+1 Frameshifting as a Novel Mechanism to Generate a Cryptic Cytotoxic T Lymphocyte Epitope Derived from Human Interleukin 10

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Abstract
Recent data indicate that some cytotoxic T cells (CTLs) recognize so-called cryptic epitopes, encoded by nonprimary open reading frame (ORF) sequences or other nonclassical expression pathways. We describe here a novel mechanism leading to generation of a cryptic CTL epitope. We isolated from the synovial fluid of a patient suffering from a Reiter’s syndrome an autoreactive T cell clone that recognized cellular IL-10 in the HLA-B*2705 context. The minimal IL-10 sequence corresponding to nucleotides 379–408 was shown to activate this clone, upon cotransfection into COS cells with the DNA encoding HLA-B*2705, but the synthetic peptide deduced from this sequence did not stimulate the clone. Using a site-directed mutagenesis approach, we found that this clone recognized a transframe epitope generated by an internal +1 frameshifting in the IL-10 sequence and so derived partly from ORF1, partly from ORF2. We defined that +1 frameshifting was induced by a specific heptamer sequence. These observations illustrate the variety of mechanisms leading to generation of cryptic epitopes and suggest that frameshifting in normal cellular genes may be more common than expected.

Key words: frameshift • epitope • CTL • IL-10 • autoimmunity

Introduction
It has been generally assumed that degradation of full-length protein supplies the majority of epitopes presented by MHC molecules to the T cell antigen receptors. Yet, recent data indicate that some CTL epitopes in viral, tumor, and model systems are generated through nontraditional pathways of gene expression, when unexpected events take place during transcription, splicing or translation (for a review, see reference 1). Examples include peptides encoded by cryptic translational reading frames, in some cases even without upstream AUG initiation codons (2–6), derived from reverse strand transcription (7) or from improper splicing of introns (8).

Here we describe a new mechanism leading to generation of a class I CTL epitope. In the course of an extensive analysis of CD8 T cell responses to Epstein-Barr virus (EBV) within synovial fluid-derived T cells from patients suffering from chronic arthritis (9, 10), we observed a response to BCRF1, the EBV-encoded IL-10, in the HLA-B*2705 context (10). On account of the strong homology between BCRF1 and human IL-10 (11), we presently searched for possible cross-reactive T cell responses against IL-10 in these patients. This led to the isolation of an autoreactive CD8 T cell clone that recognized a cryptic epitope derived from cellular IL-10, in the HLA-B*2705 context. We show here that this transframe epitope, derived partly from ORF1 partly from ORF2, resulted from an internal +1 frameshifting in the IL-10 sequence.

Materials and Methods

T Cells. The CD8 synovial T cell line from patient RS2 was expanded in vitro, under polyclonal activation conditions and clones were obtained by seeding synovial T cells at 0.3 cells per well as described previously (9).

Expression Vectors and Production of Modified cDNAs. Genomic HLA-B*2705 was cloned into pcDNA3 (Invitrogen). cDNA encoding full-length or truncated BCRF1 or human IL-10 was cloned into pcI-neo (Promega). Truncated IL-10 or BCRF1 cDNAs, generated by PCR amplification, were cloned into pcI-neo. A pFlag expression vector was constructed to allow the expression of

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shorter constructs by inserting the sequence encoding the tag Flag in the pCI-neo expression vector. The Flag sequence contains, at the 5’ end, a favorable Kozak sequence for translation initiation and a methionine start codon. A pFlag-IL-10 Δ4 and a pFlag-BCRF1 Δ4 vectors were also constructed. IL-10 and BCRF1 site-directed mutants were generated by introducing the desired point mutation in oligonucleotide primers in the IL-10 Δ4 and BCRF1 Δ4 sequences, respectively. All constructs were checked by sequencing.

Transfections and T Cell Stimulation Assays. Transfection into COS cells was performed by the DEAE-dextran chloroquine method as described previously (9). Transfectants were tested after 48 h for their ability to stimulate TNF-α production by T cells. 5 × 10^5 cells from a given T cell clone were added to transfected COS cells, culture supernatants were harvested 6 h later and tested for TNF content by measuring culture supernatant cytotoxicity to WEHI 164 clone 13 in a colorimetric assay (9). C1R cells were stably transfected with HLA-B*2705 genomic DNA and IL-10 or BCRF1 cDNA, using neomycin and zeocin as selection markers.

