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FRAMESHIFT PEPTIDE-DERIVED T-CELL EPITOPES: A SOURCE OF NOVEL TUMOR-SPECIFIC ANTIGENS

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Microsatellite instability (MSI) caused by defective DNA mismatch repair (MMR) is a hallmark of hereditary nonpolyposis colorectal cancers (HNPCC) but also occurs in about 15% of sporadic tumors. If instability affects microsatellites in coding regions, translational frameshifts lead to truncated proteins often marked by unique frameshift peptide sequences at their C-terminus. Since MSI tumors show enhanced lymphocytic infiltration and our previous analysis identified numerous coding mono- and dinucleotide repeat-bearing candidate genes as targets of genetic instability, we examined the role of frameshift peptides in triggering cellular immune responses. Using peptide pulsed autologous CD40-activated B cells, we have generated cytotoxic T lymphocytes (CTL) that specifically recognize HLA-A2.1-restricted peptides derived from frameshift sequences. Among 16 frameshift peptides predicted from mutations in 8 different genes, 3 peptides conferred specific lysis of target cells exogenously loaded with cognate peptide. One peptide derived from a (−1) frameshift mutation in the TGFβIIR gene gave rise to a CTL bulk culture capable of lysing the MSI colorectal cancer cell line HCT116 carrying this frameshift mutation. Given the huge number of human coding microsatellites and assuming only a fraction being mutated and encoding immunologically relevant peptides in MSI tumors, frameshift protein sequences represent a novel subclass of tumor-specific antigens. It is tempting to speculate that a frameshift peptide-directed vaccination approach not only could offer new treatment modalities for existing MSI tumors but also might benefit asymptomatic at-risk individuals in HNPCC families by a prophylactic vaccination strategy.

Key words: *DNA mismatch repair; microsatellite instability; frameshift peptides; tumor antigens; T-cell epitopes*

The accumulation of genetic alterations and resulting mutant proteins represents a major obstacle for tumor cells to escape immune surveillance. It has thus been hypothesized that mutant proteins or derived peptides must exist that are capable of eliciting specific cellular immune responses. In fact, CTL have been reported that recognize peptides of mutant or aberrantly expressed proteins such as p21/ras, MAGE-1, oncogenic fusion proteins,[1–3] products from alternative open reading frames[4–6] and also frameshift mutated APC.[7] These tumor antigens originally have been identified in tumors showing chromosomal instability.[8]

Much less is known about the immunogenicity of tumor cells that show more subtle genetic alterations such as small deletions and insertions in repetitive DNA sequences, termed microsatellites.[9,10] Microsatellite instability in these tumor cells is due to deficient DNA mismatch repair caused by germline and/or sporadic mutations in at least 5 different MMR genes,[11] leading to high spontaneous mutation rates. More than 90% of HNPCC and about 15% of sporadic cancers of different organs show MSI.[9,12] If instability affects microsatellites in coding regions of expressed genes, encoded proteins are often truncated and frequently contain unique frameshift peptide sequences at their C-terminus. Several coding microsatellites bearing genes are affected by frameshift mutations in MSI tumors including TGFβRII, IGFIIR, hMSH3, hMSH6, BAX, Caspase-5 and TCF.[13–18] However, MSI tumors of different organs clearly show major differences in mutation frequency of these coding microsatellite-bearing genes. In addition, genes containing coding microsatellites of identical length and type display different mutation frequencies. These 2 observations strongly suggest that coding region MSI is a selective process contributing to tumor development. Since sporadic as well as hereditary colorectal MSI tumors have a better prognosis than MSS tumors,[19] it has been speculated that the accumulation of frameshift proteins and derived peptides might induce a cellular immune response that accounts for the improved survival. Interestingly, enhanced peritumoral lymphocytic infiltration in MSI tumors has been observed in several studies.[20,21] In addition, a significantly higher number of activated CTL around carcinoma cells, which is one of the histopathological features of HNPCC, has recently been reported in MSI vs. MSS tumors as revealed by CD3 immunostaining and granzyme B expression.[22] As a first step to analyze the role of frameshift peptides in host immune response against MMR-deficient cells, we have previously performed a systematic database search to identify coding microsatellite-containing genes that are affected by frameshift mutations.[23] In this study, we identify mutation-derived HLA-A2.1-restricted frameshift peptides capable of inducing expansion of T cells. These T-cell lines are shown to be activated and can lyse peptide-loaded cells in a peptide-specific manner. Most significantly, 1 peptide induced a T-cell line capable of lysing an MSI tumor cell line expressing this frameshift peptide endogenously. These data present for the first time functional evidence for the role of frameshift peptides in immune response against MMR-deficient tumor cells.

