

Antitumor Efficacy of Vaccinia Virus-modified Tumor Cell Vaccine¹

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ABSTRACT

The antitumor efficacies of vaccinia virus-modified tumor cell vaccines were examined in murine syngeneic MH134 and X5563 tumor cells. UV-inactivated vaccinia virus was inoculated i.p. into C3H/HeN mice that had received whole body X-irradiation at 150 rads. After 3 weeks, the vaccines were administered i.p. 3 times at weekly intervals. One week after the last injection, mice were challenged i.p. with various doses of syngeneic MH134 or X5563 viable tumor cells. Four methods were used for preparing tumor cell vaccines: X-ray irradiation; fixation with paraformaldehyde for 1 h or 3 months; and purification of the membrane fraction. All four vaccines were effective, but the former two vaccines were the most effective. A mixture of the membrane fraction of untreated tumor cells and UV-inactivated vaccinia virus also had an antitumor effect. These results indicate that vaccine with the complete cell structure is the most effective. The membrane fraction of UV-inactivated vaccinia virus-absorbed tumor cells was also effective. UV-inactivated vaccinia virus can react with not only intact tumor cells but also the purified membrane fraction of tumor cells and augment antitumor activity.

INTRODUCTION

TATA³ have been demonstrated in various tumor systems (1), but they usually have only weakly immunogenic effects on syngeneic tumors. Many attempts have been made to augment the immunogenicity of tumors (2-9). Lindenmann and Klein (10) found that influenza virus rendered the host resistant to Ehrlich ascites tumor after inducing destruction of the tumor cells. Wallack *et al.* reported that VOV was useful in the treatment of human cancer (11-13). Hamaoka *et al.* induced cytotoxic T-lymphocyte production against a syngeneic tumor in mice by priming with a hapten followed by immunization with hapten-conjugated tumor cells (14-16). We examined the effect of use of VV to modify TATA in the model described by Hamaoka *et al.* and succeeded in inducing resistance to MH134 and X5563 syngeneic tumors in C3H/HeN mice (17, 18). Then we demonstrated that UV-VV absorption alone was as effective as VV infection (19). In a preliminary study, we compared the antitumor effects of UV-VV TCV and VOV, finding that UV-VV TCV was the more effective (20). In the present study, we examined the antitumor effects of four different preparations of UV-VV-modified TCV.

MATERIALS AND METHODS

Experimental Animals. Female C3H/HeN mice were purchased from Charles River Laboratory, Kanagawa, Japan, and used at 6-8 weeks of age.

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³ The abbreviations used are: TATA, tumor-associated transplantation antigens; VV, vaccinia virus; TCV, tumor cell vaccine; UV-VV, UV-inactivated VV; UV-VV TCV, UV-VV-absorbed TCV; PF TCV, paraformaldehyde-fixed TCV; UV-VV Mfr, membrane fraction of UV-VV-absorbed tumor cells; Mfr, membrane fraction of untreated tumor cells; TLD₅₀, 50% tumor lethal dose; cTLD₅₀, comparative TLD₅₀; VOV, vaccinia oncolysate vaccine; MEM, Eagle's minimal essential medium; VSA, VV-specific antigen.

Tumors. Two syngeneic tumor cell lines, MH134 (from a CCL₄-induced hepatoma in a C3H mouse) and X5563 (from a spontaneous myeloma in a C3H/He mouse), were used in ascitic forms. Both cell lines were maintained and passaged i.p. in C3H/HeN mice at 10-day intervals.

Vaccinia Virus. The Ikeda strain of VV, formerly used as a seed virus for smallpox vaccine in Japan, was grown in HeLa cells. The virus was harvested and purified as described by Joklik (21) with slight modifications (22). VV was inactivated with a 15-W germicidal UV lamp (257.7 nm, 2 × 10⁴ erg/cm²/s) at a distance of 6 cm for 10 min (UV-VV).

UV-VV-absorbed Tumor Cell Vaccine. Tumor cells were incubated with UV-VV at a virion dose equivalent to a multiplicity of infection of 10 at 37°C for 2 h. Then they were washed once with MEM, suspended in MEM at a concentration of 10⁷ cells/0.1 ml, and X-irradiated at 10⁴ rads.

Paraformaldehyde-fixed UV-VV-absorbed Tumor Cell Vaccine. UV-VV was incubated in the same way as UV-VV TCV with tumor cells and then the cells were fixed with 2% paraformaldehyde at 4°C for 1 h [PF TCV(1h)] or 3 months [PF TCV(3m)]. After fixation, the cells were washed twice with MEM and suspended in MEM at a concentration of 10⁷ cells/0.1 ml.

