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ADDITIONAL EVIDENCE FOR THE AUGMENTED INDUCTION OF TUMOR-SPECIFIC RESISTANCE IN VACCINIA VIRUS-PRIMED MICE BY IMMUNIZATION WITH VACCINIA VIRUS-MODULATED SYNGENEIC TUMOR CELLS

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SUMMARY The augmenting effect of vaccinia virus infection of tumor cells on induction of tumor-specific resistance was examined in mice. C3H/HeN mice were primed intraperitoneally (ip) with live vaccinia virus after whole-body irradiation with 250 rad of X-rays. Three weeks later the mice were immunized ip 3 times at weekly intervals with syngeneic murine hepatoma MH134 or spontaneous myeloma X5563 which had been infected in vitro with vaccinia virus and subsequently irradiated with 7000 rad of X-rays. One week after the third immunization, the mice were challenged with 1×10^5 viable cells of MH134 or X5563 ip or 1×10^6 tumor cells intradermally (id). On ip challenge with viable MH134 cells all mice that had not been pretreated died within 3 weeks due to ascites tumor outgrowth, whereas all mice that had been vaccinia virus-primed and immunized with vaccinia virus-infected MH134 cells survived. On ip challenge with X5563 cells, the percentage survival of vaccinia virus-primed and vaccinia virus-modified tumor-immunized mice was 80%. On id challenge with MH134 and X5563 tumor cells, in un-treated mice tumors grew to more than 5 mm in diameter within 3 weeks, whereas 90% and 60%, respectively, of the mice that had been vaccinia virus-primed and immunized with vaccinia virus-infected tumor cells showed no tumor outgrowth. Pretreatment by only immunization with vaccinia virus-infected cells or vaccinia virus-priming and immunization with virus non-infected tumor cells were not effective for preventing induction of tumor-resistance to either ip or id challenge with MH134 or X5563 tumor cells. Moreover, no cross-resistance between MH134 and X5563 was observed, indicating that the tumor-resistance induced by this protocol was tumor-specific.

INTRODUCTION

Tumor-associated transplantation antigens (TATA) have been demonstrated in various tumor systems (Klein, 1966). However, the TATA on syngeneic tumor cells are generally only weakly immunogenic.

There have been several attempts to induce resistance to tumor cells using various viruses. Lindenmann (1965) and Lindenmann and Klein (1967) found that influenza virus rendered the host resistant to Ehrlich ascites tumor after inducing destruction of the tumor cells. Asada (1974) showed that intravenous injection of mumps virus diminished the symptoms of cancer patients. Wallack et al. (1977; 1981) tested lysates of tumor cells infected with vaccinia virus in tumor bearing-patients. However, the effects of virus application in tumor-immunotherapy are generally weak and the underlying mechanisms are unknown.

Recently, Hamaoka et al. (1979) developed a model system to augment host-immune responses to syngeneic tumor cells that eradicate tumor-outgrowth and Fujiwara et al. (1980) analyzed the mechanisms underlying this tumor-eradication. They succeeded in increasing the generation of cytotoxic T cell activity against tumor cells by priming mice with a hapten-self component and subsequently immunizing the mice with hapten-modified tumor cells. They used 2, 4, 6-trinitrophenol (TNP) as a hapten to modify the surface of syngeneic tumor cells. Using the same line of approach, Takatsu et al. (1980) modified tumor cells with a purified protein derivative (PPD) from *Mycobacterium tuberculosis*, and demonstrated that immunization with PPD-coupled tumor cells induced potent tumor-eradication activity in tumor-bearing *Mycobacterium*-primed mice. These studies showed that effective preimmunization with antigens that become attached to the tumor cell surface is a prerequisite for such augmented induction of tumor-specific immunity.

Vaccinia virus had been extensively used for immunization against smallpox, and its immunological memory persists for throughout life. Moreover, this virus has a broad host range of infection, and easily modulates the surface of tumor cells by infection. Thus, we previously attempted to use vaccinia virus as a tumor-modifying antigen in the model system of Hamaoka et al., and succeeded in inducing resistance to a syngeneic tumor, MH134 (from a CCl_4-induced hepatoma in a C3H mouse) in C3H/He mice (Wu et al., 1981).