Abbreviations: CD40 Bs, CD40-activated B cells; CTL, cytotoxic T lymphocyte; ELISpot, enzyme-linked immunospot; FACS, fluorescence-activated cell sorting; FI, fluorescence index; HLA, human leukocyte antigen; HNPCC, hereditary nonpolyposis colorectal cancers; MHC, major histocompatibility complex; MMR, mismatch repair; MSI, microsatellite instability; MSS, microsatellite stability; NK, natural killer; OGT, O-GlcNAc transferase; PBMNC, peripheral blood mononuclear cell; TGFβIIR, transforming growth factor beta receptor type II.

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MATERIAL AND METHODS

Tumor cell lines

All tumor cell lines were obtained from ATCC and grown in RPMI 1640 medium supplemented with 10% FCS, 2 mmol/L L-glutamine and antibiotics. HLA-A2-expressing cell lines included SW480, HCT116, LS174T, LS180 (all colon carcinoma) and T2 cells (174xCEM.T2 hybridoma, TAP1 and TAP2 deficient). The proerythroblastic HLA-A2.1-negative cell line K562 was used as a target cell in cytotoxicity assays to test for natural killer (NK) activity. All media and supplements were from Gibco BRL (Eggenstein, Germany) unless stated otherwise.

CD40 ligand system for the culture of normal human peripheral blood B cells

The culture of CD40 ligand-activated B cells was performed as described.[24] Briefly, B cells from peripheral blood mononuclear cells (PBMNC) were stimulated via NIH/3T3 cells stably expressing human CD154 (t-CD154). Lethally irradiated t-CD154 (100 Gy) were plated on 6-well plates (0.4×10^5 cells/well) and cultured overnight. After rinsing with PBS, PBMNC were added (2×10^6 cells/ml) in Iscove's MDM (Gibco BRL) in the presence of IL-4 (2 ng/ml; R&D, Wiesbaden, Germany) and cyclosporin A (5.5×10^{-7}M), supplemented with 10% human AB serum, 5 μg/ml insulin, 50 μg/ml transferrin and 15 μg/ml gentamicin. Every 3 to 5 days, cells were transferred to new plates containing fresh irradiated t-CD154 cells.

Peptides and HLA-A2.1-binding assay

Peptides displaying HLA-A2.1-binding motifs were selected by taking advantage of specific computer programs (http://bimas.dcrt.nih.gov/molbio/hla_bind/[25] and http://134.2.96.221/scripts/MHCServer.dll/home.htm[26]). Peptides were purchased from the peptide synthesis unit of the DKFZ. Stock solutions (10 mg/ml in DMSO) were stored at −70°C and diluted to 1 mg/ml in PBS before use. T2 cells were pulsed with 50 μg/ml peptide and 5 μg/ml β2-microglobulin (Sigma, Deisenhofen, Germany) overnight at 37°C. The expression of HLA-A2.1 was then analysed by flow cytometry using MAb BB7.2 followed by incubation with FITC-conjugated (ab′)2 goat anti-mouse Ig.[27]

T-cell purification

Peripheral blood was obtained from a healthy HLA-A2.1$^+$ donor and collected in heparinized tubes. PBMNC were isolated by Ficoll-density gradient centrifugation. Whole CD3$^+$ T cells were isolated from PBMNC by magnetic depletion of non-T cells using the MACS Pan T-Cell Isolation Kit (Miltenyi, Bergisch Gladbach, Germany) according to manufacturer's instructions. Preparations contained at least 97% of CD3$^+$ cells as assessed by immunophenotypic analysis.

Induction of peptide-specific cytotoxic T lymphocytes (CTL)

CD40 Bs of a HLA-A2.1$^+$ donor were incubated with peptide (10 μg/ml) and human β2-microglobulin (3 μg/ml; Sigma) in serum-free Iscove's DMEM medium for 1 hr at room temperature, washed twice to remove excess of peptide, were irradiated (30 Gy) and added to purified CD3$^+$ autologous T cells (>97% CD3$^+$) at a ratio of 4:1 (T:CD40 Bs) in Iscove's MEM containing 10% human AB-serum, supplements (1:100) and hIL-7 (10 ng/ml, R&D). Cells were plated at a density of 2×10^6 T cells/well in 1 ml of medium. After 3 days in culture, they were fed with 1 ml complete medium. For restimulation of T cells, this was repeated weekly. IL-2 was first given at day 21 (10 IU/ml, R&D), also at day 24, and from day 28 on only hIL-2 was used instead of hIL7.