Membrane Fraction of UV-VV-absorbed Tumor Cells. Tumor cells were incubated with UV-VV at a virion dose equivalent to a multiplicity of infection of 10 at 37°C for 4 h. Then the membrane fraction was purified as described by Ichihashi (23). The cells were allowed to swell in 20 mM Tris buffer, pH 8.4, for 10 min and then homogenized in a Dounce homogenizer. The homogenate was centrifuged (6 × 10³ g, 10 min), and the supernatant was recentrifuged (1 × 10⁵ g, 1 h). The resulting pellet was sonicated, layered on a discontinuous sucrose gradient (10 and 42%), and centrifuged (3.7 × 10⁴ g, 1 h). The membrane fraction was collected from the boundary between the 10 and 42% sucrose, washed once (1 × 10⁵ g, 1 h), suspended in phosphate-buffered saline at a concentration equivalent to 10⁷ cells/0.1 ml, and stored at -80°C until use (UV-VV Mfr). Mfr was purified by the same method.

Immunization Schedule. As shown in Fig. 1, mice received whole body X-irradiation at 150 rads and i.p. injection of UV-VV equivalent to 10⁷ plaque-forming units. After 3 weeks, vaccines were injected into the mice i.p., 3 times at weekly intervals, and viable tumor cells were injected i.p. 1 week after the third vaccination. The control group received only challenge with tumor cells. All the mice were observed for 8 weeks after challenge, and the TLD₅₀ was calculated by the method of Reed and Muench (24). The ratio of the TLD₅₀ in the vaccine-treated group to that in the control group, designated as the cTLD₅₀, was used as an index of antitumor efficacy (25).

RESULTS

Antitumor Effect of UV-VV TCV. Two tumor cell lines, MH134 and X5563, were used. Each type of vaccine was prepared from both MH134 and X5563 tumor cells. The mortality rates of subgroups of five C3H/HeN mice each after i.p. challenge with MH134 tumor cells are shown in Table 1. In the control group, all mice died when challenged i.p. with 1 × 10¹ or 1 × 10² tumor cells. The TLD₅₀ was 10^{0.5}. In the UV-VV TCV-treated group, all mice survived i.p. challenge with 1 × 10⁵ or 1 × 10⁶ tumor cells, and all mice died when challenged with 1 × 10⁷ tumor cells. The TLD₅₀ was 10^{6.5}.

Table 1 Comparison of mortality rates of C3H/HeN mice immunized with UV-VV TCV and PF TCV

Immunization	Challenge dose (1×10^6 cells)	No. of deaths/no. of mice inoculated		Mean time to death (days)		TLD ₅₀ ($\times 10^6$)	
		MH134	X5563	MH134	X5563	MH134	X5563
None	1	5/5	5/5	29.8	31.2	0.5	0.5
	2	5/5	5/5	22.6	23.0		
UV-VV TCV	5	0/5	0/5	18.2	19.0	6.5	6.6
	6	0/5	0/5				
	7	5/5	4/5				
PF TCV(1h)	1	0/5	0/5	20.0	20.0	6.4	6.4
	2	0/5	0/5				
	3	0/5	0/5				
	4	0/5	0/5				
	5	0/5	0/5				
	6	1/5	1/5				
	7	5/5	5/5				
PF TCV(3m)	3	1/5	4/5	23.0	31.5	6.0	5.0
	4	1/5	1/5	24.0	24.0		
	5	1/5	1/5	20.0	20.0		
	6	1/5	3/5	18.0	17.7		
	7	5/5	5/5	17.8	16.8		

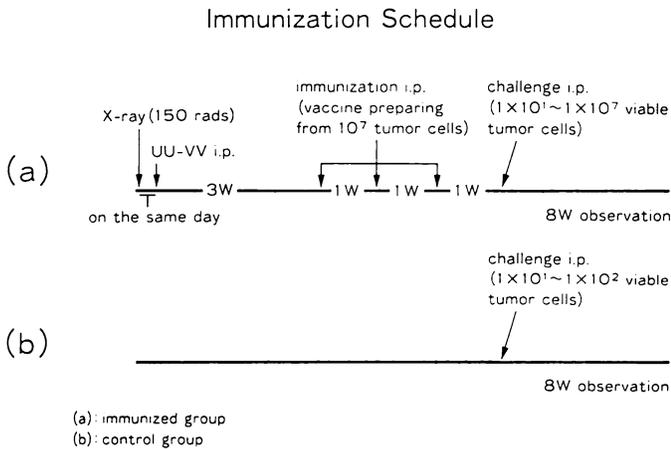


Fig. 1. Immunization schedule. (a) immunized group. (b) control group. For details, see "Materials and Methods."

The mortality rates after i.p. challenge with X5563 tumor cells are also shown in Table 1. The TLD₅₀ values of the control group and UV-VV TCV-treated group were $10^{0.5}$ and $10^{6.6}$, respectively.