Peptide Stimulation Assays. Characterization of the IL-10/B27 epitope was achieved by assaying the ability of various peptides (Genosys) loaded at various nanomolar concentrations on HLA-B*2705 BLCL (i) to induce lysis of these BLCL by T cell clone 35 as described previously (9) and (ii) to trigger TNF-α release by clone 35 T cells. 3 × 10^5 cells from a given T cell clone were added to transfected COS cells, culture supernatants were harvested 6 h later and tested for TNF content by measuring culture supernatant cytotoxicity to WEHI 164 clone 13 in a colorimetric assay (9). C1R cells were stably transfected with HLA-B*2705 genomic DNA and IL-10 or BCRF1 cDNA, using neomycin and zeocin as selection markers.

Reporter Luciferase Assays. Frameshift reporter plasmids were generated by inserting primers containing the IL-10 sequence (CTTCCCT) or a random irrelevant sequence (GAAGCCTT) in the Firefly luciferase gene in +1 frame (pIL10-FF and pRandom-FF) and in the Renilla luciferase gene in 0 frame (pIL10-RL and pRandom-RL). These constructs were cloned into pCI-neo vector and critical regions were checked by sequencing. 100 ng of a Firefly luciferase plasmid together with 100 ng of the corresponding Renilla luciferase control were cotransfected into COS cells. Luciferase activities of the cell lysates were determined 48 h after the transfection by using the Dual Luciferase Assay System (Promega) and a Berthold Junior luminometer (PerkinElmer Instruments). pGL3-Control (Firefly) and pRL-SV40 (Renilla) vectors (Promega) were used to control transfection efficiency.

Results

Isolation of an Autoreactive CD8 T Cell Clone Recognizing Human IL-10 in the HLA-B*2705 Context. In the course of an analysis of CD8 T cell responses to EBV within synovial fluid-derived T cell lines from patients suffering from chronic arthritis, we showed that the CD8 synovial T cell line from a patient with a Reiter’s syndrome (patient RS2), recognized COS cells cotransfected with DNAs coding for HLA-B*2705 and BCRF1 (10). Owing to the high homology between BCRF1 and human IL-10 (85% homology if the leader sequence is excluded), we looked for possible cross-reactive T cell responses against BCRF1 and IL-10. To this end, 80 clones derived from the CD8 synovial T cell line from patient RS2 were screened for recognition of COS cells transfected with HLA-B*2705 DNA and with BCRF1 or IL-10 cDNA. One clone (#35), produced TNF-α when incubated with COS cells cotransfected with IL-10 cDNA and the DNA coding for HLA-B*2705 (Fig. 1 B). By contrast, this clone did not recognize COS cells expressing HLA-B27 or IL-10 alone, or BCRF1/B27 (Fig. 1 and 2). Similarly, significant cytolytic activity (Fig. 2) and TNF release (data not shown) by clone 35 were both activated after a short-term coculture with C1R B cells stably transfected with expression vectors coding for IL-10 and HLA-B*2705.

To identify the minimal IL-10 epitope recognized by clone 35, several truncated IL-10 variants (Δ1, Δ2, and Δ3) were transfected into COS cells along with the HLA-B*2705 DNA. Reactivity of the T cell clone 35 to the transfected COS cells was measured in a TNF release assay (Fig. 1). As IL-10 Δ1 (1–414), but not IL-10 Δ2 (1–375), sequence stimulated T cells, we generated

Figure 1. Mapping of the IL-10 cDNA sequence encoding the antigenic peptide recognized by clone 35. (A) Schematic representation of IL-10 and BCRF1 sequences. The IL-10 full-length and truncated cDNAs are marked in black. The IL-10 Δ3 and Δ4 PCR products were cloned into a Flag-pCI-neo vector. The BCRF1 cDNA sequence is represented by cross-hatched boxes. The Flag sequence is represented by open boxes. Nucleotides are numbered from the start codon. (B) The various constructions shown in A were tested for their ability to induce clone 35 TNF production after transfection into COS cells along with a plasmid construct encoding HLA-B*2705. Constructions with IL-10 sequences, in which the thymine at position 394 was deleted (FS 394), were also included in the assay.
the small IL-10 Δ3 fragment (355–414), containing an internal ATG start codon, which induced clone 35 activation, though suboptimally. To increase T cell clone 35 activation, we added a Flag tag in front of the shortest construct. The H9004 construct was cloned into a pCI-neo Flag expression vector, thus expressed as a Flag-IL-10 H9004 fusion protein, and optimal response of clone 35 was then obtained. A minimal Flag-IL-10 H9004 construction (379–408), coding for 10 amino acids, was also capable to induce clone 35 activation (Fig. 1) and was used for further analysis. In contrast, neither the BCRF1 full-length cDNA nor the pFlag-BCRF1 H9004 (358–387), homologous to the IL-10 H9004 stimulatory sequence, activated clone 35. This was rather unexpected because the amino acid sequence of the IL-10 stimulatory region was 100% homologous to the corresponding sequence in BCRF1 (Fig. 3 A).

