Cancer Immunol Immunother (1998) 47:21–31 © Springer-Verlag 1998

ORIGINAL ARTICLE

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Induction of HLA-unrestricted and HLA-class-II-restricted cytotoxic T lymphocytes against MUC-1 from patients with colorectal carcinomas using recombinant MUC-1 vaccinia virus

Received: 22 October 1997 / Accepted: 24 April 1998

Abstract We recently reported that immunization with a recombinant MUC-1 vaccinia virus (rVMUC-1) protected C57BL/6 mice from challenge with DF3/MUC-1-positive syngeneic tumors. To elucidate whether anti-MUC-1 tumor immunity, especially MUC-1-specific cytotoxic T lymphocytes (CTI), can be induced in cancer patients by rVMUC-1, we stimulated the peripheral blood lymphocytes from patients with DF3/MUC-1$^+$ or DF3/MUC-1 colon carcinomas using the autologous monocytes infected with rVMUC-1 (rVAMN). The stimulated T lymphocytes from two patients with DF3/MUC-1-positive colorectal carcinomas (rVPY + T and rVPW + T) demonstrated HLA-unrestricted cytotoxicity against MUC-1, whereas those from the patient with DF3/MUC-1-negative colon carcinoma (rVPA-T) did not. The HLA-unrestricted cytotoxicity was demonstrated by the CD8$^+$ T cells possibly recognizing an epitope present on the tandem repeats. Adoptive immunotherapy who performed three times with patient PY, at 4-week intervals. The adoptive transfer of the first stimulated lymphocytes, demonstrating a high level of HLA-unrestricted cytotoxicity against MUC-1, resulted in the significant reduction of the liver metastasis of patient PY. However, HLA-unrestricted cytotoxicity against MUC-1 was extremely reduced at the second transfer and finally eliminated at the third, whereas the CD4$^+$ T cells demonstrating HLA-class-II-restricted cytotoxicity against MUC-1 predominantly proliferated at the third adoptive immunotherapy treatment. The liver metastasis and the serum levels of tumor markers (carcinoembryonic antigen CA19-9) demonstrated a rapid and marked increment after the second transfer and especially after the third. These results suggest that the HLA-unrestricted cytotoxic CD8$^+$ T cells against MUC-1, induced in patients with DF3/MUC-1$^+$ colorectal carcinomas using rVMUC-1, correlate with the antitumor activity in vivo.

Key words DF3/MUC-1 · Vaccinia virus · Immunotherapy · CTL · Colon cancer

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Introduction

MUC-1 is a type I transmembrane glycoprotein, consisting mainly of repeats of 20 amino acids, that contains five *O*-linked glycosylation sites: serine and threonine [14, 23]. MUC-1 protein core is normally hindered by a large number of carbohydrate side-chains that are predominantly attached to the molecule by *O*-glycosidic linkages to serine or threonine residues. Following malignant transformation of ductal epithelial cells, MUC-1 glycoprotein is underglycosylated to express MUC-1-related tumor-associated antigens (TAA) such as DF3, sialyl-Lewis^a (CA19-9), and sialyl-Lewis^X [4, 5, 8, 16]. These TAA, possibly recognized as cryptic epitopes by an immunosurveillance system, are found to elicit a humoral and cellular immune response in cancer patients [6, 11, 19, 20, 22]. Finn and co-workers [6, 20] reported that MUC-1-specific cytotoxic T lymphocytes (CTL) were induced from tumor-infiltrating T lymphocytes of mammary and pancreatic cancer patients, that recognized one of the MUC-1-related cryptic peptide epitopes, SM-3, present on tandem repeats of the MUC-1 molecule and lysed SM-3-expressing cells in an HLA-unrestricted manner. One of the MUC-1-related cryptic epitopes, DF3/MUC-1, was preferentially expressed on primary colon adenocarcinoma cells [3], and previous studies using a combination of DF3 and a related mAb, 115D8 (CA153), also demonstrated elevated serum levels in 61% of patients with colorectal carcinomas [15]. Thus, it is conceivable that DF3/MUC-1 may elicit an immune response in colorectal cancer patients. However, there are, to our knowledge, no reports of evidence

of induction of MUC-1-reactive CTL from patients with colorectal carcinomas.

Several reports have revealed the efficacy of recombinant vaccinia viruses in generating effective immune responses against the inserted TAA, such as human melanoma-associated antigen p97 [18] and carcinoembryonic antigen (CEA) [21]. The tumor cells expressing those antigens were rejected, when mice immunized with these recombinant vaccinia viruses were challenged. The rejection of tumor expressing TAA, seen in animals immunized with recombinant vaccinia virus, was attributed to the TAA-specific CTL induced by these recombinant viruses. Recombinant MUC-1 vaccinia virus was also constructed and the mice immunized with recombinant MUC-1 vaccinia virus showed an ability to inhibit the growth of murine syngeneic tumors bearing MUC-1 antigen, although the existence of CTL directed against MUC-1 antigen was not proven [1]. We recently reported that vaccination of C57BL/6 mice with rVMUC-1 rendered them resistant to the growth of subsequently transplanted DF3/MUC-1^+ syngeneic tumors and elicited a cell-mediated anti-MUC-1 immune response [2]. This animal model indicates the possibility of immunotherapy targeting DF3/MUC-1, using recombinant MUC-1 vaccinia virus.

In this study, we attempted to elucidate whether MUC-1-specific CTL can be efficiently induced from the PBL (peripheral blood lymphocytes) of patients with DF3/MUC-1^+ or DF3/MUC-1^- tumors by rVMUC-1. The PBL from the patients with colorectal carcinomas were stimulated in vitro with autologous monocytes infected with rVMUC-1, and the cytotoxicity of the stimulated T lymphocytes against MUC-1 was analyzed, using MUC-1^+ autologous and allogeneic cells as target cells.

Materials and methods

Cells

Human pancreatic cancer cell lines (CAPAN-1 and PANC-1) and a human colon cancer cell line (HT-29) were maintained in RPMI-1640 medium supplemented with 200 mg/l ampicillin, 100 mg/l kanamycin, and 10% fetal bovine serum (FBS).

The amphotropic packaging cell line PA317 and murine colon adenocarcinoma cell line MC-38 were obtained from Dr. Jeffrey Schlom (National Cancer Institute, NIH, Bethesda, Md.). BS-C-1, an African green monkey kidney cell line, was used for propagation of wild-type and recombinant vaccinia virus. These cell lines were maintained in RPMI-1640 medium with 10% FBS, 2 mM glutamine, 0.1 mM nonessential amino acids and 0.1 mM sodium pyruvate.

Ab

DF3 mAb was obtained from Donald kufe (Dana-Farber Cancer Institute, Boston, Mass.). Rabbit polyclonal antibody against NAB-1 peptide epitope, 20 amino acid peptides of the MUC-1 molecule containing cytokine-like sequences [26], present upstream from the transmembrane region of the molecule, was made for detecting NR-MUC-1 protein, i.e. MUC-1 protein devoid of the tandemly repeated region, and designated as pAb NAB-1. Briefly, rabbits were given subcutaneous injections of 0.4 mg keyhole-limpet hemocyanin-conjugated NAB-1 peptide (VHDVETQFN-QYKTEAASRYNLTISDVSV) in Freund's complete adjuvant (Wako, Osaka, Japan), followed by a booster injection (0.2 mg NAB-1 peptide) in Freund's incomplete adjuvant (Wako, Osaka, Japan) four times at 2-week intervals. The serum from the immunized rabbits was collected and used as the polyclonal antibody NAB-1. Mouse IgG1, W6/332 (anti-HLA class I), CR3/43 (anti-HLA-DR, DQ, DP), anti-human CD4, and anti-human CD8 mAb were obtained from Dako (Dako Japan K. K., Kyoto, Japan). Anti-(human CD14), anti-(human CD33), and anti-(human HLA class II) (HLA-DR, -DQ, and -DP) were obtained from Beckton Dickinson Co. Ltd. (Mountain View, Calif. and, together with anti-(human CD80) and anti-(human CD86) mAb from Ancell (Ancell Corporation, Bayport, USA), were used in flow cytometry for characterization of the autologous monocytes derived from patients with colorectal carcinomas.