Enzyme-linked immunospot (ELISpot) assay

ELISpot assays were performed as described elsewhere.[28] Briefly, nitrocellulose 96-well plates (Multiscreen; Millipore, Bedford, MA) were covered with mouse anti-human IFN-γ MAb (Mabtech, Nacha, Sweden) and blocked with serum containing medium. Varying numbers of effector cells were plated in triplicate with 3.5×10^4 peptide-loaded T2 cells per well as targets. After incubation for 18 hr, plates were washed, incubated with biotinylated rabbit anti-human IFN-γ second antibody, washed again, incubated with streptavidin-coupled alkaline phosphatase, followed by a final wash. Spots were detected by incubation with NBT/BCIP (Sigma) for 45 min, reaction was stopped with water, then after drying spots were counted using the KS-ELISpot reader (Zeiss Kontron, Göttingen, Germany).

Chromium release assay

Standard chromium release assays were performed as described.[29,30] Tumor target cells were labeled with 100 μCi [^{51}Cr]-sodium chromate for 1 hr at 37°C. For peptide recognition, T2 cells were incubated overnight at 37°C with 5 μg/ml peptide, washed and subsequently labeled. For each experimental condition, cells were plated in V-bottomed 96-well plates with 10^3 target cells/well in triplicate. Varying numbers of CTL were added to a final volume of 200 μl and incubated for 4 hr at 37°C. Spontaneous and maximal release was determined in the presence of medium alone or of 1% NP-40. Supernatants (100 μl/well) were harvested and counted in a gamma counter. The percentage of specific lysis was calculated as follows: 100% × [experimental release − spontaneous release] ÷ [maximal release − spontaneous release].

Antigen specificity was further determined by cold target inhibition using nonradiolabeled T2 loaded with cognate peptides or with the irrelevant HLA-A2.1-binding P68-peptide as negative control. The ratio of cold to hot target was 50:1. HLA-A2.1-specificity was further determined by blocking studies using MAb BB7.2 (hybridoma supernatant) for blocking of HLA-A2, MAb W6/32 (hybridoma) for blocking of MHCI and isotype antibodies (IgG2b) as control. Cells were incubated for 30 min with 10 μg/ml of MAb before seeding in 96-well plates.

Immunofluorescence analysis

For analysis of HLA-A2 expression, $1–5 \times 10^5$ tumor cells were washed in PBS and incubated with primary MAb BB7.2 for 20 min on ice. After washing twice in PBS, cells were incubated for 20 min with the secondary FITC-conjugated goat anti-mouse IgG antibody. Cells were then washed twice and subsequently resuspended in PBS containing 1% paraformaldehyde. CD40 Bs and T cells were examined for expression of the following surface markers: CD3 (UCHT1), CD45RA (HI100), MHC I (W6/32) and MHC II (12G6) with unlabeled primary antibodies and FITC-labeled second antibody; CD4 (RPA-T4), CD8 (RPA-T8), CD19 (HIB19), CD23 (M-L233), CD45RO (UCHL1), CD50 (TU41), CD58 (1C3 AICD58.6), CD80 (BB1/B7-1), CD86 (B70/B7-2) and CD102 (B-T1; Serotec, Oxford, UK) were directly FITC conjugated, whereas CD25 (M-A251) was PE conjugated. Isotype-matched MAb was used as a negative control. All antibodies and signal detection reagents were obtained from Pharmingen (San Diego, CA), unless stated otherwise.

RESULTS

Identification of HLA-A2.1-restricted peptides

Since instability of coding microsatellites causes frameshift mutations in affected target genes and, upon expression, leads to frameshift protein sequences, we were interested in determining whether derived frameshift peptides represent potential antigens that would make MSI tumor cells particularly sensitive to CTL attack. As a first approach to identify such frameshift peptides, we used 2 publicly available computer-based peptide-motif scoring systems for predicting theoretical binding values for HLA-A2.1-restricted peptides of frameshift protein sequences corresponding to mutations in coding microsatellites of the TGFβRII gene and 15 additional genes that we had found to be unstable in mismatch repair-deficient tumor cells.[23] For 8 of these mutated genes, 16 frameshift peptides with moderate (theoretical score >10 for at least 1 program) to high (theoretical score >100 for Ken Parker's program and >20 for SYFPEITHI) HLA-A2.1 binding values

were identified (Table I). Mutations in coding microsatellites of the remaining genes either led to translational termination without generating frameshift protein sequences and thus lacking HLA-A2.1 binding motifs, or the predicted binding values obtained with both computer programs were low (theoretical score <10 for both programs). Peptides of these gene products were not further investigated. To confirm the theoretical HLA-A2.1 binding properties of the peptides on a functional basis, we performed T2 stabilization assays,[27] including the Influenca Virus matrix protein and the growth-regulated protein P68 as positive binding controls (Table I).