Clone 35 Recognizes a Cryptic Epitope Generated by an Internal Frameshifting in the IL-10 Sequence. The nonamer peptide deduced from the minimal IL-10 Δ3 sequence, HRFLPCENK, and three related peptides (HRFLPCENKS, HRFLPCENKS, CHRLPCENKS), were screened for their ability to sensitize HLA-B*2705 EBV-B cells to lysis by clone 35 and for optimal response of clone 35 was then obtained. A minimal Flag-IL-10 Δ4 construction (379–408), coding for 10 amino acids, was also capable to induce clone 35 activation (Fig. 1) and was used for further analysis. In contrast, neither the BCRF1 full-length cDNA nor the pFlag-BCRF1 Δ4 (358–387), homologous to the IL-10 Δ4 stimulatory sequence, activated clone 35. This was rather unexpected because the amino acid sequence of the IL-10 stimulatory region was 100% homologous to the corresponding sequence in BCRF1 (Fig. 3 A).

Thus, the recognition of an epitope modified by N- or O-glycosylation (data not shown).

Of the large number of constructions cloned into the pFlag-IL-10 Δ4 expression vector, one was fortuitously generated, in which the thymine at position 394 was missing (IL-10 Δ4 FS-394). This mutation, which originated from a contaminant in the primer preparation used for PCR, resulted in an internal frameshift and consequently yielded a peptide encoded partly by ORF1 partly by ORF2 (Fig. 3 B). Surprisingly, this construction strongly induced TNF release by clone 35 in a COS transfection assay (Fig. 1 B). The synthetic nonamer peptide deduced from this sequence (HRFLPVKTRA), when loaded on HLA-B*2705 + EBV-B cells, clearly activated clone 35 as shown by lytic activity and TNF release (Fig. 4). Half-maximal lysis by the CTL clone was obtained at <10 nM concentration. The deletion of nucleotide 394 in the full length IL-10 cDNA (IL-10 Δ4) also led to clone 35 activation (Fig. 1 B).
Definition of the Frameshift Site in the IL-10 Sequence. To determine if a precise nucleotidic sequence favored frameshifting, we first synthesized a set of peptides whose sequence was deduced from systematic +1 frameshifting in the relevant IL-10 nucleotidic sequence (nucleotides 379–408). It appeared that all the +1 frameshifts between positions 389–395, which yielded two distinct peptides, HRFLPVKTR (frameshift at positions 389–394) and HRFLPLKTR (frameshift at position 395), were able to optimally induce clone 35 activation, as shown by both TNF release and cytotoxicity assays (Fig. 4). This targeted the sequence 389 to 395 as the likely frameshift site.

We generated various BCRF1/IL-10 chimeric constructs to try either to induce recognition of BCRF1 or to abrogate IL-10 recognition (Fig. 3 C). The results obtained supported a key role of the CCC triplet in IL-10 sequence in frameshift induction. In fact, silent mutation of codon CCC to CCG in the IL-10 sequence abrogated activation of clone 35 (Fig. 3 C). We also succeeded in modifying the corresponding BCRF1 sequence such as to induce activation of clone 35, thus confirming that the CCC codon was critical. Although the wild-type BCRF1 nucleotidic sequence did not lead to clone 35 activation in COS assay, the BCRF1 sequence in which the thymine at position 373 was deleted activated clone 35. However, the peptide deduced from this BCRF1 frameshifted sequence (HRFLPVKTR) was less efficient to activate clone 35 than the IL-10 transframe peptide (HRFLPKVTR) (Fig. 4), most probably due to the arginine to lysine replacement at position 7. The mutation of GAG to GAA in the BCRF1 sequence did not lead to clone 35 activation in COS assay, as shown by both TNF release and cytotoxicity assays (Fig. 4). This targeted the sequence 389 to 395 as the likely frameshift site.