Immunohistochemistry

The avidin-biotin-peroxidase complex (ABC) method [17] was employed, using the Vectastain ABC kit (Vector Laboratories Inc., Burlingame, Calif). Briefly, sections were deparaffinized in xylene and redehydrated in graded solutions of ethanol. After the endogenous peroxidase activity had been quenched in absolute methanol containing 0.3% (w/v) hydrogen peroxide for 30 min, nonspecific binding was blocked by treatment with 1.5% (v/v) normal horse serum (Vector) for 30 min. Primary DF3 mAb were applied to the sections and each specimen was incubated in a moist chamber for 2 h at room temperature. After the sections had been washed three times in 0.05 mol/l phosphate-buffered saline (PBS), biotinylated anti-(mouse Ig) (Vector) was applied at a dilution of 1:200. The sections were again incubated for 50 min at room temperature. Freshly prepared ABC reagent (Vector) was applied and incubated for 60 min following three washes in PBS. The localization of the DF3 epitope was visualized by incubating the sections for 5 min in freshly prepared 0.05 mol/l TRIS HCl (pH 7.6) containing both 0.02% (w/v) 3,3-diaminobenzidine tetrahydrochloride (Nacalai Tesque Inc., Kyoto, Japan) and 0.03% (w/v) hydrogen peroxide.

Transfection and transduction of the R-MUC-1 and the NR-MUC-1 genes in pLNSX

The 2-kbp (2×10^3 base pairs) MUC-1 cDNA containing ten tandem repeats (R-MUC-1) was constructed and inserted into the retroviral vector pLNSX (PLNSX-R-MUC-1) as described before [2]. PLNSX-R-MUC-1 contains signal sequences, ten tandem repeats of the MUC-1 gene, and 3′ unique sequences downstream from tandem repeats containing the transmembrane region.

To construct MUC-1 cDNA devoid of tandem repeats of MUC-1 (NR-MUC-1), a fragment of MUC-1-Y DNA [27] was produced by the polymerase chain reaction and inserted into retroviral vector pLNSX. This was designated as PLNSX-NR-MUC-1 and contained signal sequences, no tandem repeats, and a transmembrane region.

The PLNSX-R-MUC-1 or the PLNSX-NR-MUC-1 genes were transfected into packaging cell line PA317, using Lipofectin (Gibco BRL, Gaithersburg MD) according to the manufacturer's instructions. The cells were then harvested, spread into 60-mm dishes, and incubated with 200–500 μg/ml G418 for 3 weeks. Clones of PA317 cells containing the R-MUC-1 or NR-MUC-1 gene were identified by Northern blotting and Western blotting analysis using mAb DF3 and polyclonal antibody NAB-1. The retrovirus-containing supernatants of R-MUC-1-transfected PA317 cells were collected and used to transduce MC-38 cells and HT-29 cells (R-MC-38 and R-HT-29 respectively). The NR-MUC-1-transduced MC-38 cells were designated as NR-MC-38. Following transduction, MC-38 cells and HT-29 cells were cloned by isolating a single colony. Clones of MUC-1-transduced MC-38 and HT-29 cells were

identified by Western blotting and FACS analysis, using mAb DF3 and pAb NAB-1.

PBL from patients PA and PY were Epstein-Barr (EB)-virus-immortalized using supernatant from the marmoset cell line MCUV5 (M-PA-EB and M-PY-EB, respectively). The autologous EB-virus-immortalized B cells from patients PA and PY were transduced with the retrovirus-containing supernatants of R-MUC-1-transfected PA317 cells and designated as M + PA-EB and M + PY-EB respectively. Since flow cytometry showed that M + PA-EB and M + PY-EB cells expressed both DF3 and NAB-1 epitopes (data not shown), they were used as autologous target cells expressing MUC-1-related epitopes for ^{51}Cr-release assay.

Recombinant MUC-1 vaccinia virus (rVMUC-1)

Recombinant MUC-1 vaccinia virus (rVMUC-1), containing R-MUC-1 cDNA containing ten tandem repeats, was generated as described before [12]. Briefly, the modified gene encoding R-MUC-1 cDNA was inserted into a plasmid vector for the insertion of foreign genes into vaccinia virus. The resulting plasmid contained the R-MUC-1 gene under the control of the vaccinia virus 40K early/late promoter flanked by DNA sequences including the vaccinia K1L host-range gene required for growth of vaccinia virus on rabbit kidney RK13 cells. A derivative of the Wyeth strain of vaccinia that was used as the parental virus lacked a functional K1L gene and thus could not efficiently replicate on RK13 cells. The recombinant vaccinia virus generated via homologous recombination was selected by growth on RK13 cells.

FACS analysis

To determine the cell-surface phenotype of CTL and to detect MUC-1 protein on cultured cells, indirect immunofluorescence assays were performed as described before [24]. Briefly, cells were incubated with saturating amounts of mAb for 30 min on ice, then washed twice with PBS and incubated with fluorescein-labeled goat anti-(mouse IgG) (Kirkegaard and Perry, Gaithersburg, Md.) or fluorescein-labeled goat anti-(rabbit IgG) (Kirkegaard and Perry, Gaithersburg, Md.) for 30 min on ice. After two washes with PBS, cells were analyzed by a FACScan flow cytometer (Becton-Dickinson Co. Ltd., Mountain View, Calif.).

In vitro stimulation of PBL from patients with colorectal carcinomas

Mononuclear cells were isolated from the peripheral blood of patients with colorectal carcinomas using the blood cell separator, the Haemonetics V50 (Haemonetics Corporation, Braintree, Mass.) and purified with lymphocyte separation medium (ICN Biomedical Inc., Costa Mesa, Calif.) These cells were collected in a flask and incubated for several hours. The adherent monocytes were infected at a multiplicity of infection of 1–2 with rVMUC-1 and incubated for 12 h to express MUC-1-related epitopes. The infected cells were washed three times with RPMI-1640 medium supplemented with 10% FFP (fresh frozen plasma), and UV-irradiated by exposure to a UV-light source for 10 min in the presence of psoralin to inactivate the vaccinia virus. A portion of the infected cells were added to BSC-1 cells and, after 12 h incubation, the number of vaccinia viral plaques formed on BSC-1 cells were counted, to confirm whether vaccinia virus was completely inactivated. However, plaques were not observed at every stimulation. The infected cells were then incubated with 100 μg/ml mitomycin C (Kyowa Hakko, Tokyo, Japan) for 20 min at 37 °C and washed three times with RPMI-1640 medium containing 10% FFP. The lymphocytes were cocultured with the rVMUC-1-infected monocytes at a lymphocyte-to-stimulator ratio of 50–100:1 for 5 days without interleukin-2 (IL-2), and then incubated for 7 more days with CP-2 medium containing 100 U/ml IL-2 (Nikken Biomedical Lab., Kyoto, Japan).

For adoptive immunotherapy, the stimulated lymphocytes [$(0.5–1.0) \times 10^8$ cells] described above, after three washes with physiological saline (Ohtsuka pharmacology Co., Osaka, Japan), were suspended in 50 ml physiological saline containing 350 000 JRU (Japan Reference Unit) (Shionogi Pharmacology Co., Osaka, Japan). Patient PY had the catheter inserted percutaneously through the femoral artery, and the stimulated lymphocytes were administered into the proper hepatic artery through the catheter. The blood pressure and body temperature of the patient were monitored for 24 h after the adoptive immunotherapy. However, no significant side-effects, except a slightly high temperature (37–38 °C), were observed. Patient PY received adoptive immunotherapy three times at 4-week intervals, and the mononuclear cells were isolated from the peripheral blood of patient PY at every adoptive immunotherapy treatment (AI). To evaluate the efficiency of the AI, the size of the liver metastasis present on the anterior superior segment (S8) of the liver was measured in two dimensions by computer tomography (CT) 1–2 weeks after administration of the stimulated lymphocytes, and the volume was calculated by the following formula: $\text{width}^2 \times \text{length}/2$. Serum levels of CEA and CA19-9 were measured 1 week before and 1 week after AI. T lymphocytes, purified from these stimulated lymphocytes by immunomagnetic beads coated with anti-CD3 mAb (Nihon Dynal K.K., Tokyo, Japan), were tested for cytotoxicity, and the stimulated lymphocytes were characterized by flow cytometry at every AI.