Here, we extended the principle of the previous studies and also analyzed various experimental parameters for effective induction of tumor-specific immunity using 2 lines of syngeneic tumor cells, MH134 and X5563 (from a spontaneous myeloma in a C3H/He mouse). Our results showed that immunization of virus-primed mice with vaccinia vrius-infected tumor cells induced potent tumor-specific resistance in both the MH134 and X5563 tumor system. This indicates the potential applicability of vaccinia virus to the above generalized protocol for augmented induction of tumor-specific immunity.

MATERIALS AND METHODS

1. *Mice*

Female, 5-week-old C3H/HeN mice were purchased from Charles River Japan Inc. (Kanagawa) or kindly supplied from Fujisawa Pharmaceutical Co. (Osaka), and used at the age of 6 weeks.

2. *Tumor cells*

Two cell lines of syngeneic tumors, MH134 (from a CCl_4-induced hepatoma in a C3H mouse) and X5563 (from a spontaneous myeloma in a C3H/He mouse) were used in ascites form. Both lines were maintained and passaged intraperitoneally in C3H/He mice at 10-day intervals.

3. *Vaccinia virus*

The Ikeda strain of vaccinia virus, an attenuated live vaccine for smallpox used in Japan, was grown

in chorioallantoic membranes (CAM) of chick embryos. Infected CAM was homogenized in Eagle's minimum essential medium (MEM) (1 sheet of CAM/ml) and the homogenate was centrifuged at 3000 rpm at 4 C for 15 min. The supernatant fluid was kept at −80 C as stock virus. The infectious titer of the stock virus was usually 2×10^8 plaque forming units (PFU)/ml.

4. *Infection of tumor cells*

Tumor cells collected from ascites of C3H/HeN mice were washed once with MEM, or in the case of X5563 cells, were suspended in hemolysing solution (155 m*M* NH_4Cl, 10 m*M* $KHCO_3$, and 1 m*M* Na_2-EDTA) to remove contaminating red blood cells before washing with MEM. Tumor cells were then infected with vaccinia virus at a multiplicity of infection (moi) of 10 in plastic dishes of 10 cm diameter at 37 C for 2 h. During the incubation period, the dishes were gently shaken at 15 min intervals. After incubation, the cells were washed once with MEM and resuspended in fresh MEM supplemented with 5% calf serum, and incubated at 37 C for an additional 6 h with gentle shaking at intervals. Then the cells were collected by centrifugation and adjusted to 2×10^7 cells/ml with MEM. Usually the viability of vaccinia virus-infected cells was more than 90%.

5. *Tumor-immunization of mice and challenge with tumor cells*

The experimental protocol is shown schematically in Fig. 1. Mice in group 1 received 250 rad whole-body X-ray-irradiation and intraperitoneal (ip) injection of 1×10^7 PFU of vaccinia virus on the same day as X-ray-irradiation (vaccinia virus-priming). After 3 weeks, they were immunized ip 3 times at weekly intervals with 1×10^7 tumor cells, which had been infected in vitro with vaccinia virus and then irradiated with 7000 rad of X-rays. Approximately 60% and 50% of the vaccinia virus-infected MH134 and X5563 tumor cells expressed viral cell surface antigens.

Besides the above fully treated mice, five other groups of mice (groups 2–6) were set up as controls: Group 2 did not receive vaccinia virus-priming, group 3 was immunized with uninfected tumor cells, group 4 did not receive vaccinia virus-priming and was immunized with non-infected tumor cells, group 5 was not immunized with tumor cells, and group 6 received no treatment. Each group consisted of