Induction of T cell lines using peptide-pulsed autologous CD40Bs

We next investigated whether frameshift peptides selected upon their theoretical binding scores are also capable of stimulating T-cell growth *in vitro*. CD40 Bs were generated from PBMNC of a healthy HLA-A2.1$^+$ donor using CD154-expressing fibroblasts. After 14 days, analysis of surface markers showed high levels of expression of MHC class I and II molecules, costimulatory and adhesion molecules (CD80, CD86, CD50, CD58 and CD102) and the B-cell activation markers CD19 and CD23 (data not shown), clearly demonstrating the generation of highly activated B cells, considered to be efficient antigen-presenting cells. These autologous CD40 Bs were pulsed with synthetic frameshift peptides and used to stimulate weekly autologous CD3$^+$ T cells. T cells stimulated with MP peptide-loaded CD40 Bs or unloaded CD40 Bs were used as positive and negative controls, respectively. We generated independent T-cell lines with each of the peptides listed in Table I. All cell lines showed at least a 3-fold increase in cell number by day 35, but no correlation between HLA-A2.1 binding scores of a cognate peptide and the potential to stimulate T-cell growth was observed. Two T-cell lines that had been stimulated with frameshift peptides FSP02 and FSP06 for 3 months showed more than a 200-fold and 600-fold expansion, respectively.

To quantitate antigen-specific cells in expanded T-cell cultures, the number of IFN-γ-secreting T cells after stimulation with the cognate peptides was evaluated weekly starting at day 28. As shown in Figure 1, the MP peptide was most efficient in generating IFN-γ-secreting cells compared with lack of reactive T cells in cultures stimulated with unloaded CD40 Bs. At day 35, 4 of 16 frameshift peptide-stimulated T-cell lines demonstrated IFN-γ release (Fig. 1; FSP02, FSP05, FSP06, FSP11) and 2 weeks later, 3 of these 7 T-cell lines still remained reactive containing up to 11% IFN-γ-releasing cells (data not shown). These 3 T-cell lines had been stimulated with frameshift peptides FSP02, FSP06 and FSP11, respectively, and were chosen for further analysis.

To characterize the phenotype of proliferating T cells, expression of surface markers was analyzed by flow cytometry (data not shown). There were no significant differences between the 3 T-cell lines analyzed; almost all cells were CD3$^+$, consisting of about 10% of CD4- and about 90% of CD8-expressing cells. The majority of T cells ($>80\%$) expressed the activation markers CD25 and CD69 and displayed an activated or memory phenotype, as judged by the expression of CD45RO. Only a small number of cells ($<20\%$) remained in a naive state, as judged by the expression of CD45RA. These flow cytometry data together with the ELISpot results demonstrate that frameshift peptides are capable of stimulating highly activated CD8$^+$ T cells when presented in an immunogenic manner.

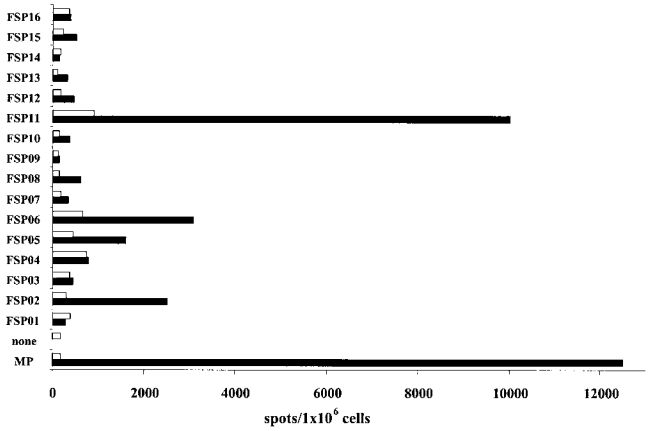

FIGURE 1 – ELISpot analysis of FSP and control T-cell lines after 4 rounds of restimulation (day 35). Titrated amounts of T cells were incubated overnight with 3.5×10^4 peptide-loaded T2 cells per well. The number of IFN-γ-releasing activated T cells (spots) among the total number of cells analyzed (10^6) is depicted for each frameshift peptide (black bars). Reactivity against peptide YLLPAIVHI from the nuclear protein P68 served as a negative control and is indicated (open bars).