Antitumor Effect of PF TCV. The mortality rates after i.p. challenge with MH134 tumor cells are shown in Table 1. The TLD₅₀ values of the groups treated with PF TCV(1h) and PF TCV(3m) were $10^{6.4}$ and $10^{6.0}$, respectively. The mortality rates after i.p. challenge with X5563 tumor cells are also shown in Table 1. The TLD₅₀ values of the PF TCV(1h)- and PF TCV(3m)-treated groups were $10^{6.4}$ and $10^{5.0}$, respectively. The antitumor activity of PF TCV(1h) was almost the same as that of UV-VV TCV. As judged by mortality rates, the antitumor activity of PF TCV(3m) was less than that of PF TCV(1h) against X5563 ($P < 0.05$ by Fisher's statistical analysis), but not significantly different from the latter against MH134.

Antitumor Effect of Membrane Fraction Vaccine. The membrane fraction of untreated tumor cells (Mfr), the membrane fraction of UV-VV-absorbed tumor cells (UV-VV Mfr), and a mixture of UV-VV and Mfr were injected into mice i.p. The UV-VV(s.c.) + Mfr(i.p.)-treated group received s.c. injection of UV-VV and i.p. injection of Mfr.

The mortality rates after i.p. challenge with MH134 tumor cells are shown in Table 2. The TLD₅₀ values of the groups

treated with Mfr, UV-VV Mfr, UV-VV + Mfr, and UV-VV(s.c.) + Mfr(i.p.) were $10^{1.8}$, $10^{5.6}$, $10^{5.4}$, and $10^{1.8}$, respectively. The mortality rates after i.p. challenge with X5563 tumor cells are also shown in Table 2. The TLD₅₀ values of the groups treated with Mfr, UV-VV Mfr, UV-VV + Mfr, and UV-VV(s.c.) + Mfr(i.p.) were $10^{3.4}$, $10^{5.2}$, $10^{5.4}$, and $10^{1.3}$, respectively. Thus UV-VV Mfr and UV-VV + Mfr had almost the same effects and were more effective than Mfr and UV-VV(s.c.) + Mfr(i.p.). The differences in the mortality rates of the groups treated with UV-VV Mfr and Mfr, UV-VV Mfr and UV-VV(s.c.) + Mfr(i.p.), UV-VV + Mfr and Mfr, and UV-VV + Mfr and UV-VV(s.c.) + Mfr(i.p.), respectively, were all significant at $P < 0.05$ by Fisher's statistical analysis.

Comparative TLD₅₀. The cTLD₅₀ is the ratio of the TLD₅₀ in a vaccine-treated group to that in the control group and is an index of the antitumor efficacy of a vaccine that can be used to compare the effectiveness of vaccines in different experiments (25). The cTLD₅₀ values of the vaccines tested in this work are shown in Table 3.

DISCUSSION

Previous studies showed that vaccinia virus-modified tumor cell vaccine induced tumor-specific resistance to MH134 and X5563 tumors. The specificity of resistance *in vivo* was demonstrated by cross-challenge with these two tumor cell lines; resistance was observed only when the same tumor cells were used for immunization and challenge (17). Responses of cytotoxic T-lymphocytes to X5563 and MH134-specific antibody *in vitro* were also reported (16).

In this study, UV-inactivated vaccinia virus-modified tumor cell vaccine was prepared by four methods, and the antitumor effects of the preparations were examined using the cTLD₅₀ values and mortality rates as indices of effects.

UV-VV TCV and PF TCV(1h) were the most effective. These two vaccines had been treated by X-ray irradiation and fixation with paraformaldehyde, respectively. In these vaccines, the cell structure is complete, and VSA was detected on the cell surface (data not shown). VSA becomes distributed over the entire cell surface on fusion of the virus envelope with the plasma membrane (26). The M_r 32,000 VSA is highly immunogenic against mice (27). We observed cocapping of VSA and MH134-specific

Table 2 Comparison of mortality rates of C3H/HeN mice immunized with Mfr, UV-VV Mfr, UV-VV + Mfr, and UV-VV (s.c.) + Mfr (i.p.)