CTL assays

The stimulated lymphocytes were tested for cytolytic activity as described before [21]. Briefly, target cells were labeled for 1 h by incubating 1×10^6 target cells in 100 μCi sodium [^{51}Cr]chromate (NEN Research Products, Boston, Mass.) at 37 °C. Cells were washed three times in RPMI-1640 medium supplemented with 10% FCS and dispensed into wells of 96-well round-bottom plates (1×10^4 cells/well) (Falcon 3077, Oxward, Calif.). All assays were set up in triplicate for each E:T ratio tested, with 200 μl/well. After centrifugation of the plates at 800 g for 5 min, the cells were incubated for 6 h at 37 °C. Supernatants were harvested in a supernatant collection system (Skatron, Sterling, Va.) and assayed in a gamma counter. The percentage specific lysis was calculated according to the following formula: [(mean ^{51}Cr release in experimental samples − mean spontaneous ^{51}Cr release)/(mean maximal ^{51}Cr release−mean spontaneous ^{51}Cr release)] × 100. Spontaneous release was measured by incubating target cells in medium alone and maximum release was obtained by adding 1% Triton X-100 in PBS to target cells.

Antibody-blocking studies were performed by incubating either target cells or effector cells, as indicated, with monoclonal antibody for 45 min at 37 °C prior to their use in a cytotoxicity assay.

Statistical analysis

Significant differences were determined using the paired or unpaired Student's *t*-test. Any *P* value below 0.05 was considered to be statistically significant.

Results

Expression of DF3/MUC-1 on the resected specimen of patients with colorectal carcinomas

The immunohistochemical localization of DF3/MUC-1 epitope in the resected specimens derived from patients with colorectal carcinomas (patients PA, PW and PY), was investigated using mAb DF3. Figure 1 shows that,

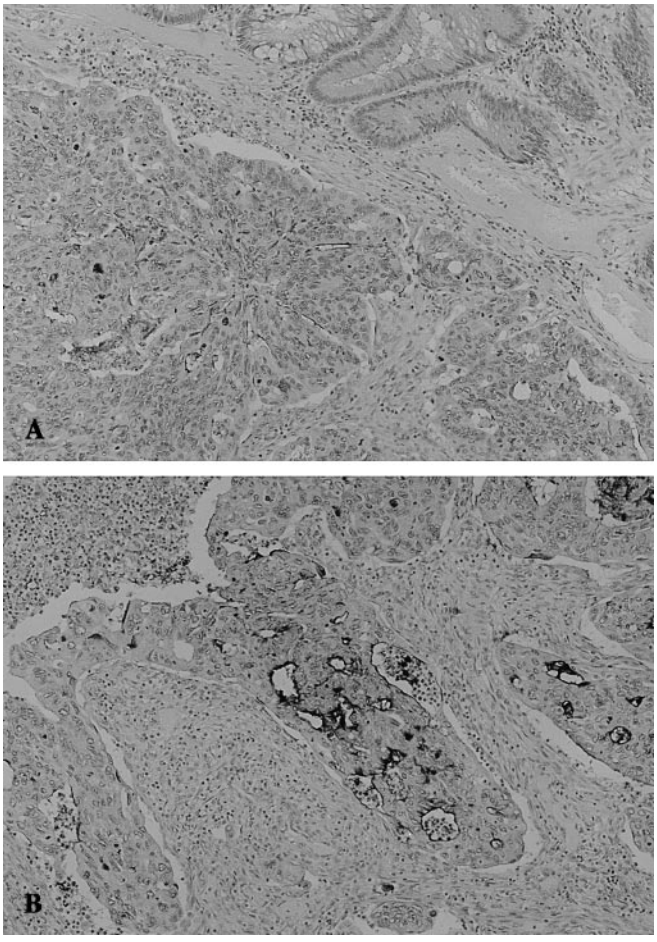


Fig. 1A, B Immunohistochemical staining with mAb DF3 of human colon carcinoma tissue sections. The sections were taken from surgical specimens from patient PW (**A** original magnification × 40) and patient PY (**B** original magnification × 200). The normal colonic epithelial tissue adjacent to malignant colonic tissue is negative for mAb DF3. The membranes of glandular lumens and the luminal secretions were immunoreactive to mAb DF3

in the primary tumor of patient PW (Fig. 1A) and patient PY (Fig. 1B), the surface of the carcinoma cells forming glands was positive for mAb DF3 and that the glands surrounded by carcinoma cells were filled with a DF3-positive excretion, although normal intestinal mucosa adjacent to carcinoma cells was not stained by mAb DF3. Thus, the DF3/MUC-1 epitope was expressed preferentially on malignant cells of the primary tumor of patients PY and PW (Fig. 1), but not detected on those of patient PA (data not shown).

Autologous monocytes infected with rVMUC-1 as APC

The phenotype of the adherent cells isolated from peripheral blood of patients with colorectal carcinomas was analyzed by flow cytometry. The cells expressed high levels of CD14, CD33, CD86, HLA class I (HLA-ABC), and HLA class II (HLA-DR, -DP, and -DQ), but a very low level of CD80 (Fig. 2A), which is characteristic of macrophages. The expression of a panel of markers on the monocytes was not significantly influenced by infection with rVMUC-1 (Fig. 2A).

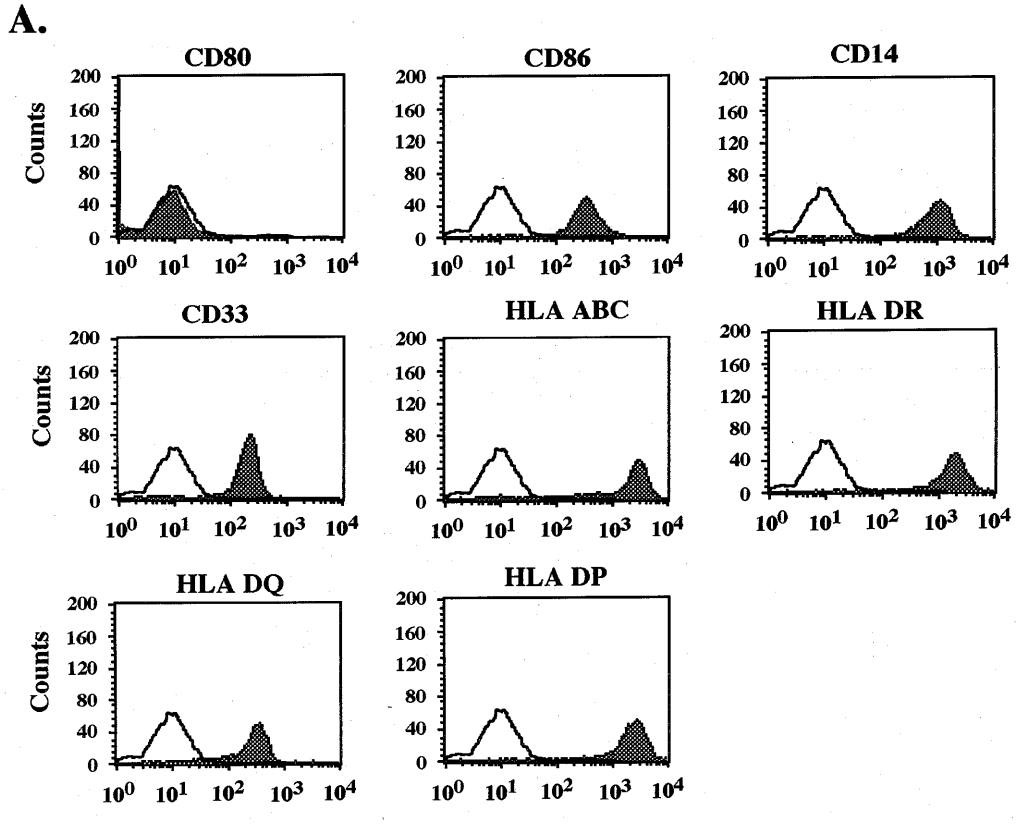
The monocytes infected with rVMUC-1 expressed high levels of MUC-1-related epitopes, DF3 and NAB-1, consisting of 20-amino-acid sequences with the predicted cytokine-receptor-like sequences [26] (Fig. 2B).