TABLE I – FRAMESHIFT AND CONTROL PEPTIDES USED IN THIS STUDY

Protein	Accession number[1]	Name	Peptide[2]	Theoretical scores[3]		Fluorescence index[4]
				Ken Parker	SYFPEITHI	
Influenza matrix protein	AAA43682	MP	^{57}-GILGFVFTL	408	30	0.93
Growth-regulated protein P68	226021	P68	^{128}-YLLPAIVHI	551	30	2.96
TGF-betaRII	AAA61164	FSP01	^{128}-SLVRLSSCV	70	23	0.01
TGF-betaRII	AAA61164	FSP02	^{131}-RLSSCVPVA	5	19	0.65
TGF-betaRII	AAA61164	FSP03	^{135}-CVPVALMSA	1	14	0.80
HPDMPK	CAA71862	FSP04	^{136}-LLHSAPTPSL	36	25	0.82
HPDMPK	CAA71862	FSP05	^{129}-FLSASHFLL	570	21	0.43
HPDMPK	CAA71862	FSP07	^{125}-RVFFFYQHL	39	15	0.33
OGT	AAB63466	FSP06	^{128}-SLYKFSPFPL	397	23	0.47
D070	BAA11534	FSP08	^{35}-KIFTFFFQL	1593	21	0.80
D070	BAA11534	FSP09	^{68}-ALLPAGPLT	28	21	0.21
D070	BAA11534	FSP10	^{69}-LLPAGPLTQT	29	20	0.52
U79260	AAB50206	FSP11	^{59}-TLSPGWSAV	118	25	0.98
U79260	AAB50206	FSP12	^{83}-ILLPQPPEWL	362	26	2.32
Sec63	AAC83375	FSP13	^{551}-RQMESLGMKL	33	15	0.63
MAC30X	AAA16188	FSP14	^{198}-VEMPTGWLL	20	14	0.28
MAC30X	AAA16188	FSP15	^{198}-VEMPTGWLLV	14	15	0.34
FLT3L	U29874	FSP16	^{113}-FQPPPAVFA	13	10	1.43

[1]Protein or nucleotide accession numbers are indicated.–[2]Position of the start amino acid in the protein is indicated.–[3]Predicted binding scores to HLA-A2.1 using computer-assisted analysis.–[4](Mean fluorescence with peptide − mean fluorescence without peptide)/(mean fluorescence without peptide). Results are representative of 2 experiments.

Recognition of target cells is peptide specific and HLA-A2.1-restricted

To test whether these activated $CD8^+$ T cells represent CTL and are capable of recognizing specific peptides, cytotoxicity assays were performed using peptide loaded T2 cells. As shown in Figure 2, established CTL lines lysed frameshift peptide-loaded T2 target cells to a higher percentage than T2 cells presenting the control peptide P68. It should be noted that all 3 frameshift peptide-stimulated CTL lines examined showed only little background lysis, and specific lysis was comparable to that achieved with CTL raised against the positive control peptide MP. Since these CTL failed to lyse the proerythroblastic HLA-A2.1-negative cell line K562, NK activity could be excluded (data not shown). Antigen specificity of lysis was further determined in a cold target inhibition experiment. As depicted in Figure 3*a*, lysis of FSP02-coated T2 cells could be completely inhibited in the presence of unlabeled FSP02-loaded T2 targets. Lysis was not affected by cold T2 cells coated with irrelevant peptide P68 in comparison with the positive control, *i.e.*, lytic activity without cold target blocking. These data suggest antigen specificity and MHC restriction of the cytotoxic activity (FSP02 shown). To examine whether target cell lysis occurs in a HLA-A2.1-restricted manner, blocking experiments were performed using monoclonal anti-HLA-A2 and anti-MHCI antibodies. Incubation of peptide-pulsed T2 cells with either anti-HLA-A2 or anti-MHC I antibodies resulted in about 50% inhibition of target cell lysis, whereas the isotype control had no effect (data not shown). Taken together, these results clearly demonstrate antigen specificity and HLA-A2.1-restriction of the CTL lines raised against frameshift peptides.

