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Tumor Cells Treated with Vaccinia Virus Can Activate the Alternative Pathway of Mouse Complement

Nobutaka Wakamiya,[1,4] Noriko Okada,[2] Yu-Lan Wang,[1] Tetsuya Ito,[1] Shigeharu Ueda,[1,5] Shiro Kato[1] and Hidechika Okada[3]

[1]Department of Pathology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565, [2]Department of Microbiology, Fukuoka University School of Medicine, Fukuoka 814-01 and [3]Department of Molecular Biology, Medical Biotechnology Research Institute, Nagoya City University School of Medicine, Mizuho-cho, Nagoya 467

Vaccinia virus has been shown to render mouse tumor cells highly immunogenic. Since we have demonstrated that induction of complement activating capacity on guinea pig tumor cells by Sendai virus infection causes the tumor cells to become immunogenic, we assumed that vaccinia virus infection of mouse tumor cells might render them reactive with homologous mouse complement. Therefore, murine tumor cells, MH134 and X5563, infected with vaccinia virus (VV) were incubated with mouse plasma and C3 deposition was determined by staining with fluorescein isothiocyanate-labeled anti-C3. We found that VV-infected tumor cells possess the ability to activate the alternative complement pathway (ACP) of murine complement. For induction of complement activating ability, at least a 3 h incubation of the infected MH134 cells was required indicating that the generation of ACP-activating capacity on MH134 infected with VV is time-dependent. Furthermore, ultraviolet-irradiated vaccinia virus was able to induce ACP-activating capacity on tumor cells as well.

Key words: Murine tumors — Vaccinia virus — Alternative pathway

Wallack *et al.*[1] claimed that vaccinia virus (VV[6]) infection of murine tumor cells made the cells immunogenic to the syngeneic host, as does influenza virus.[2] We have also used VV and UV-inactivated VV (UV-VV) as a murine tumor-modifying antigen and succeeded in inducing resistance to syngeneic murine tumors MH134 and X5563, in C3H/HeN mice.[3–5] It has been suggested that VV antigens on cell membranes may contribute as an antigenic determinant with which helper T cells interact to augment the immune response to tumor cells.[6,7] On the other hand, we have demonstrated that Sendai virus infection of guinea pig tumor cells induces complement activating ability (CAA) on the cells which could play a role in the enhancement of their immunogenicity.[8–11] We supposed that VV-infected tumor cells might also gain the capacity to activate murine complement resulting in increased immunogenicity. Furthermore, we have found that VV infection of human tumor cells causes the cells to become reactive with human complement via the alternative pathway.[12] Therefore we have studied reactivity to the murine alternative complement pathway (ACP) of murine tumor cells before and after VV treatment.

MATERIALS AND METHODS

Diluents Gelatin Veronal-buffered saline (GVB) contained 0.1% gelatin (Difco, Detroit, Michigan), 148 m*M* NaCl and 10 m*M* barbiturate buffer (pH 7.4). Mg-EGTA-GVB was prepared by mixing 9 volumes of GVB and 1 volume of a solution containing 20 m*M* $MgCl_2$ and 100 m*M* ethyleneglycol-bis(β-aminoethyl ether)-N,N′-tetraacetate (EGTA); the pH was adjusted to 7.4. Mg-EGTA-GVB was used as a diluent for the complement reaction via the ACP. Ethylenediaminetetraacetate (EDTA)-GVB (pH 7.4) was prepared by mixing 9 volumes of GVB and 1 volume of 100 m*M* EDTA.

Virus The Ikeda strain of vaccinia virus, formerly used as the seed virus of smallpox vaccine in Japan, was grown in HeLa cells. The virus was harvested from infected HeLa cells and purified as described by Joklik[13] with slight modifications.[14] The purified virus was kept at −80°C as stock vaccinia virus (VV). The infectious titer of the stock virus was usually 1×10^9 plaque-forming units (PFU)/ml. VV were ultraviolet (UV)-irradiated

[4] To whom correspondence should be addressed.

[5] Present address: Department of Preventive Medicine, Research Institute for Microbial Diseases, Osaka University, Osaka 565.