Induction of MUC-1-specific CTL

The PBL from patients PY and PW with DF3/MUC-1-positive colorectal carcinomas and from patient PA with DF3/MUC-1-negative colon carcinoma were stimulated with autologous monocytes infected with rVMUC-1 (rVAMN) for 12 days, as described in Materials and methods. T lymphocytes were isolated from the stimulated PBL of patients PA, PW, and PY, by immunomagnetic beads coated with an anti-CD3 mAb (rVPA-T, the rVPW + T, and the rVPY + T respectively), and then tested for cytotoxicity against autologous EBV-B cells from patients PA, PW, and PY (M-PA-EB, M-PW-EB, and M-PY-EB respectively) and MUC-1^{+}-autologous EBV-B cells from patients PA, PW, and PY (M + PA-EB, M + PW-EB, M + PY-EB respectively), as described in Material and methods. The rVPY + T cells were highly lytic for M + PY-EB cells, but not for M-PY-EB cells (Fig. 3A). The rVPW + T cells also displayed a similar result to the rVPY + T cells. (data not shown). In contrast, the rVPA-T cells were not lytic for either M-PA-EB or M + PA-EB cells, but were highly lytic for autologous EBV-B cells infected with wild-type vaccinia virus (V-Wyeth) (Fig. 3B). Both the rVPY + T and rVPW + T cells also demonstrated cytotoxicity against autologous EBV-B cells infected with V-Wyeth (data not shown). These results suggest that stimulation using autologous monocytes infected with rVMUC-1 can induce cytotoxic T lymphocytes recognizing MUC-1 only from patients with DF3/MUC-1-positive colorectal carcinomas. The following experiments were performed using the rVPA-T, the rVPY + T, and the rVPW + T cells.

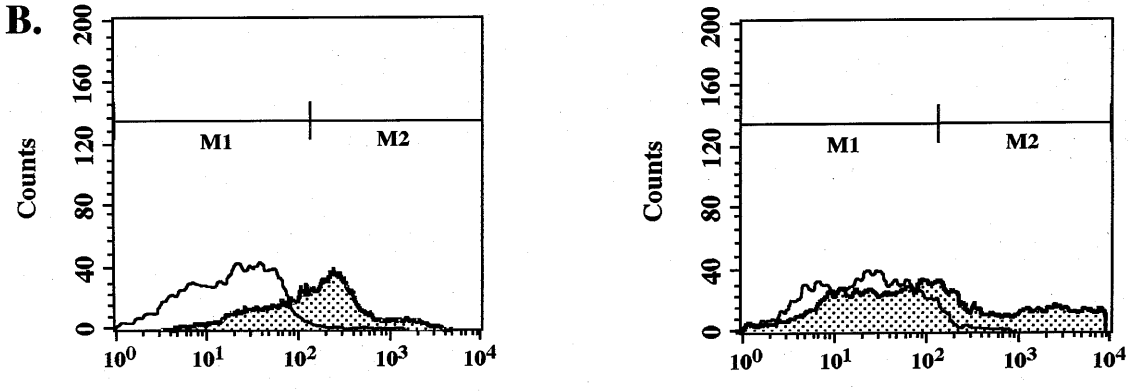
HLA-unrestricted killing by the MUC-1-specific CTL (rVPW + T and the rVPY + T)

Since one of the most interesting features of MUC-1-specific CTL, as reported before [18, 22], is their HLA-unrestricted cytotoxicity, we investigated an association of HLA molecules with the MUC-1-specific lysis caused by the rVPY + T and rVPW + T cells as described above. The rVPY + T and the rVPW + T cells were tested for cytotoxicity against human pancreatic cancer cell lines (CAPAN-1 and PANC-1) expressing high levels of DF3 and NAB-1, human colon cancer cell line HT-29



rVMUC-1	CD80	CD86	CD14	CD33	HLA ABC	HLA DR	HLA DP	HLA DQ
(-)	1.07	59.04	65.50	64.51	98.0	71.11	70.89	48.73
(+)	1.62	77.44	72.69	72.60	99.8	81.79	82.41	57.73

Values are expressed as % positive cells.



DF-3

Marker	Events	% Total	Mean	Peak Ch
All	9692	96.92	786.74	31
M1	4186	41.86	39.87	31
M2	5522	55.22	1350.93	193

NAB-1

Marker	Events	% Total	Mean	Peak Ch
All	9673	96.73	708.31	154
M1	6072	60.72	43.71	102
M2	3621	36.21	1819.65	154

Fig. 2 A FACScan cell-cytometric analysis of the rVMUC-1-infected monocytes from patient PY, using anti-CD80, anti-CD86, anti-CD14, anti-CD33, anti-HLA class I, and anti-HLA class II mAbs. *Solid area* reactivity with each mAb; *open area* reactivity with fluorescein-labeled goat anti-(mouse IgG). Expression of these molecules on the rVMUC-1-infected monocytes is compared with that on the non-treated monocytes. **B** Expression of the DF3 antigen (*left*) and the NAB-1 peptide epitope (*right*) on the rVMUC-1-infected autologous monocytes. The reactivity of mAb DF3 and pAb NAB-1 with the monocytes was determined by flow cytometry. *Solid area* reactivity with mAb DF3 and pAb NAB-1; *open area* reactivity with IgGl-isotype-identical control antibody (*left*) and control whole rabbit antibody (*right*)