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To test the ability of this sequence to induce a +1 frameshift in COS cells, we generated a luciferase reporter plasmid (pIL10–FP), in which the IL-10 heptamer slippery site, followed by the sequence encoding Firefly luciferase, was cloned into pCI-neo so that a +1 frameshift was required for the luciferase to be translated. The heptamer IL-10 sequence was also cloned upstream of the Renilla luciferase in 0 frame. After cotransfection into COS cells, luciferase activities were monitored and the efficiency of the IL-10 sequence to induce +1 frameshift was estimated by the ratio Firefly activity/Renilla activity. Luciferase activities generated from cells harboring pRandom plasmids (with a random heptamer) were monitored as a negative control to determine the background level of nonprogrammed +1 frameshifting. The results of these experiments demonstrated a significant frameshifting induced by the IL-10 motif, when compared with the random plasmid (Fig. 5).

In an attempt to define at what step (i.e., DNA synthesis, transcription or translation) the frameshift occurred, we sequenced a large number of cDNA clones (150) corresponding to the minimal activating IL-10 sequence (nucleotides 379–408). To this end, the pFlag-IL-10 Δ4 expression vector was transfected into COS cells (2 × 10⁶) for 48 h. RT-PCR was performed from DNA-free RNA, followed by automated sequencing. Sequence alignment for the 150 cDNA clones showed that none of them had deletion, mutation or insertion, thus suggesting that the frameshift is probably occurring at the translational level.

Discussion

Several different forms of aberrant gene expression have been shown to lead to the generation of a variety of MHC class I–restricted epitopes (1–8). Here we describe the reactivity of an HLA-B²²⁷⁰⁵-restricted CTL clone, isolated from the synovial fluid of a patient suffering from a Reiter’s syndrome, that recognized a transframe epitope generated by a +1 frameshifting in IL-10 sequence. The +1 frameshift described here appears as a programmed frameshift which occurred systematically in COS transfection assays. This frameshift also occurred in EBV-B cells as indicated by experiments using C1R B27/IL-10 transfectants. The rather inefficient generation of transframe epitope in B cell IL-10 transfectants, when compared with COS cells, could possibly be due to downregulation of the transporter associated with antigen processing (TAP) in these stable transfectants, due to the high level of IL-10 (12), thus leading to decreased presentation of antigens. The nonamer synthetic peptide (HRFLPVKTR) deduced from the frameshifted IL-10 sequence stimulated the CTL clone at nanomolar range of concentration, indicating that this peptide is likely to be the actual peptide. To our knowledge, this is the first
equimolar quantities of each clone (Pool). 2 d later, luciferase activity/or random sequence for ciferases activities of the cell lysates were measured. Efficiency of the IL-10 and pRandom- plasmid encoding the IL-10 sequence CTTCCCT (boxed sequence), followed by the sequence encoding the Firefly luciferase (bold) was cloned into pCI-neo vector. In pIL10-FF, the IL-10 sequence was replaced by a random irrelevant sequence. In these two constructs, to which was added a G (indicated by an arrow), a +1 frameshift is required for the Firefly luciferase to be trans- lated. In contrast, in pIL10-RF and in pRandom-RF, the reporter se- quence of the Renilla luciferase is in 0 frame. (B) 100 ng of three plasmid clones (#1, #2, and #3) corresponding to pIL10-FF or pRandom-FF constructs were cotransfected into COS cells along with their control plasmid encoding the Renilla luciferase in frame, respectively pIL10-RF and pRandom-RF. A similar experiment was performed by mixing equimolar quantities of each clone (Pool). 2 d later, Firefly and Renilla lu- ciferases activities of the cell lysates were measured. Efficiency of the IL-10 or random sequence for +1 frameshift was estimated by the ratio Firefly luciferase activity/Renilla luciferase activity.