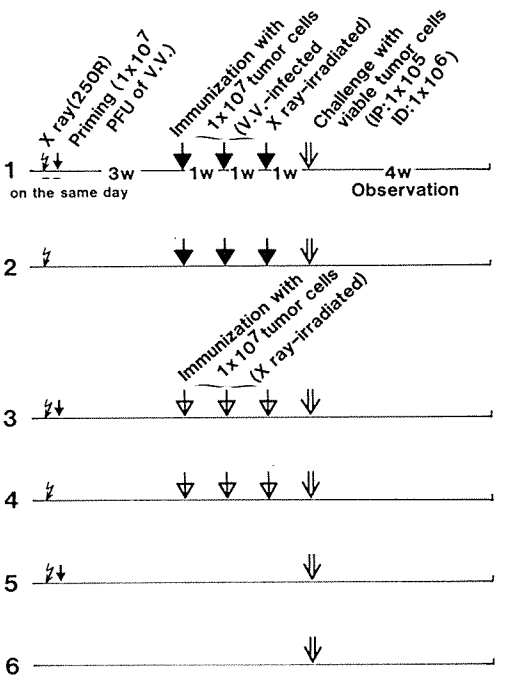

FIGURE 1. Experimental protocol. Numbers on the left denote experimental groups. V. V.: vaccinia virus. For details, see the Materials and Methods.

10 mice, unless otherwise stated.

These pretreated or un-treated mice were challenged ip with 1×10^5 viable tumor cells or intradermally (id) with 1×10^6 viable tumor cells 1 week after the third immunization, and tumor growth was observed for at least 4 weeks after challenge with tumor cells.

RESULTS

1. *Demonstration of systemic effect of augmented induction of tumor-resistance on immunization with MH134 tumor cells*

Previously, we demonstrated that a combination of priming with live vaccinia virus and immunization with vaccinia virus-infected MH134 cells rendered the host resistance to challenge with viable MH134 cells (Wu et al., 1981). In the previous study, we also found that whole-body irradiation with 250 rad of

X-rays to eliminate host suppressor cell activity before inoculation of vaccinia virus was a prerequisite for induction of augmented resistance of the mice to challenge with tumor cells.

In the present study, we extended previous finding and also analyzed various experimental conditions for effective induction of resistance of mice to tumor cell challenge using 6 experimental groups (Fig. 1). Groups 1–5 received 250 rad whole body X-ray-irradiation, while group 6 served as a control for confirming the tumorigenicity of the tumor cells used.

Result are summarized in Fig. 2. All mice in group 6 died due to ascites tumor outgrowth within 3 weeks after ip challenge with 1×10^5 viable MH134 cells. In sharp contrast, all mice in group 1 that had been primed with vaccinia virus and then immunized with vaccinia virus-infected MH134 cells showed complete resistance to ip challenge with viable MH134 cells. Mice in group 2, which had been immunized with infected MH134 cells alone without vaccinia virus-priming, did not show significant resistance to challenge. This indicates that priming with vaccinia virus before immunization with vaccinia virus-infected tumor cells is a prerequistite for the effective induction of resistance to syngeneic tumor cell challenge. In fact, vaccinia virus-priming only in group 5 did not augment induction of tumor resistance. Immunization of vaccinia virus-primed mice with uninfected tumor cells in group 3 also did not induce tumor resist-

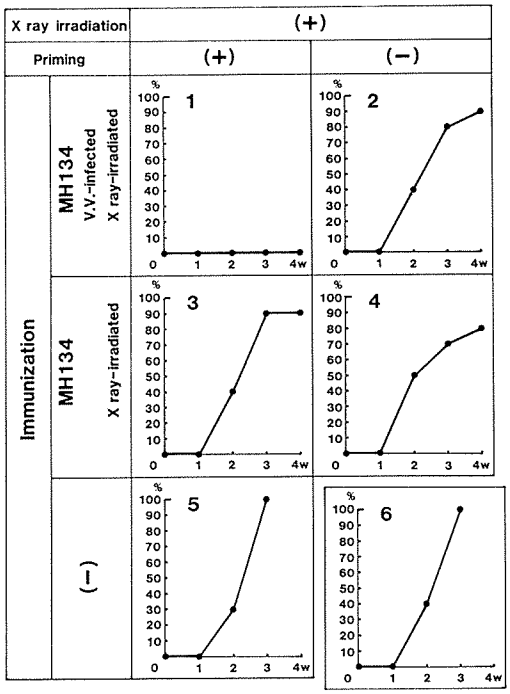

FIGURE 2. Cumulative mortalities after ip challenge with MH134 cells. Numbers in the upper left corner of each pannel denote the experimental groups shown in Fig. 1. Each group consisted of 10 mice. Mice were challenged ip with 1×10^5 viable MH134 cells. Ordinates represent cumulative mortalities and abscissae, the time (weeks) after tumor cell challenge.