[6] Abbreviations: ACP, alternative complement pathway; CAA, complement activating ability; CTL, cytotoxic T lymphocytes; EDTA, ethylenediaminetetraacetate; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-N,N′-tetraacetate; FITC, fluorescein isothiocyanate; GVB, gelatin Veronal-buffered saline; MEM, Eagle's minimum essential medium; moi, multiplicity of infection; MP, mouse plasma; PFU, plaque-forming units, PFUeq, PFU equivalent; PMN, polymorphonuclear leukocytes; UV, ultraviolet; VV, vaccinia virus.

(2×10^4 erg/cm^2/sec) for 5 min to prevent virus replication in infected cells. The amount of UV-irradiated VV (UV-VV) was expressed in terms of PFU equivalent (PFUeq) which indicates the plaque-forming ability before UV-irradiation.

Cells Two cell lines of syngeneic tumor, MH134 (from a CCl_4-induced hepatoma in a C3H mouse) and X5563 (from a spontaneous myeloma in C3H/He mouse) in an ascitic form were used. X5563 tumor cells normally express H-2 antigens, while our laboratory's MH134 tumor cells have lost these antigens. Both cell lines were maintained by intraperitoneal passage in C3H/HeN mice at 10-day intervals.

Tumor cells treated with VV or UV-VV Tumor cells collected from the ascites of C3H/HeN mice were washed once with Eagle's minimum essential medium (MEM), suspended in a hemolyzing solution (155 m*M* NH_4Cl, 10 m*M* $KHCO_3$ and 1 m*M* Na_3-EDTA) to remove contaminating red blood cells and then washed with MEM. Tumor cells were then infected with VV at a multiplicity of infection (moi) of 10 at 37°C for 3, 6, 9, 12 and 24 h. They were also treated with UV-VV at a virion dose equivalent to an moi (PFUeq VV) of 10 at 37°C for 4 and 12 h.

Complement Healthy C3H/HeN mice were bled by heart puncture and the blood was diluted immediately with an equal volume of Mg-EGTA-GVB or EDTA-GVB. Blood cells were removed by centrifugation and mouse plasma (MP) in Mg-EGTA-GVB or EDTA-GVB was prepared as a complement source. About 50% of mouse blood by volume is blood cells, and therefore MP used was at a 1/3 dilution. MP in Mg-EGTA-GVB can be used as a source of complement reaction via the ACP. MP in EDTA-GVB was used as a control where both the classical and the alternative complement pathways are inhibited.

Fluorescein isothiocyanate anti-C3 methods Tumor cells treated with MP in Mg-EGTA-GVB at 37 for 30 min were washed twice with EDTA-GVB containing 0.1% sodium azide (EDTA-GVB-Azide). Complement activation by the test cells was evaluated in terms of the deposition of C3 molecules (C3b, etc.) on the cell membranes as follows. Washed cells were incubated with 15 μl of 10 mg/ml normal sheep IgG at 37°C for 5 min to block Fc receptors, if any, and then treated with 20 μl of fluorescein isothiocyanate (FITC) labeled anti-mouse C3 (FITC anti-C3) (Cappel, Cochranville, PA) in EDTA-GVB-Azide. After incubation at 37°C for 30 min, cells were washed and the extent of FITC staining was determined under a UV microscope and by flow cytometry. Flow cytometric analysis was performed on a FACS IV analyzer (Becton Dickinson, Oxnard, CA) with immunofluorescence measured on a logarithmically amplified scale.

Detection of viral antigens on VV-infected cells Virus-infected tumor cells were treated with anti-VV rabbit serum in EDTA-GVB-Azide at 37°C for 30 min and were washed twice with EDTA-GVB-Azide. The cells were incubated with 15 μl of 10 mg/ml normal sheep IgG at 37°C for 5 min and then treated with 20 μl of FITC-labeled anti-rabbit IgG and rhodamine-labeled anti-rabbit IgG in EDTA-GVB-Azide. After a 30 min incubation at 37°C, the cells were washed twice with EDTA-GVB-Azide and examined under an ultraviolet microscope and by flow cytometry as described above.