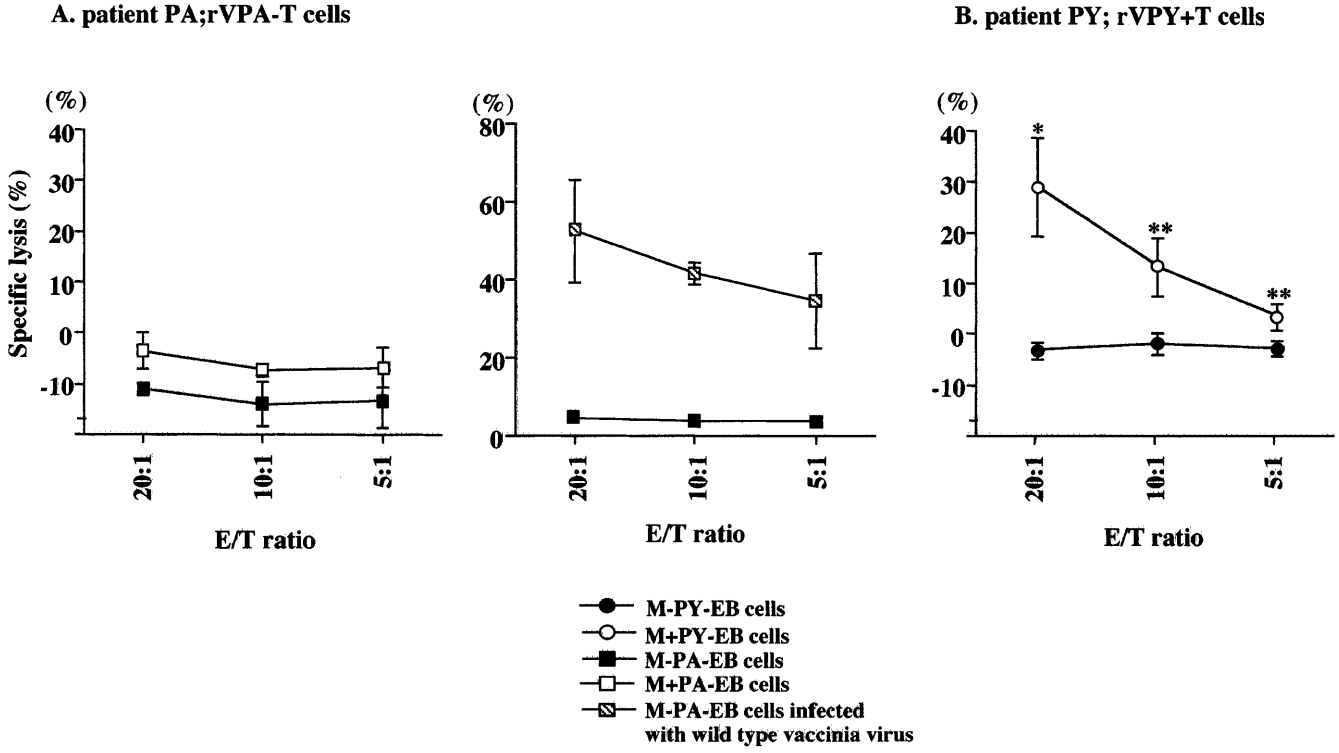


Fig. 3A, B Cytotoxicities of the stimulated T cells from patient PA (rVPA-T; **A**) and patient PY (rVPY + T; **B**) against the following target cells: EBV-B cells from patient PA (M-PA-EB; ■) and from patient PY (M-PY-EB; ●), and MUC-1^{+}-EBV-B cells from patients PA and PY (M + PA-EB) □, and M + PY-EB ○, respectively). *Hatched squares* M-PA-EB cells infected with wild-type vaccinia virus. $P^{*} = 0.005$; $P^{**} < 0.05$, as compared to cytotoxicity of the rVPY + T cells against M-PY-EB cells, unpaired Student *t*-test. *Symbols* mean percentage cytotoxicity; *bars* SD

expressing a low level of NAB-1 but not DF3, and mouse colon adenocarcinoma cell line MC-38 expressing neither DF3 nor NAB-1 (Table 1). The rVPY + T cells demonstrated moderate to high levels of cytotoxicity against DF3/MUC-1-positive allogeneic human cell lines, CAPAN-1 and PANC-1 cells (HLA-A2 compatible), but no cytotoxicity against the DF3/MUC-1-negative allogeneic human cell line HT-29 (HLA-B17-compatible) (Table 1). Although the rVPW + T cells shared no HLA-A/B/C antigen with CAPAN-1, PANC-1, or HT-29 cells, as shown in Table 1, high levels of lysis of CAPAN-1 and PANC-1 cells by the rVPW + T cells was also observed. Further, the rVPW + T and the rVPY + T cells, while not lytic for native HT-29 cells expressing no DF3/MUC-1, became significantly lytic for R-HT-29 cells expressing DF3/MUC-1 (Table 1). In contrast, the rVPA-T cells did not lyse either DF3/MUC-1-positive allogeneic cells, CAPAN-1 or PANC-1 cells (HLA-A2 compatible), or DF3/MUC-1-negative

Table 1 Cytotoxic activity of the rVMUC-1-stimulated T cells against human and mouse cell lines. Values are the means for triplicate wells of the percentage lysis against each cell line + SD. Percentage of cells judged positive by flow cytometry: + + + 71%–100%, + + 31%–70%, + 11%–30%, – less than 10%

Target cells	MHC phenotype	DF3	NAB-1	Lysis of target cells (%) by effector cells (effector/target ratio = 20:1)		
				Patient PY; rVPY + T (DF3/MUC-1; +) A2, 33; B17, 46; Cw1, 3	Patient PW; rVPW + T (DF3/MUC-1; +) A24, 26; B54, 61; Cw1, 3	Patient PA; rVPA-T (DF3/MUC-1; –) A24 (9), 2; B7, 59; Cw1, 7
CAPAN-1	A2, 9; B13, 17	+ + +	+	17.6 ± 5.7	48.2 ± 4.8	2.3 ± 1.9
PANC-1	A2, 1; B39	+ + +	+	47.5 ± 3.7	44.5 ± 3.7	2.0 ± 2.1
HT-29	A1, 3; B12, 17; Cw5	–	+	1.6 ± 0.5	1.3 ± 0.5	1.8 ± 0.7
R-HT-29	A1, 3; B12, 17; Cw5	+	+	15.8 ± 3.2*	11.2 ± 7.6*	3.1 ± 0.9
MC-38 (mouse cell line)	H-2 K*	–	–	2.1 ± 1.4	0.4 ± 0.4	4.1 ± 1.5

* $P < 0.001$ compared to cytotoxic activity against HT-29 cells

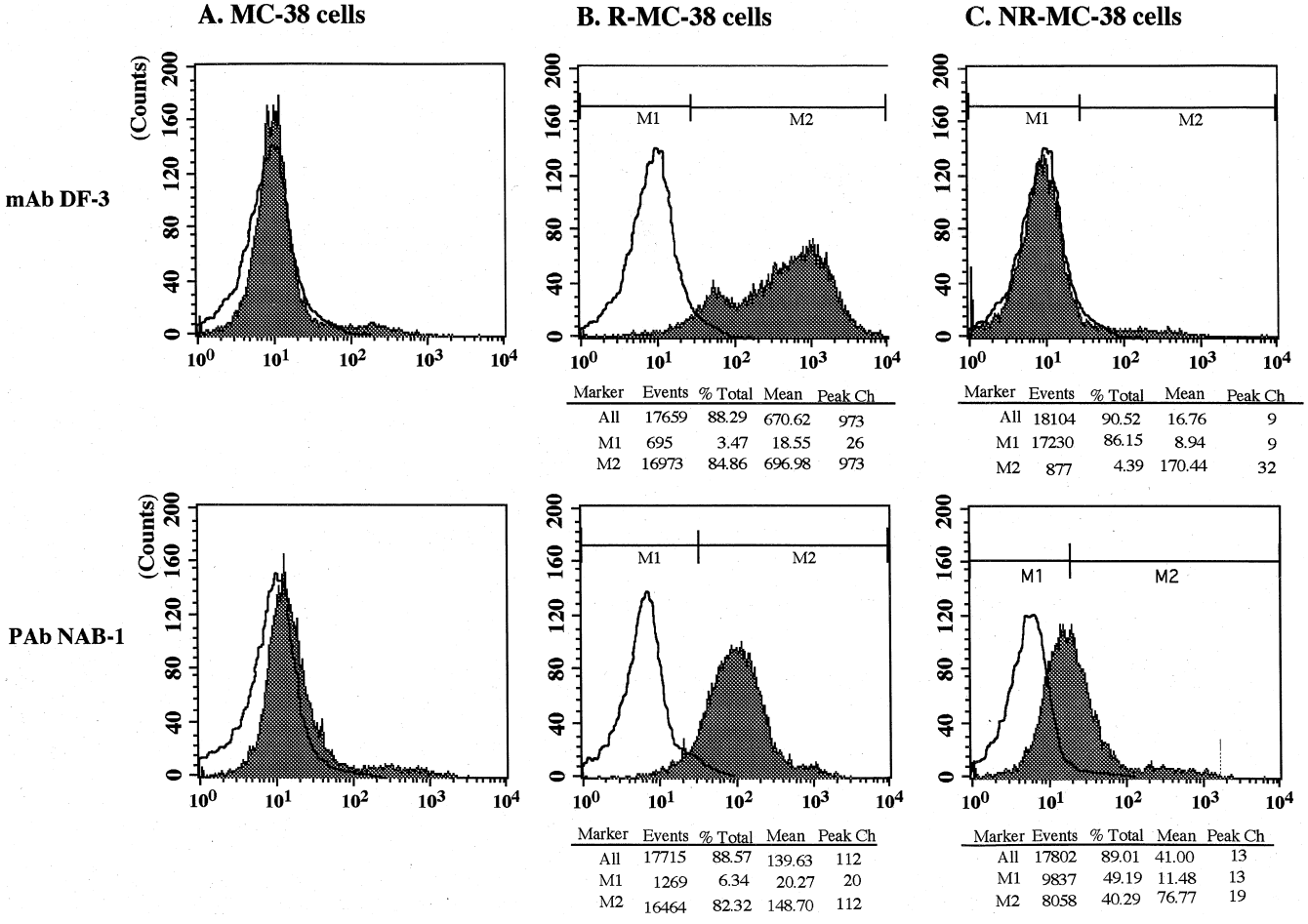


Fig. 4A–C Expression of the DF3 (*upper panel*) and the NAB-1 epitopes (*lower panel*) on MC-38 cells (**A**), R-MC-38 cells (**B**) and NR-MC-38 cells (**C**). The reactivity of mAB DF3 and pAb NAB-1 with these cell lines was determined by flow cytometry. *Solid areas* reactivity with mAb DF3 (*upper panel*) and pAb NAB-1 (*lower panel*); *open areas* reactivity with IgG1-isotype-identical control antibody (*upper panel*) and whole rabbit antibody as the control (*lower panel*)