Lysis of a MSI colon carcinoma cell line

Finally, we analyzed the capacity of the CTL lines to lyse tumor cells expressing endogenously processed frameshift proteins and peptides derived thereof. Among the MSI colon carcinoma cell lines available to us, HCT116 was recently found to express TGFβRII with a (−1) shift[31] and U79260 with a (−4) shift.[23] This cell line was chosen as target to examine reactivity of the CTL lines that had been raised against frameshift peptides FSP11 (derived from U79260) and FSP02 (TGFβRII). In addition, the 2 MSI colon carcinoma cell lines LS174T and LS180 expressing a (−1) shift in the coding microsatellite of O-GlcNAc transferase (OGT) were used as targets for the FSP06- (derived from OGT) stimulated CTL effector line. As a negative control, the MSS colon carcinoma cell line SW480 was included in the analysis. Although all 4 colon carcinoma cell lines have been reported to express HLA-A2.1, reexamination by flow cytometry revealed high levels of HLA-A2.1 expression on SW480 and HCT116 cells but no or only low expression on LS174T and LS180, even after treatment with IFN-γ. In the cytotoxicity experiments performed, the CTL line raised against FSP11 failed to lyse HCT116 cells both in the presence or absence of IFN-γ (data not shown). However, the FSP02-specific CTL line showed significant lytic activity against the HCT116 target (Figs. 3*b*, 4*b*). This lysis could be blocked by adding cold T2 targets coated with peptide FSP02 (Fig. 3*b*), thus excluding alloreactivity and demonstrating tumor cell specificity

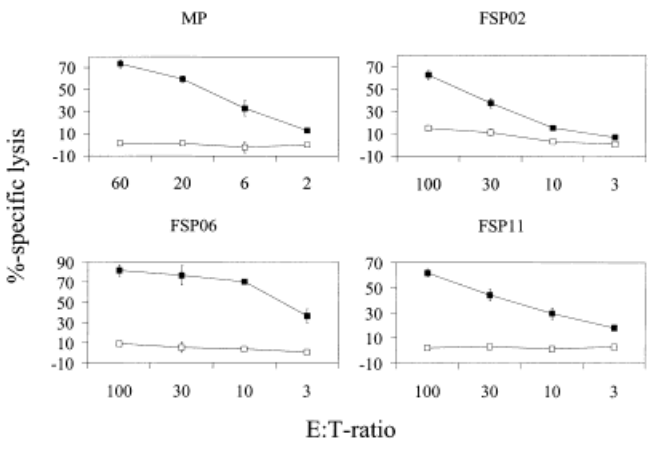

FIGURE 2 – Cytotoxic activity of CTL lines after 4 to 6 rounds of restimulation with the peptides MP, FSP02, FSP06 and FSP11. T2 cells were incubated overnight with the cognate peptides or the control peptide P68 and labeled with [^{51}Cr]-sodium chromate for 1 hr. Killing of target cells at different effector to target cell ratios (E:T) is shown for the control (open squares) and cognate peptides (closed squares). Results are displayed as the mean and standard deviation from 3 replicate wells.

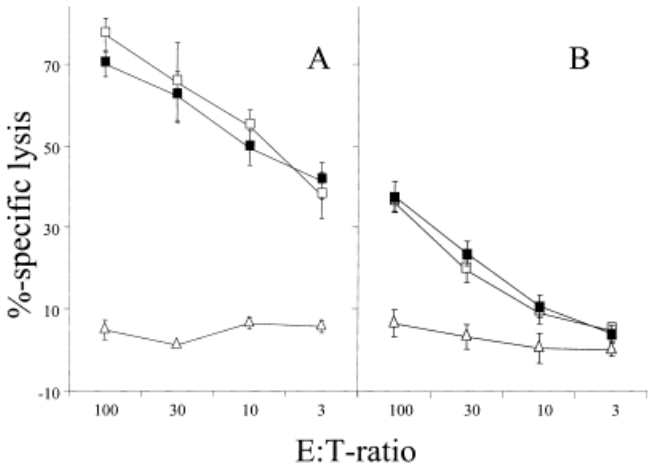

FIGURE 3 – Frameshift peptide-specific and HLA-A2-restricted lysis of target cells. The antigen specificity of the FSP02 CTL line was tested in the presence of unlabeled cold targets, T2 cells pulsed either with FSP02 (open triangles) or with an irrelevant peptide (P68, open squares) at an inhibitor:target ratio of 50:1. Lysis without cold targets is shown as a control (closed squares). *(a)* Reactivity against FSP02 peptide loaded, labeled T2 targets. *(b)* Reactivity against HCT116 tumor cell targets. All data are shown as the mean and standard deviation from 3 replicate wells.