Neutralization test by mouse plasma Fifty microliters of 1×10^9 PFU/ml VV strain Ikeda was mixed with 150 μl of MP or heat-inactivated (56°C, 1 h) MP in Mg-EGTA-GVB or EDTA-GVB. After a 1 h incubation at 37°C, the reaction mixtures were diluted in plain MEM and 0.1 ml of each dilution was spread on Vero cells in a 24-well plate (Falcon; Becton Dickinson) which was then incubated at 37°C for 1 h to allow the virus to be adsorbed on the cells. Following removal of the virus solution, 2 ml of agar medium consisting of MEM, 2% fetal bovine serum, and 0.8% agar (Difco) was added. The plate was incubated at 37°C in a CO_2 incubator for 3 days, and was then overlaid with a second agar medium consisting of MEM, 0.8% agar and 0.1% neutral red. After a 2 h incubation, the number of plaques was counted.

RESULTS

ACP-activating capacity of tumor cells infected with VV We have previously demonstrated that a virus-induced antigen is expressed on the surface of cells infected with VV.[15, 16] Cell surface antigen induced by VV was quantitatively analyzed by flow cytometry. At the same time, C3 molecules deposited on the cell membranes through incubation with MP in Mg-EGTA-GVB as a result of ACP activation were examined by flow cytometry (Fig. 1). Appreciable amounts of C3 deposition ability were observed on MH134 cells 3 h after VV infection. C3 deposition was detected on MH134 cells infected with VV in parallel with the viral cell surface antigen expression. The amount of cells infected with VV was over 90% 24 h after infection, while the amount of cells which could show C3 deposition following MP treatment was only 11.2%. Therefore, cells infected with VV do not always possess ACP-activating capacity as detectable by the FITC-anti-C3 method. C3 deposition was seen as a patchy distribution of fluorescein on the membranes of infected cells.

Reactivity of MP with three tumors infected with VV MH134 and X5563 are murine tumor cell lines. Each tumor's viability was greater than 95% as determined by the dye exclusion test with trypan blue. All cells infected with 10 PFU/cell were cultured overnight (12 h) and

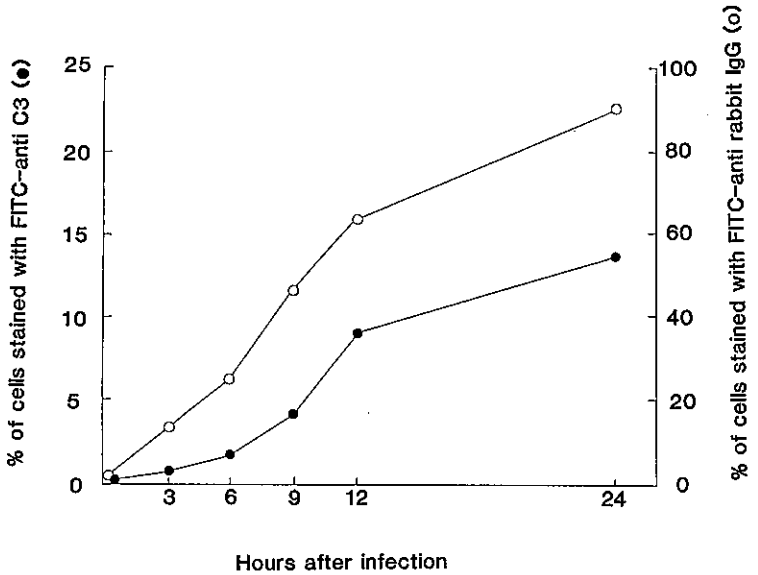

Fig. 1. Time-dependent induction of complement reactivity on MH134 cells following VV-infection. MH134 cells were infected with VV at an moi of 10 and incubated at 37°C for the indicated periods. The VV-infected MH134 cells were stained with FITC-anti C3 following treatment with MP in Mg-EGTA-GVB (●) as well as with FITC-anti rabbit IgG following treatment with anti-VV rabbit serum (○). Amounts of stained cells were measured by flow cytometry.

