Cancer Immunol Immunother (1987) 25: 7–9

Cancer Immunology Immunotherapy

Sensitization of human tumor cells to homologous complement by vaccinia virus treatment

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Summary. Although cell membranes have potent inhibitors which protect the activation of complement on the self cell membranes, some viruses have been shown to activate complement via the alternative pathway on the virus-infected cells. Tumour cells have been made reactive to homologous complement following treatment with such viruses and became highly immunogenic to syngeneic host guinea pigs and mice. Vaccinia virus (VV) made murine tumour cells highly immunogenic thus generating complement activating capacity on the infected cells. Since it has been suggested that VV can make some human tumour cells immunogenic to the cancer patients, we examined VV to see if the virus also has the capacity to make human tumour cells reactive with homologous human complement. Our present results indicate that not only is this the case but ultraviolet-treated VV also has the same effect.

Key words: Complement – Immunogenicity

Introduction

Although the alternative complement pathway can be activated in the absence of antibodies or foreign substances [5], activation on the membranes of homologous normal cells is strictly regulated [9, 11, 13]. It has been demonstrated that restriction of the complement reaction on homologous cell membranes is due to the presence of membrane inhibitors which preferentially inhibit the alternative pathway of homologous complement [9, 11, 13]. Since those membrane inhibitors also exist on most tumour cells, activation of the alternative complement pathway homologous to those tumour cells is also suppressed. However, some virus infections have been demonstrated to induce reactivity to the alternative complement pathway [4, 7, 8, 10, 12, 16] on the infected cells. Tumour cells which have become reactive to complement following such virus treatment would induce inflammation around the tumour cells as a result of complement activation which generates chemotactic factors and vascoactive peptides [2]. In the locus of inflammation, polymorphonuclear leucocytes (PMN) and macrophages are activated to release mediators which stimulate lymphocyte response [6, 14]. Furthermore, the viral antigens on the virus-treated cells might work as antigenic determinants for helper T cells which support the generation of cytotoxic T killer cells [15]. Therefore, we expected that the tumour cells in the inflammation locus might be efficiently recognized by the immune system. Our experiments on guinea pig tumour cells [13] have demonstrated that this would be the case. Furthermore, vaccinia virus (VV), which can make murine tumour cells highly immunogenic [15], has been demonstrated to make the infected mouse tumour cells reactive with homologous mouse complement [17]. Since VV has been shown to increase the immunogenicity of human tumour cells to the autologous host in some patients [1], we investigated if VV has the capacity to make human tumour cells reactive with homologous human complement.

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Materials and methods

Virus. The Ikeda strain of VV, formerly used as a seed virus for smallpox vaccine in Japan, was grown in HeLa cells and purified after Joklik [3] with a slight modification. The purified virus had an infectious titer of 1×10^9 plaque forming units (PFU)/ml and was frozen at $-80°$ C until use. Ultraviolet (UV)-irradiated VV (UV-VV) was prepared by irradiation with UV of 2×10^4 erg/cm^2/per s for 5 min. The amount of UV-VV was expressed in terms of PFU equivalent (PFUeq) which indicates the PFU before UV irradiation.

Cells. C24 is a human melanoma cultured cell line which had been cloned from Seki melanoma in the 24th generation by Dr. M. Shimoyama (National Cancer Center, Tokyo). The C24 cells were maintained in RPMI 1640 medium (Microbiological Ass., Bethesda, Md.) supplemented with 10% fetal calf serum (FCS) (Microbiological Ass., Bethesda, Md.), glutamine and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin).

Treatment of tumour cells with VV or UV-VV. C24 cells washed with plain RPMI 1640 were infected with VV at 10 PFU/cell and incubated at 37° C. Alternatively, C24 cells were incubated with UV-VV at 10 PFUeq/cell at 37° C for 4 and 12 h.

Diluents. Gelatin veronal buffered saline (GVB) contained 0.1% gelatin, 148 m*M* NaCl and 10 m*M* veronal 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* $MgCl_2$ ans 100 m*M* ethyleneglycol-bis(β-amino-

ethyl ether)*N,N'*-tetraacetate (EGTA). EDTA-GVB was a mixture of 9 volumes of GVB and 1 volume of 100 m*M* ethylenediaminetetraacetate (EDTA).

Human plasma. A healty 2-year-old Rh (+) child with blood type 0 was selected as a source of plasma complement since his serum contained no anti-VV antibody detectable either by the haemagglutination inhibition test with chicken erythrocytes or by the immunofluorescein test with acetone-fixed vero cells (a cell line derived from green monkey kidney cells) which had been infected with VV. The child was bled by venipuncture with his parent's agreement and used as a source of complement for in vitro experiments. The blood was diluted immediately with an equal volume of Mg-EGTA-GVB or EDTA-GVB. The blood cells were removed by centrifugation and human plasma (HP) in Mg-EGTA-GVB and EDTA-GVB was prepared. The HP was at a 1:3 dilution in the respective diluents since the haematocrit of the blood was approximately 50%. The HP was aliquoted and frozen at −80° C unitl use.