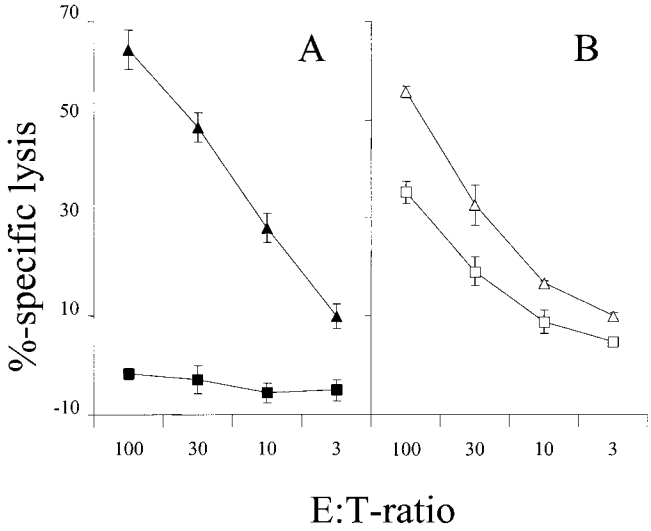

FIGURE 4 – The FSP02 CTL bulk culture was tested against HLA A2.1-positive colon carcinoma cell lines in the presence (triangles) or absence (squares) of exogenously added peptide FSP02. *(a)* Reactivity against MSS SW480 cells. *(b)* Lytic activity against MSI HCT116 target cells. Results are shown as the mean and standard deviation from 3 replicate wells.

of the FSP02 CTL line. To examine whether tumor cell lines show different susceptibilities to CTL lysis, we compared CTL-mediated lysis of HCT116 and SW480 in the presence and absence of exogenously added peptide FSP02 (Fig. 4). In contrast to HCT116, unloaded SW480 cells remained refractory to CTL attack. Peptide loading caused a high level of SW480 tumor cell lysis but only moderately increased HCT116 lysis. The lysis of HCT116 cells, expressing the (−1) frameshift variant of TGFβRII clearly demonstrates that frameshift peptides can promote specific T-cell priming and expansion, thereby generating CTL, capable of lysing frameshift protein expressing tumor cells.

DISCUSSION

In our study, we aimed to test the ability of frameshift peptides derived from mutated and expressed microsatellites to induce productive T-cell response. In our *in vitro* system, we used CD40-activated B cells as antigen-presenting cells to generate autologous peptide-specific T cells.[24,27] These T cells conferred MHC class I-specificity as well as lysis of peptide-coated target cells. One particular T-cell line also lysed a MSI colon carcinoma cell line, endogenously expressing the cognate antigen.

MSI colorectal carcinomas have better survival rates,[10,19,32] higher numbers of infiltrating cytotoxic lymphocytes within neoplastic epithelial structures[21] and higher percentage of tumor cells undergoing apoptotic cell death. These findings are consistent with the presence of antitumor cytotoxic immune responses.[22] Reactivity of T cells against tumors is mediated by recognition of tumor-specific antigens. Several groups of tumor-specific antigens have been described including shared antigens (MAGE family), tissue-specific differentiation antigens (gp100, tyrosinase, MelanA/Mart, CEA), overexpressed self-proteins (Her2/neu, wild-type p53, hTERT), viral gene products (HPV E6 and E7, EBNA-1) and novel antigens resulting from mutated proteins (CDK4, β-catenin, p53, p21/ras, bcr-abl).[33,34] With respect to tumor biology, this latter group is generally considered as the most interesting one, since the resulting mutant proteins are unique to tumor cells and directly contribute to tumorigenesis. Moreover, mutation-derived epitopes should be foreign to the immune system without encountering pre-existing tolerance. In the past, T-cell-defined antigenic epitopes have been identified for those mutation-derived antigens that arise by point mutations or translocations.[1–3] Frameshift mutations affecting coding microsatellite-containing genes in MMR-deficient tumor cells have the potential to generate an even higher antigenicity since in the majority of cases the whole frameshifted part of the amino-acid sequence can serve as antigenic determinant. T-cell epitopes resulting from frameshifts that are derived from alternative translation initiation sites have also been described.[4–6] Our study for the first time not only identified T-cell epitopes derived from frameshift mutations affecting coding microsatellites but also clearly demonstrated the immunogenic potential of the TGFβRII (−1) frameshift-derived peptide RLSSCVPVA (FSP02). It remains to be investigated whether the suggested immunogenic potential of frameshift peptides applies as a general rule to all MSI tumors of different organs and is causally related to the known histopathologic features, although for colorectal and endometrial tumors, such tumor-associated lymphocytes have been described.[20,21] However, no association between MSI status and intratumoral lymphocyte infiltration has been found in gastric carcinomas.[35] Although our data were obtained from a small number of frameshift peptides and mutated coding microsatellite containing genes, the overall number of such target genes in MSI tumor cells must be enormous and should provide a unique and novel source of tumor-specific antigens. In fact, preliminary data from our lab indicate that several hundred to thousand coding mononucleotide repeats exist in the human genome and at least some of them are affected by microsatellite instability.[23]

