these early clinical observations are to be confirmed, immunization of selected cancer patients against *RAGE*-derived antigens could be considered.

Specific cancer immunotherapy using defined antigens may involve a variety of modalities, including vaccines containing peptides or proteins. These modalities are presently tested in melanoma with *MAGE*-derived antigens (Marchand et al. 1995). Although *HLA-B7* is present in about 22% of Caucasians, the RAGE-1.B7 antigen will not allow immunization of a significant number of RCC patients because of the very low frequency of expression of *RAGE1* in kidney tumor samples. However, *RAGE1* is more frequently expressed in sarcomas, bladder tumors, and melanomas, and *RAGE1*-based vaccines may therefore prove useful for the treatment of a limited number of patients bearing tumors of these types.

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