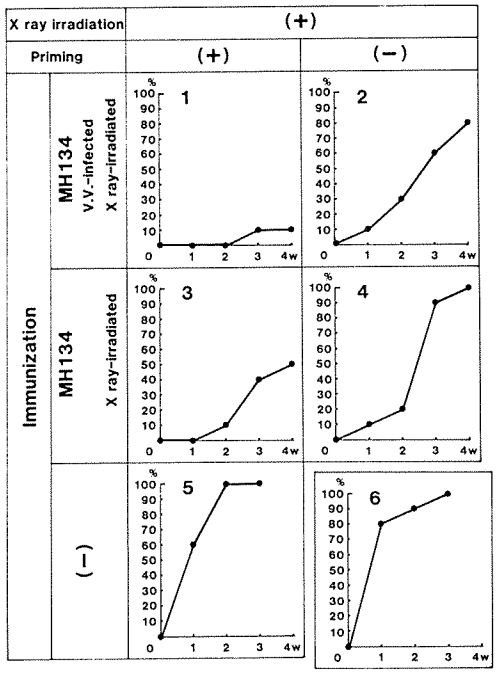

FIGURE 3. Cumulative incidences of tumor outgrowth after id challenge with MH134 cells. Mice were challenged id in the back with 1×10^6 viable MH134 cells. The ordinates shows the cumulative incidence of tumors of more than 5 mm in diameter. See legend to Fig. 2 for other details.

ance.

These results unequivocally demonstrated a critical role of a combinatin of ip priming with vaccinia virus and ip immunization with vaccinia virus-infected tumor cells for augmenting induction of resistance to ip challenge with syngeneic tumor cells.

To determine whether the augmented resistance to the syngeneic tumor cells induced by vaccinia virus in the above protocol was restricted to the immunization site or was systemic, we challenged 6 groups of mice that had received the same pretreatments as for Fig. 2 with 1×10^6 MH134 viable cells intradermally (id) in the back.

Figure 3 summarizes the cumulative tumor-bearing ratios in the 6 groups after id challenge. Again the mice in group 1 showed nearly complete resistance and survived. Mice in groups 5 and 6 developed tumors of more than 5 mm in diameter within 3 weeks after challenge. Mice in groups 2 and 3 showed only partial resistance, as on ip challenge, and eventually died due to outgrowth of the tumors.

2. *Induction of immune resistance to X5563 tumor cells*

To determine whether the above protocol utilizing vaccinia virus was also effective for other syngeneic tumor lines, we conducted a similar experiment using X5563 tumor cells. Figure 4 shows the cumulative mortalities after ip challenge with 1×10^5 viable cells of X5563, and Fig. 5 shows the percentages of tumor-bearing mice after id challenge with 1×10^6 viable cells of X5563. Although re-

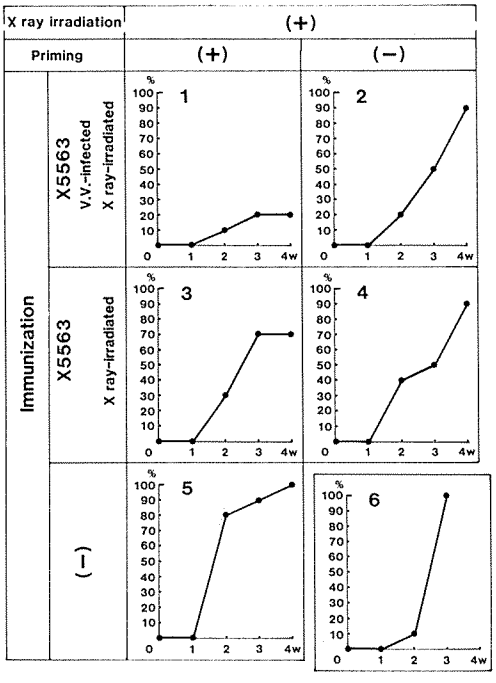

FIGURE 4. Cumulative mortalities after ip challenge with X5563. Mice were challenged ip with 1×10^5 viable X5563 cells. See legend to Fig. 2 for other details.