Table I. FITC-anti C3 Staining Following Mouse Plasma Treatment of MH134 and X5563 Cells Infected with Vaccinia Virus

Cells[a]	Virus	Cells stained with FITC-anti C3 (%)[b, c]	
		Mg-EGTA-GVB	EDTA-GVB[d]
MH134	+	11.2	1.2
	−	0	0
X5563	+	8.8	0.9
	−	0	0

a) These cells were infected with VV at an moi of 10, followed by a 12 h incubation at 37°C.
b) No staining with FITC-anti C3 was observed on uninfected tumor cells following treatment with mouse plasma.
c) Cells stained with FITC-anti C3 were measured by flow cytometry.
d) Diluents used for reaction with mouse plasma before FITC-anti C3 staining.

washed prior to analyzing their reactivity with MP in the presence or absence of Mg^{2+}. C3 deposition was observed after reaction with MP in Mg-EGTA-GVB on those tumor cells which had been treated with VV (Table I).

Neutralization of VV with MP No antibody activity to VV was detected in the mouse serum used as a source of complement by the hemagglutination-inhibition test with chicken erythrocytes or by indirect immunofluorescence using acetone-fixed Vero cells which had been infected with VV. Furthermore, to examine whether VV itself reacts directly with complement, the effect of the MP treatment of VV on their infectivity was determined. VV (5×10^7 PFU in 50 μl) was incubated at 37°C for 1 h with 150 μl of MP in Mg-EGTA-GVB. Table II shows that there was essentially no reduction in infectivity. These results show that virions of VV have no capacity to interact with complement directly via the ACP.

Tumor cells treated with UV-irradiated VV Since VV was not inactivated by direct interaction of the ACP, viral proteins on the infected cells should not be responsible for ACP activation. Furthermore, the induction of ACP reactivity on MH134 infected with VV is time-dependent, and therefore, newly generated membrane molecules may be responsible for the induction. To prevent the generation of progeny viruses on tumor cells, VV was ultraviolet (UV) irradiated as described in "Materials and Methods." To determine the inducibility of ACP reactivity by the UV-VV, MH134 cells were treated with UV-VV at 1, 10 and 100 PFU eq/cell at 37°C for 4 h and were examined for ACP-activating capacity using the FITC anti-C3 method. Table III shows that UV-VV are capable of inducing ACP reactivity on MH134 and that the ACP reactivity is dependent on the amount of UV-VV added. Therefore the production of new progeny viruses on cell membranes is not an essential requirement for ACP activation. In addition, further incubation for up to 12 h of the UV-VV treated cells did not bring about an increase in the population of complement-reactive cells.

DISCUSSION

It has been reported that tumor cell lysate infected with VV has the ability to induce specific immunity

Table II. Absence of Inhibitory Effect of Mouse Plasma on Vaccinia Virus Plaque Formation

Group[a]	Mouse plasma[b]	Diluent[c]	Number of plaques ($\times 10^6$)[d]
1	Fresh	Mg-EDTA-GVB	59±11.2
2	Heated	Mg-EGTA-GVB	51±9.1
3	Fresh	EDTA-GVB	63±21
4	Heated	EDTA-GVB	58±13.4
5	None[e]	PBS	54±8.0

a) Vaccinia virus (5×10^7 PFU in 50 μl) was used for each group.
b) Fresh or heat-inactivated mouse plasma.
c) Diluent used to prepare mouse plasma.
d) Numbers are mean plaque numbers ± standard deviation for four determinations.
e) Vaccinia virus was incubated with PBS alone.

Table III. Percent of Cells Stained with FITC-anti-C3 after Mouse Plasma Treatment of MH134 Cells Absorbed with UV-irradiated Vaccinia Virus

PFUeq/cell[a]	% staining after MP treatment in	
	Mg-EGTA-GVB	EDTA-GVB
0	0	0
1	1.9	0
10	3.0[b]	0
100	7.5[c]	0

a) MH134 cells were adsorbed with various amounts of UV-VV at 37°C for 4 h.
b) No stained cells were observed when MH134 cells had been treated with UV-VV of 10 PFUeq/cell for 1 h.
c) 4.8% of cells were stained when MH134 cells had been treated with UV-VV of 100 PFUeq/cell for 1 h.