Immunization	Challenge dose (1 × 10 ⁿ cells)	No. of deaths/no. of mice inoculated		Mean time to death (days)		TLD ₅₀ (×10 ⁿ)	
		MH134	X5563	MH134	X5563	MH134	X5563
None	1	3/5	5/5	29.3	30.0		
	2	4/5	5/5	23.0	22.6	1.0	0.5
Mfr	1	0/5	0/5				
	2	4/5	2/5	28.8	27.0		
	3	4/5	0/5	21.8			
	4	5/5	4/5	21.2	21.3	1.8	3.4
	5	5/5	5/5	16.2	18.2		
	6	5/5	5/5	17.0	15.2		
	7	5/5	5/5	13.8	11.8		
UV-VV Mfr	1	0/5	0/5				
	2	0/5	0/5				
	3	0/5	0/5				
	4	0/5	0/5				
	5	0/5	2/5		20.5	5.6	5.2
	6	5/5	5/5	18.2	18.6		
	7	4/5	5/5	17.5	17.2		
UV-VV + Mfr	1	0/5	0/5				
	2	0/5	0/5				
	3	0/5	0/5				
	4	0/5	0/5				
	5	1/5	1/5	19.0	24.0	5.4	5.4
	6	5/5	5/5	18.0	19.6		
	7	5/5	5/5	16.6	16.6		
UV-VV (s.c.) + Mfr (i.p.)	1	2/5	4/5	28.5	30.3		
	2	5/5	3/5	28.2	26.3		
	3	3/5	4/5	21.3	23.3		
	4	3/5	5/5	20.3	20.8	1.8	1.3
	5	5/5	5/5	17.2	18.0		
	6	4/5	4/5	16.3	14.8		
	7	5/5	5/5	14.0	11.4		

Table 3 Comparison of cTLD₅₀ values (10ⁿ) of tumor cell vaccines

Immunization	MH134	X5563
UV-VV TCV	6.0	6.1
PF TCV(1h)	5.9	5.9
PF TCV(3m)	5.5	4.5
UV-VV Mfr	4.6	4.7
Mfr	0.8	2.9
UV-VV + Mfr	4.4	4.9
UV-VV(s.c.) + Mfr(i.p.)	0.8	0.8

surface antigen (data not shown). In UV-VV TCV and PF TCV(1h), VSA seems to be closely associated with TATA on the membrane of cells whose cell structure is not destroyed. We suppose that this situation is advantageous for interaction of the antigen-presenting cell with the cell membrane. In a preliminary study, we found that the cTLD₅₀ values of VOV challenged with MH134 and X5563 were 10^{4.9} and 10^{3.9}, respectively (20). VOV, which was a preparation of sonicated, VV-infected tumor cells containing all the fractions of VV-infected tumor cells, and UV-VV Mfr, which was a purified membrane fraction of UV-VV-absorbed tumor cells, had similar effects. This finding suggests that the effective fraction of UV-VV-modified TCV is the membrane fraction.

UV-VV Mfr and UV-VV + Mfr had almost the same effect, but UV-VV(s.c.) + Mfr(i.p.) had no effect. UV-VV Mfr was a membrane fraction of UV-VV-absorbed tumor cells and contained VSA (data not shown), the association of which with TATA would result in augmentation of immunogenicity against tumor cells. Mfr was a membrane fraction of untreated tumor cells. The UV-VV + Mfr-treated group received an i.p. injection of a mixture of UV-VV and Mfr, whereas the UV-VV(s.c.) + Mfr(i.p.) group received a s.c. injection of UV-VV and i.p.

injection of Mfr; thus there was no association between VSA and TATA. Therefore, our findings suggest that the association of VSA with TATA was essential for augmenting the antitumor activity. Lindenmann and Klein (10) found that merely mixing egg-grown virus with homogenates of uninfected tumor cells did not augment the immunogenicity of the tumor cell extracts, thus excluding the possibility that the virus acts simply as an adjuvant. The mechanism by which a virus augments the immunogenicity of TATA is unknown but has been suggested to be mainly by acting as a helper antigen (28). VSA exists in close proximity to TATA and during the process of response to VSA, the host immune system responds more strongly to adjacent TATA than in the absence of VSA. We found that UV-VV + Mfr had the same effect as UV-VV Mfr, clearly indicating that VSA acted as a helper antigen not only on intact tumor cells but also on membrane fragments.

VSA in close association with TATA presumably augments the immunogenicity of TATA on the intact cell structure. Previously, we demonstrated that infection of murine or human tumor cells with VV induced the cells to react with their complement via the alternative pathway (29, 30). The activation of the alternative complement pathway induced by VV or UV-VV occurred only at the surface of VV-infected or UV-VV-absorbed tumor cells. Therefore, the intact cell structure is essential for activation of the alternative complement pathway and recognition of the helper antigen by the antigen-presenting cell. Paraformaldehyde-fixed vaccine is reported to be effective for inducing a humoral and cellular immune reaction against human immunodeficiency virus 1 (31). Our results also showed that fixation with paraformaldehyde for 1 h did not affect the antitumor activity.

In a preliminary experiment, we used this protocol in tumor-

bearing mice. The mice were inoculated i.p. or s.c. with 1×10^5 MH134 tumor cells, and beginning 1 week later, UV-VV TCV was injected i.p. three times at weekly intervals. However, all the mice finally died of tumors. Healthy mice and tumor-bearing mice showed quite different augmentations of antitumor immunity. We think this immune protocol would be useful for preventing metastasis after surgical operation with nonspecific adjuvant therapy.

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