allogeneic cells HT-29 cells (HLA-non-compatible) (Table 1). These results suggested that the lysis by rVPY + T and rVPW + T cells was dependent upon the expression of DF3/MUC-1 on the target cells, but not upon the target cells having an HLA phenotype (Table 1).

To confirm HLA-unrestricted recognition of the rVPY + T and the rVPW + T cells, mouse colon adenocarcinoma cell line MC-38 (H-2K^b), hardly sharing a MHC with humans, was transduced with the retrovirus-containing supernatant of R-MUC-1-transfected PA317 cells (R-MC-38), and used as target cells for the ^{51}Cr-release assay. Flow cytometry, using mAb DF3 and pAb NAB-1, showed that R-MC-38 cells expressed high levels of both DF3 and NAB-1 epitopes, although native MC-38 cells did not express either DF3 or NAB-1 epitopes (Fig. 4A, B). The rVPY + T and the rVPW + T cells were highly lytic for DF3/MUC-1-positive R-MC-38 cells, but not for mock MC-38 cells: MC-38 cells transduced with empty retroviral vectors (Fig. 5, Table 2). Further, preincubation of the rVPY + T cells with anti-MHC class I mAb resulted in no inhibition of lysis against R-MC-38 cells (Fig. 6B). In contrast, the rVPA-T cells failed to lyse either mock MC-38 or R-MC-38 cells (Fig. 5, Table 2). Thus, these results revealed that the rVPY + T and the rVPW + T cells lysed DF3/MUC-1$^+$ target cells in an HLA-unrestricted manner.

Epitope recognized by the HLA-unrestricted CTL

It has been reported that the T cell epitope recognized by an HLA-unrestricted CTL against MUC-1 is present on tandem repeats of MUC-1 molecules [20]. To investigate whether the HLA-unrestricted CTL (rVPY + T and rVPW + T) were also recognizing an epitope on tandem repeats, we constructed NR-MC-38 cells expressing MUC-1 protein devoid of tandem repeats, as described in Materials and methods. Flow cytometry showed that the NR-MC-38 cells expressed no measurable DF3/MUC-1 epitope, but did express a moderate level of NAB-1 epitope (Fig. 4C). The killing of NR-MC-38 cells by the rVPY + T cells was extremely reduced compared to that of R-MC-38 cells (Fig. 6A), which suggested that the T cell epitope recognized by the rVPY + T cells was

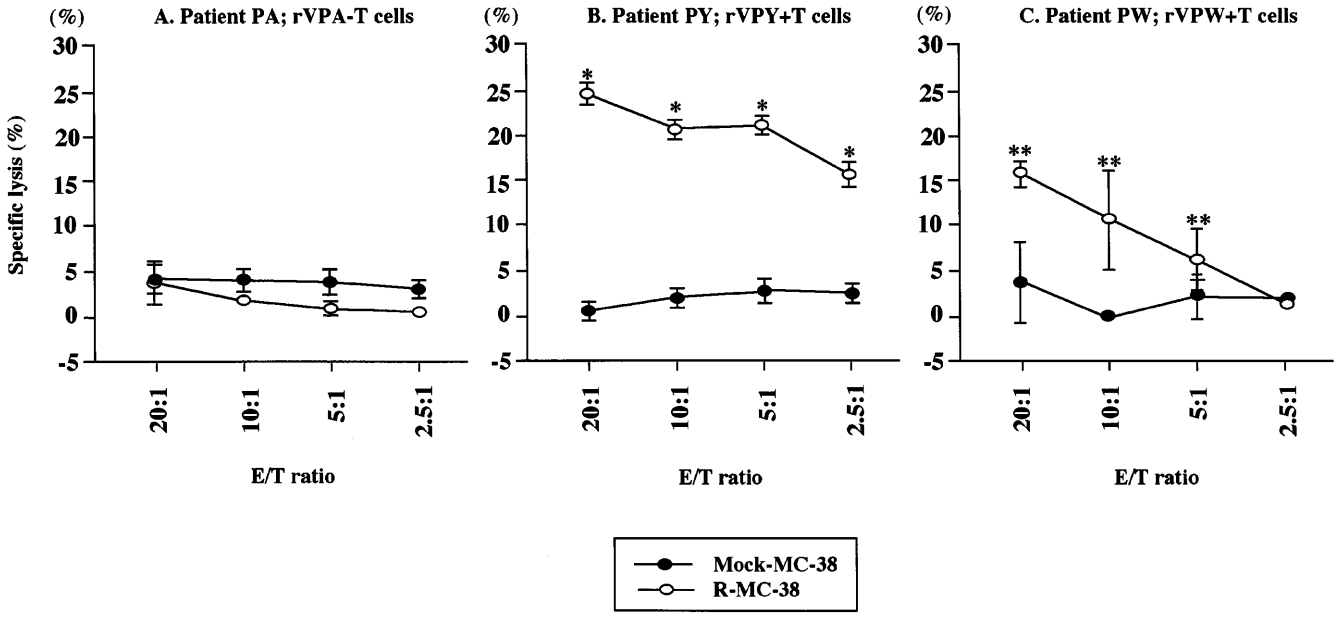


Fig. 5A–C Cytotoxicities of rVPA-T (**A**), rVPY + T (**B**), and rVPW + T (**C**) cells against mock MC-38 (●) and R-MC-38 (○). *Mock MC-38* MC-38 cells transduced with empty retroviral vectors. $P^* < 0.001$ and $P^{**} < 0.05$, as compared to cytotoxicity against mock MC-38 cells at each E:T ratio, unpaired Student's *t*-test. *Symbols* mean percentage cytotoxicity; *bars* SD

within tandem repeats. To determine the more precise position of the predicted T cell epitope, the rVPY + T cells were preincubated with mAb DF3, and then tested for cytotoxicity against R-MC-38 cells. This treatment can completely block the MUC-1-specific lysis of the rVPY + T cells against R-MC-38 cells (Fig. 6B).

$CD8^+$ T cells are responsible for the HLA-unrestricted killing of MUC-1

To investigate which subset of T-cells was involved in the effector phase of the HLA-unrestricted cytotoxicity against DF3/MUC-1, the rVPY + T cells were preincubated with anti-(human CD4) and anti-(human CD8) mAb, and then tested for cytotoxicity to MC-38 and R-MC-38 cells. An anti-(human CD8) mAb can block lysis by the rVPY + T cells, whereas preincubation of the rVPY + T cells with an anti-(human cD4) mAb had no effect on the lysis of R-MC-38 cells. These results suggested that the $CD8^+$ T cells were responsible for the HLA-unrestricted lysis (Fig. 6C), and stabilization of effector target interaction might require accessory interaction of the CD8 molecule with MHC class I molecule on the target cells, even in the case of MHC-unrestricted lysis [20].