Figure 5. Efficiency of the IL-10 heptamer sequence to induce +1 frameshifting. (A) Plasmids used to estimate +1 frameshifting induced by the IL-10 sequence in a dual reporter luciferase assay. In pIL10-FF, the IL-10 sequence CTTCCT (boxed sequence), followed by the sequence encoding the Firefly luciferase (bold) was cloned into pCI-neo vector. In pRandom-FF, the IL-10 sequence was replaced by a random irrelevant sequence. In these two constructs, to which was added a G (indicated by an arrow), a +1 frameshift is required for the Firefly luciferase to be translated. In contrast, in pIL10-RF and in pRandom-RF, the reporter se- quence of the Renilla luciferase is in 0 frame. (B) 100 ng of three plasmid clones (#1, #2, and #3) corresponding to pIL10-FF or pRandom-FF constructs were cotransfected into COS cells along with their control plasmid encoding the Renilla luciferase in frame, respectively pIL10-RF and pRandom-RF. A similar experiment was performed by mixing equimolar quantities of each clone (Pool). 2 d later, Firefly and Renilla lu- ciferases activities of the cell lysates were measured. Efficiency of the IL-10 or random sequence for +1 frameshift was estimated by the ratio Firefly luciferase activity/Renilla luciferase activity.

Evidence that such a mechanism contributes to the generation of a T cell epitope.

The question arises of whether the frameshift is the result of errors at the level of DNA replication, transcription or translation. The slipping of DNA polymerase on the heptameric slippery site during DNA synthesis seems unlikely because if that occurred, the mutation would result in a polymorphism in the IL-10 gene. Sequencing of a large number of cDNA clones did not reveal any IL-10 mRNA molecule missing one nucleotide, thus suggesting that the frameshift could result from +1 ribosomal frameshift in intact RNA molecules. Examples of programmed ribosomal frameshifting have been described. Programmed –1 ribosomal frameshift signals are among the most extensively characterized of these translational recoding phenomena. Although most examples concern viral systems, –1 frame- shifting has also been documented in bacteria (for a review, see reference 13). To date, only one example of eukary- otic mRNAs undergoing –1 frameshift has been reported (14). However, using a bioinformatic approach, Hammell et al. (15) could find that there are significant number of consensus programmed –1 ribosomal frameshift signals in the genome of all organisms for which large DNA databases exist. It has been shown that –1 frameshifting is greatly enhanced by the presence of both a particular heptamer sequence (slippery site), 5’ of the frameshift site upon which the ribosome shifts, and a sequence that forms a defined RNA secondary structure, such as an RNA pseudoknot, 3’ of the shift site, which causes the ribosome to pause over the slippery site (16). In the case of human gene expression, only one family of genes, namely anti- zyme, has been shown to undergo a +1 frameshifting for which a pseudoknot downstream in the mRNA has a stimulatory effect (17).

In the hypothesis that IL-10 +1 frameshifting occurs during translation, what could be the mechanism involved? As the frameshift occurred in a minimal sequence, corres- ponding to nucleotides 379–408, a pseudoknot is definitively not involved. We found that a specific heptamer sequence (CTT CCC T), which to our knowledge has not been described as a slippery site previously, is central for frameshift occurrence. We can hypothesize that this heptameric sequence could induce a programmed translational frameshift by either of two simple mechanisms which have been described previously in the yeast (for a review, see reference 18): (i) a true shift of the ribosome was described for the yeast S. cerevisiae retrotransposon Ty1 element (19). In that case, frameshifting is due to the ability of mRNA-bound tRNA to slip between cognate or near-cognate codons. In our model, the ribosome could shift when only the P-site is occupied by the peptidyl tRNA (the anticodon 5’ A/GAG 3’ could base-pair to both the 0-frame CCC and the +1 frame UUC codons. Translational slippage could also occur with both P- and A-sites occupied by tRNAs, the same peptidyl tRNAs would be present, and the 5’ GGG 3’ anticodon of the aminoacyl-tRNA could base pair equally well either the 0-frame CCC or +1 frame CUC codon. (ii) A second mechanism, involving the mis-insertion of the aminoacyl-tRNA into the +1 frame A-site codon, rather a tRNA slippage, has been documented for the yeast retrotransposon Ty3 (18, 20). In our model, it would be possible that the prolyl-tRNA was mis-inserted into the A-site in the wrong frame. Whatever the mecha- nism involved, these results suggest that +1 ribosomal frameshifting in normal cellular genes may be more common than expected.

The high level of expression of IL-10 in the rheumatoid synovium (21) could contribute to the activation of au- toreactive CTLs recognizing IL-10. Since a preliminary analysis suggests recognition of this cryptic epitope of IL-10 in another HLA-B27+ patient, such a response could have an important immunological impact and be of patho- physiological relevance. T cells might become activated and autoaggressive if the epitope is presented at high levels on IL-10 producing cells and this could lead in vivo to recognition and subsequent elimination of T cell regula-
tory subsets known to produce high amounts of this cytokine (22).

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