against the tumor cells.[7] Yamaguchi *et al.* and Hosokawa *et al.* found that the Friend virus-associated antigen was able to act as the helper antigen for recognition of tumor cells.[17,18] When VV-primed mice were inoculated with VV-infected tumor cells, the mice produced strong tumor-specific resistance against syngeneic murine tumors of MH134 and X5563 cells.[3-5] Further examination showed that VV played a role as the helper antigen in stimulating an increased immune response as detected by an increase in generation of cytotoxic T lymphocytes against X5563 and enhanced production of cytotoxic antibody to MH134.[6] This phenomenon was also verified by *in vivo* experiments using the Winn assay system.[7]

If the expression of antigenic determinants for helper T cells is the essential mechanism for increased immunogenicity of tumor-specific antigens on VV-infected murine tumor cells, any other virus infection and/or chemical modification of tumor cells should bring about new antigenic determinants for helper T cells and have a similar effect on immunogenicity. However, since VV infection has an extraordinarily strong increasing effect on the immunogenicity of tumor cells,[3-5] we suspected that an alternative mechanism could also be involved. As a possible mechanism, we assumed that VV might have induced complement-activating capacity on the infected tumor cells, as has been demonstrated on human tumor cells.[12] It is noteworthy that guinea pig tumor cells become highly immunogenic following Sendai virus treatment, which causes the tumor cells to react with the ACP.[8-11] If complement activation occurs on tumor cells *in vivo*, chemotactic factors and vasoactive peptides would be generated, causing inflammation at the reaction site.[19] This would have the effect of enhancing the immune response to the tumor cells. Therefore we studied the reactivity of VV-infected mouse tumor cells with the ACP. Due to the presence of membrane inhibitors preventing complement action on homologous cell membranes,[20,21] we examined the reactivity of murine tumor cells with homologous mouse complement before and after VV infection. We found that VV has the ability to induce the antibody-independent activation of complement via the alternative pathway on murine tumor cells. Therefore complement activation by tumor cells treated with VV may have stimulated the recognition of the tumor cells by the host's immune system. The generation of chemotactic factors by complement activation on virus-infected cells would induce a local inflammation at the reaction site and bring about an accumulation of polymorphonuclear leukocytes (PMN) and macrophages. PMN and macrophages in the vicinity of tumor cells would then release mediators which stimulate differentiation of lymphocytes.[22,23] In addition, the virus-treated tumor cells would release cytokines such as interferons which effect immune responses. Therefore, further analysis is required of the immunogenicity of mouse tumor cells treated with VV in relation to ACP-activating capacity as well as virus-induced cytokines. This could provide insights into methodology for increasing immunogenicity of human tumor cells by infection with VV, enabling them to react with the homologous human alternative complement pathway. Further, to confirm the direct relationship between the increased immunogenicity and the complement activation capacity generated on the VV-treated tumor cells, we evaluated their immunogenicity in mice which had been treated with cobra venom factor to deplete complement activity *in vivo*. However, we could not get reproducible results. Although CVF treatment could suppress the induction of transplantation resistance of tumor cells, the same CVF treatment enhanced the induction of tumor immunity in other similar experiments. This variation might be due to

the polydirectional effect on immune response of complement fragments generated by CVF administration. CVF inoculation brings about extensive activation of complement via the alternative pathway which results in exhaustion of serum complement. Therefore, at the initial stage after CVF administration, macrophages and polymorphonuclear leukocytes (PMN) are activated by complement fragments such as C5a, C3a as well as C3d, and release IL-1, which stimulates T cell response. Then in the following stages, complement might become exhausted and/or macrophages and PMN might become unresponsive (anergy) to complement fragments. From another standpoint, such variable effects of CVF treatment on immunogenicity of VV-treated tumor cells might indicate that the complement reaction has an appreciable effect on immune response. Finally, the complement activating capacity of VV-treated cells may result in activation of macrophages and PMLs which then stimulate T cell proliferation by releasing IL-1. VV-treatment generates virus-related antigenic determinants on the cell surface which become available to helper T cells as reported previously. Therefore, the complement-activating capacity of the VV-treated cells will enhance immune response synergistically with the generation of antigenic sites for helper T cells. In other words, the extremely high immunogenicity of VV-treated tumor cells would be due to the synergistic effect.

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