Adoptive immunotherapy

The AI was performed three times at 4-week intervals. Lymphocytes were isolated, at every AI, from peripheral blood of patient PY, and stimulated with rVAMN as described in Materials and methods. The T cells isolated from the stimulated lymphocytes at the first AI dem-

Table 2 Cytotoxic activity of the rVMUC-1-stimulated T cells against mouse cell lines. Values are the means of triplicate wells + SD. *Mock MC-38* MC-38 cells transduced with empty retroviral vector; *IHS* (*DF3*) immunohistochemical staining of the resected specimen using mAb DF3; *Ca* cancer, *Li* liver metastasis, *Lu* lung metastasis, *Ly* lymphatic metastasis; *NR* not resected. The wild-type vaccinia virus was the Wyeth strain obtained from Jeffrey Schlom (NCI, NIH)

Patient	Type of cancer	Cytotoxic activity (%) against target cells (effector/target ratio = 20:1)			IHS (DF3)	CA19-9 (serum)
		Mock MC-38	R-MC-38	NR-MC-38		
PY	Rectal Ca + Li + Lu	0.63 ± 1.1	24.6 ± 1.55*	11.2 ± 2.2*	+	+
PW	Colon Ca + Lu + Ly	1.4 ± 2.42	15.7 ± 1.44**	7.4 ± 12.8	+	+
PA	Colon Ca + Li	4.1 ± 1.5	3.7 ± 2.4	2.9 ± 2.8	–	–
PA	Cytotoxicity against autologous EB virus-immortalized B cells infected with a wild type vaccinia virus					
	E/T ratio	20:1	10:1	5:1		
		52.4 ± 13.1	41.7 ± 2.8	34.7 ± 12.1		

* $P < 0.01$ compared with cytotoxicity of PY T cells against MC-38 cells
** $P < 0.01$ compared with cytotoxicity of PW T cells against MC-38 cells

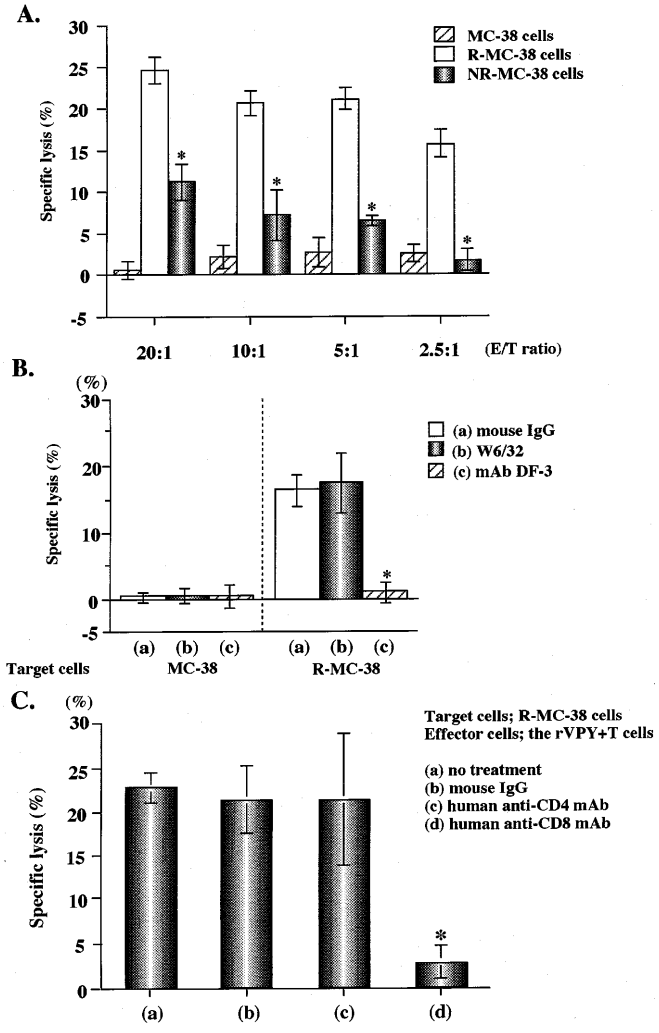


Fig. 6 **A** The cytotoxicity of rVPY + T cells against NR-MC-38 cells was compared with that against R-MC-38 cells. *Symbols* mean percentage cytotoxicity; *bars* SD. $*P < 0.01$, as compared to cytotoxicity against R-MC-38 cells at each E:T ratio, unpaired Student's *t*-test. **B** Specific target Ag recognition by the rVPY + T cells. MC-38 cells or R-MC-38 cells were incubated with (*a*) mouse IgG (30 μg/ml), (*b*) anti-(MHC class I) mAb W6/32 (30 μg/ml), and (*c*) mAb DF3 (10 μg/ml), and cytotoxic assays against MC-38 cells and R-MC-38 cells were performed. The E:T ratio was 20:1. $P* < 0.01$, as compared to values with mouse IgG, Paired Student *t*-test. *Columns* mean percentage cytotoxicity; *bars* SD. **C** Inhibition of cytotoxicity of the rVPY + T cells with antibodies against human CD4 or human CD8. The rVPY + T cells were preincubated with 10 μg/ml anti-(human CD4), 10 μg/ml anti-(human CD8) mAb, or mouse IgG as a control, and then tested for cytotoxicity against R-MC-38 cells. The E:T ratio was 20:1. $P* = 0.005$, as compared to values with mouse IgG (10 μg/ml), paired Student *t*-test. *Columns* mean percentage cytotoxicity; *bars* SD

onstrated HLA-unrestricted cytotoxicity to MUC-1$^+$ allogeneic tumor cells (R-MC-38 cells) (Fig. 7A), which was, after the second AI, finally reduced to the baseline level (Fig. 7A). Contrary to our expectation, this repetitive stimulation caused a selective proliferation of CD4$^+$T cells but not of CD8$^+$ T cells (Table 3). The stimulated T cells at the third AI lysed MUC-1$^+$-autologous EBV-B cells to a higher extent than their MUC-1-negative counterparts (Fig. 7B), which were efficiently blocked by antibodies against CD4 and HLA class II, but not by those against CD8 and HLA class I (Fig. 7C). When the stimulated T cells at the third AI were mixed with MUC-1$^+$-autologous monocytes, the cytotoxicity to MUC-1$^+$ autologous EBV-B cells was extremely reduced (Fig. 7B), suggesting that the HLA-class-II-restricted CD4$^+$ T cells cytotoxic to MUC-1 selectively lysed MUC-1$^+$-autologous monocytes as antigen-presenting cells (Fig. 7B). These results suggested that the stimulation of PBL with rVAMN caused a transient induction of HLA-unrestricted CD8$^+$ T cells cytotoxic to MUC-1, and subsequently a predominant proliferation of the HLA-class-II-restricted cytotoxic CD4$^+$ T cells possibly reacting to the same T cell epitope as the HLA-unrestricted CTL, which might suppress an efficient induction of HLA-unrestricted CTL against MUC-1 by removing autologous monocytes presenting MUC-1 Ag.

The volume of the liver metastasis on the anterior superior segment (S8) of patient PY demonstrated a 61% reduction after the first AI, but a rapid expansion especially after the third AI (Fig. 8). The volume of the liver metastasis followed a similar curve to that of serum levels of CA19-9 and CEA.

Discussion

Our working hypothesis for this study is that peripheral blood lymphocytes derived from patients with colorectal carcinomas expressing the DF3/MUC-1 epitope as a cryptic epitope should contain immune effector reactivities, perhaps at the precursor level, specific for DF3/MUC-1. This hypothesis is based on mounting evidence that the immune system of patients recognizes and responds to TAA such as MAGE-1, -2, and -3 [7], mutated p + 53 [25], and HER-2/neu [9], which are expressed by their own growing tumors. The present study revealed that the HLA-unrestricted CTL against MUC-1 were expectedly induced only in patient PY and PW, with DF3/MUC-1-positive colorectal carcinomas, but not from patient PA with a DE-3/MUC-1-negative colon carcinoma. The rVPA-T cells did not demonstrate any MUC-1-specific cytotoxicity, but were highly lytic for the autologous EBV-B cells infected with wild-type vaccinia virus (Fig. 3, Table 2), suggesting that the failure to induce HLA-unrestricted CTL against MUC-1 from patient PA was not due to an inappropriateness of this stimulation, but to an absence of susceptibility to DF3/MUC-1. Taken together, a sensitization of DF3/MUC-1-positive autologous tumors in vivo seems likely to be essential for the induction of HLA-unrestricted CTL against MUC-1.