Our results also revealed that not all candidate frameshift peptides were capable of activating specific T cells. Three of our candidate peptides (18%) induced antigen-specific CTL lines that conferred lysis of peptide-pulsed target cells in a HLA-A2.1-restricted manner. Most significantly, one particular frameshift peptide, FSP02, caused by a frameshift mutation in the (A)10 coding microsatellite of the TGFβRII gene known to be mutated in the majority of MSI colorectal tumors[36] induced CTL that were also capable of lysing the colorectal cancer cell line HCT116. The lysis was HLA-A2.1-restricted and tumor cell-specific because it could be blocked by competition with cold targets and no reactivity was seen against the MSS colon carcinoma cell line SW480 unless exogenous peptide FSP02 was added. Although not demonstrated by our experiments, transcription and translation of the mutant mRNA as well as endogenous processing and presentation of this particular peptide must have occurred in order to allow T-cell-mediated tumor cell lysis because this cell line only contains the mutant TGFβRII allele. Previous studies of this mutated TGFβR gene showed that it was highly repressed in MSI cell lines such as HCT116, as analyzed by RNase protection assay,[37] casting some doubts on the translation of the corresponding frameshift mutant protein. However, other investigators have demonstrated expression of endogenous TGFβRII in this cell line using similar methodology,[31] which is consistent with our immunological data. It is important to note that specific lysis of HCT116 tumor cells could slightly be increased by adding the cognate peptide exogenous. It is conceivable that low level of endogenous peptide is presented in a HLA-A2.1-dependent manner and might account for this observation. In contrast to FSP02, the frameshift peptide FSP06-stimulated CTL line failed to lyse MSI tumor cells carrying the corresponding frameshift mutation due to loss of HLA-A2.1-expression on these MSI cells. In the case of the FSP11-stimulated CTL line, one explanation for loss of tumor cell lysis might be due to the failure of the antigen-processing machinery unable to generate all predicted peptides.[38,39] Alternatively, these CTL might be of low avidity and do not recognize small amounts of peptide naturally presented by tumor cells.

Immune surveillance of MSI tumor cells by T cells must represent a major challenge to the host immune system. Although our data provide strong evidence for the concept of T-cell-mediated immune response towards frameshift peptide-presenting tumor cells, natural selection as the driving force of tumor evolution has to be considered.[40] Continuous exposure to frameshift peptides would be expected to concomitantly induce a strong and specific cellular immune response. As a consequence of this selection pressure, mutations in genes of the antigen processing or presenting machinery should arise in MMR-deficient tumor cells. In fact, evasion of immune surveillance by acquiring β2-microglobulin mutations has been observed at high frequency in MSI tumor cells.[41] Other possible targets of specific mutation or down-regulation of expression are TAP1/TAP2 or HLA alleles. Direct down-regulation of expression of immunogenic epitopes is also a possible mechanism of immune escape as has been shown for melanoma-associated antigens *in vivo*.[42] However, gene mutation-mediated growth advantage is essential to and is selected for specific stages of tumor development, and cells that express these aberrant proteins always will be vulnerable to CTL attack. Even if immune escape would be directed towards single-peptide epitopes, a combinatorial vaccination approach including several antigenic peptides should overcome this obstacle. The success of such a strategy depends on the identification of frameshift peptide patterns capable of mediating a strong immune response. A frameshift peptide-directed vaccination approach not only could offer new treatment modalities for existing MSI tumors but also might benefit asymptomatic at-risk individuals in HNPCC families by a prophylactic vaccination strategy. Since in our study precursor T cells recognizing frameshift peptides are shown to exist in peripheral blood of healthy donor, further studies are required to verify these findings on PBMNC and tumor infiltrating lymphocytes in patients with MSI tumors.

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