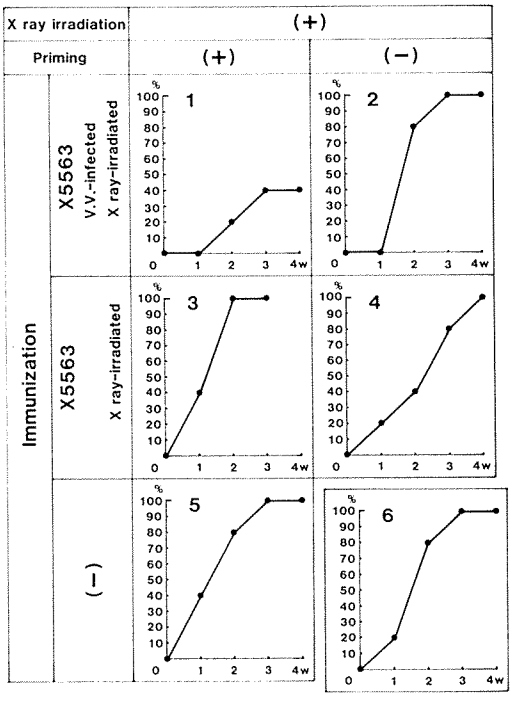

FIGURE 5. Cumulative incidences of tumor outgrowth after id challenge with X5563. Mice were challenged id in the back with 1×10^6 viable X5563 cells. Each group consisted of 5 mice. See legend to Fig. 3 for other details.

TABLE 1. *Survival rates of mice immunized with one line of tumor cells after ip challenge with another line of tumor cells*

Challenge with	Immunization with		
	MH134	X5563	None
MH134	5/5[a]	0/5	0/5
X5563	0/5	3/5	0/5

[a] No. of surviving mice/No. of mice challenged.

sistance to X5563 cells was not so great as to MH134 cells, only the mice in group 1 again showed significant resistance to tumor cell challenge.

3. *Specificity of resistance demonstrated by criss-cross challenge with two lines of syngeneic tumor cells*

Next, we challenged mice that had been fully immunized with one line of tumor cells with the other line of tumor cells to determine the specificity of resistance. Table 1 shows the survival ratios of mice after ip challenge. Mice showed significant resistance to tumor outgrowth only when the same tumor cells were used for immunization and challenge.

DISCUSSION

The present study clearly demonstrated that vaccinia virus augments induction of resistance to syngeneic tumor cells as effectively as TNP or PPD in the model system of Hamaoka et al. Here, we used two lines of syngeneic tumor cells, MH134 and X5563, and challenged mice with the tumor cells by 2 different routes, ip and id, one route being the same as for immunization, and the other different. The present study showed that resistance induced by this method with vaccinia virus was tumor-specific and systemic.

Miyamoto and Kato (1968; 1971) demonstrated that vaccinia virus-infected cells expressed virus-specific antigens on their surface. Ueda and Tagaya (1973) reported that those antigens induced skin reactions in rabbits. Oie and Ichihashi (1981) found that cell surface antigens of vaccinia virus were recognized by cytotoxic T lymphocytes in mice. The broad host range of vaccinia virus and easy modulation of the tumor cell surface by infection with vaccinia virus seem advantageous for augmenting induction of tumor-specific immunity in humans. It may be possible to modulate the cell surface of various tumors even in situ by vaccinia virus infection. Moreover, vaccinia virus has been used extensively in humans as a live vaccine against smallpox. Wallack (1981) studied the effect of application of lysates of vaccinia virus-infected tumor cells in humans. He injected oncolysates many times id into patients with various kinds of tumors and confirmed that these oncolysates were safe. Thus, it is likely that vaccinia virus-induced cell surface antigens stimulate vaccinia virus-specific helper T cells which are generated by vaccination against smallpox, thereby eliciting augmented immune responses against TATA of the tumor cells in cancer patients.

Although not shown in this report, we found by the indirect immunofluorescent technique that mice surviving challenge with MH134 cells after immunization with vaccinia virus-infected tumor cells had high titers of antibody against the surface of MH134 cells. In contrast, mice surviving challenge with X5563 cells showed TATA-specific T-cell response, but did not produce any antibody against the X5563 cell surface. We are currently analyzing the effector mechanisms underlying the specific immune resistance to these 2 syngeneic tumor cell lines.

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