The MUC-1-specific CD4$^+$ T cells efficiently induced at the third AI, are expected to play a crucial role in inducing MUC-1-specific antitumor immunity as helper T cells, because the recognition of tumor antigens by helper T cells is considered to be the initial event in the

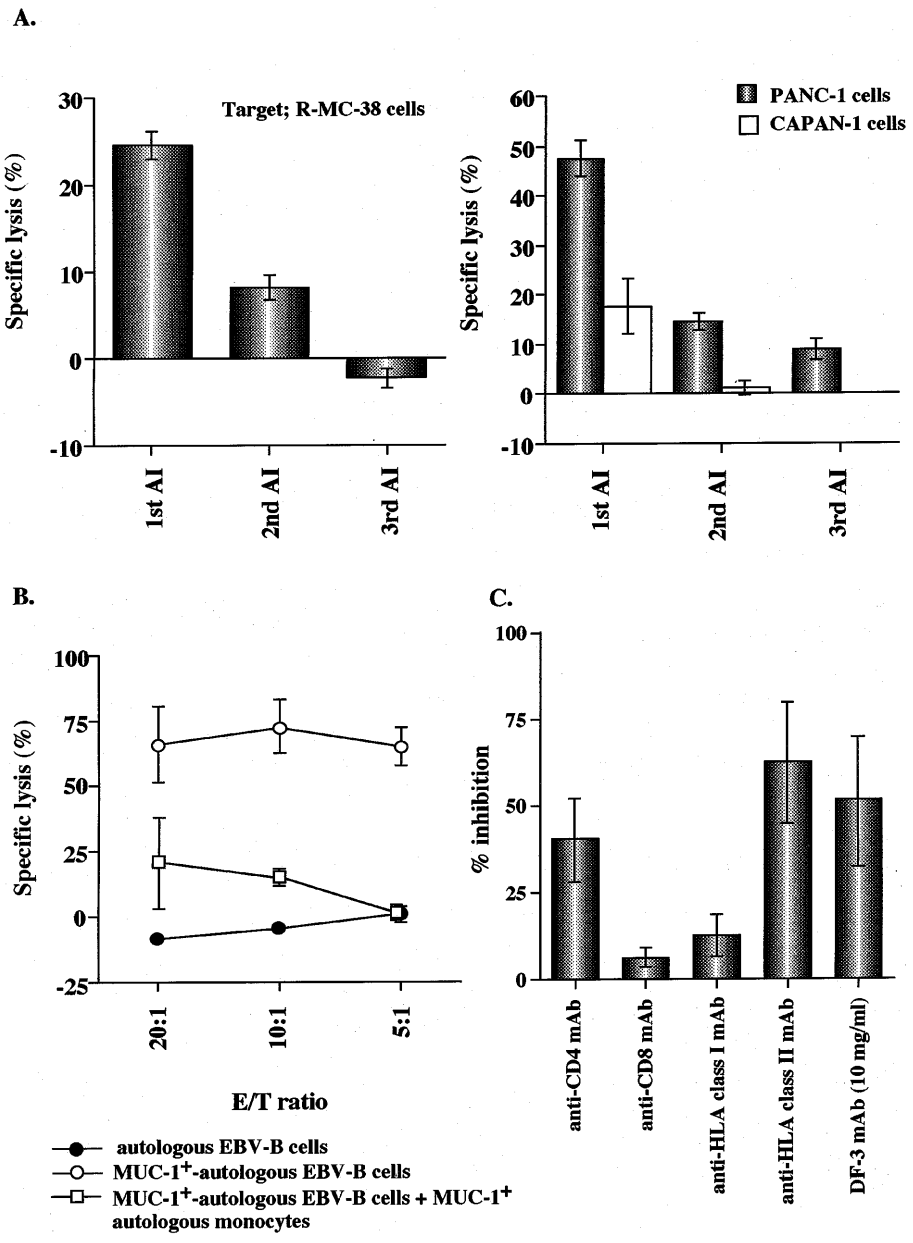


Fig. 7A–C T cells were isolated from peripheral blood lymphocytes stimulated with rVAMN at every adoptive immunotherapy treatment (AI), and tested for cytotoxicity. **A** The lysis of R-MC-38 cells, CAPAN-1, and PANC-1, was analyzed at each AI by ^{51}Cr-release assay. *Columns* mean percentage cytotoxicity; *bars* SD. **B** The cytotoxicity of the effector cells at the third AI against MUC-1$^-$ (●) and MUC-1$^+$-autologous Epston-Barr Virus B (EBV-B) cells (○). The effector cells at the third AI were mixed with autologous monocytes transduced with retrovirus-containing supernatants of R-MUC-1-transfected PA 317 cells, and tested for cytotoxicity against MUC-1$^+$-autologous EBV-B cells (□). *Symbols* mean percentage cytotoxicity; *bars* SD. **C** The effector cells at the third AI were preincubated with antibodies against CD4, CD8, HLA class I, HLA class II, and DF3 Ag, and tested for cytotoxicity against MUC-1$^+$-autologous EBV-B cells. *Columns* mean percentage cytotoxicity; *bars* SD

Table 3 Characterization of the lymphocytes from patient PY before and after stimulation with rVMUC-1. *1BS* before stimulation by first adoptive immunotherapy, *1AS* after stimulation by first adoptive immunotherapy, *ND* not done

Marker	Positive cells (%)			
	1BS	1AS	3BS	3AS
CD3	60.5	87.7	67.8	94.6
CD4	41.8	69.9	24.0	88.1
CD8	18.3	17.8	36.3	8.6
CD4/CD8	2.28	2.9	0.66	10.24
CD3*CD56	1.6	1.1	ND	2.7
CD16*CD56	12.3	5.3	3.8	0.6

cascade of antitumor immune responses [10, 12, 13]. However, the HLA-class-II-restricted CTL against MUC-1 failed to demonstrate antitumor activity in vivo; on the contrary, the liver metastasis and the serum levels of tumor markers showed steeper curves after the third AI than before the third AI.

In summary, this study has shown that (a) the HLA-unrestricted CTL against MUC-1 are induced from patients with DF3/MUC-1$^+$-colon carcinomas by rVMUC-1, and their MUC-1-specific cytotoxic activity correlates with the outcome of the adoptive immunotherapy; and (b) the HLA-unrestricted cytotoxicity is decreased following repetitive stimulation with rVMUC-1.

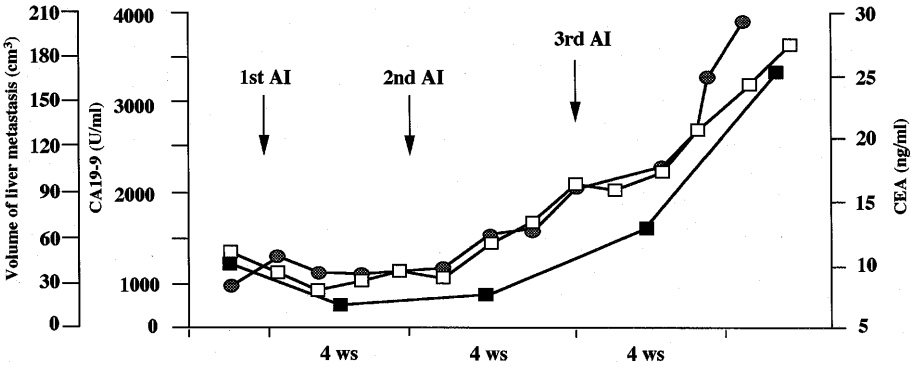


Fig. 8 The volume of the liver metastatic lesion (■) on the anterior superior segment of patient PY, and the serum levels carcinoembryonic antigen (*CEA*; ○) and CA19-9 (●) during the three rounds of adoptive immunotherapy

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