Detection of complement activation. Complement activation on test cells was evaluated by the deposition of C3 fragments on the cell membranes as a result of complement activation. The deposited C3 was detected by immunofluorescence with fluorescein isothiocyanate (FITC)-labelled antibody to C3 (FITC-antiC3) as follows. Test cells at 5×10^6/ml in GVB were incubated with an equal volume of HP in Mg-EGTA-GVB or EDTA-GVB at 37° C for 30 min and washed twice with EDTA-GVB supplemented with 0.1% sodium azide (EDTA-GVB-Azide). The washed cells were incubated with 15 µl of 10 mg/ml normal sheep IgG to block Fc receptors, if any, and then mixed with 20 µl of FITC-antiC3 (Cappel Lab., Chochranville, Pa., USA) diluted 1:10 in EDTA-GVB-Azide. After incubation at 37° C for 30 min, the treated cells were washed with EDTA-GVB-Azide and the extent of fluorescein staining was determined using an ultraviolet microscope and fluorocytometry. Fluorocytometric analysis was performed on a FACS analyzer IV (Becton Dickinson, Mountain View, Calif., USA) with immunofluorescence measured on a logarithmically amplified scale.

Direct effect on VV of HP. A 50 µl sample of 2×10^9 PFU/ml of VV was mixed with 150 µl of HP or heat-inactivated HP (56° C for 1 h) in Mg-EGTA-GVB or EDTA-GVB. After 1 h incubation at 37° C, the reaction mixtures were diluted in plain Eagle's minimum essential medium (MEM) and 0.1 ml of each dilution was spread on vero cells in a 24-well plate (Falcon, Cockeysville, Md., USA) which was then incubated at 37° C for 1 h to adsorb the virus to the cells. Following removal of virus solution, 2 ml of agar medium consisting of MEM, 2% FCS and 0.8% agar was added. The plate was incubated at 37° C in a CO_2 incubator for 3 days, and then overlayed with a second agar medium consisting of MEM, 0.8% agar and 0.1% neutral red to each well. After 2 h incubation, the number of plaques were counted.

Results

C24 cells infected with VV (10 PFU/cell) were harvested after 1, 4, 12, 24 and 48 h incubations at 37° C. The treated cells were suspended at 5×10^6/ml in Mg-EGTA-GVB or EDTA-GVB and mixed with an equal volume of HP in Mg-EGTA-GVB or in EDTA-GVB. The mixtures were incubated at 37° C for 30 min and the cells washed to determine the amount of C3 deposited on the cell membranes as a result of complement activation. As shown in Table 1, VV-infected C24 cells had the capacity to activate human complement via the alternative pathway and the capacity was time-dependent. To determine if UV-VV was also able to induce complement reactivity on tumour cells, C24 cells were treated with UV-VV for 4 h. UV-VV also had the capacity to induce reactivity to complement on the melanoma cells (Table 2). It required 4 h incubation to induce complement activating capacity on the UV-VV infected cells. However further incubation up to 24 h did not increase the capacity.

It was conceivable that the complement activating capacity on the VV-treated tumour cells might be due to the complement activation by viral particles generated on the infected cell membranes. If the VV had the capacity to activate human complement directly, VV may be inactivated by human complement. Therefore we incubated VV with HP in Mg-EGTA-GVB and evaluated the infectivity of VV after the incubation. Since essentially no reduction in infectivity was noted, VV particles had no direct reactivity to hards human complement.

Table 1. C3 deposition on vaccinia virus (VV)-infected C24 cells following human plasma treatment

Pretreatment with human plasma in	% of fluorescein isothiocyanate (FITC)-antiC3 stained cells[a] infected for					
	0[b]	1	4	12	24	48 (h)
Mg-EGTA-GVB	0	0.8	1.3	5.0	15.4	30.2 (%)
EDTA-GVB	0	0	0	0	0.5	1.0

[a] % of stained cells was determined using flow cytometry
[b] C24 cells were not mixed with VV

Table 2. C3 deposition on ultraviolet (UV) VV-treated C24 cells following human plasma treatment

Dose of UV-VV (PFUeq/cell)	FITC-antiC3 stained cells[a] following human plasma treatment in	
	Mg-EGTA-GVB	EDTA-GVB
0	0 (%)	0 (%)
1	0.8	0
10	1.7	0
100	4.8	0

[a] % of stained cells was determined using flow cytometry

Discussion

Since virus infection of tumour cells generates virus-related antigens on the infected cells, the antigens may act as antigenic determinants for helper T cells which may support the propagation and differentiation of effector T cell as well as B cells specific to the tumour antigens on the cells [15]. Furthermore, if virus infection has the additional capacity to make the infected tumour cells reactive with

homologous complement, an inflammatory reaction would occur at the inoculation site of the virus-infected tumour cells because complement activation generates inflammatory mediators such as chemotactic factors and anaphylatoxins [2]. Thus the inflammation generated might promote the host's immune response to the tumour cells since PMN and macrophages accumulated and activated at the inflammation locus release mediators such as interleukin 1 which stimulates proliferation of lymphocytes [6, 14]. Therefore, we anticipate that the complement activating capacity on tumour cell membranes induced by virus infection would efficiently stimulate the immune response to the tumour antigens caused by the effect of helper T cells which interact with the viral antigens on the cell membranes. We have demonstrated that Sendai virus-infected guinea pig tumour cells [13] became reactive with homologous complement and highly immunogenic. The effect of complement reaction on tumour cells in vivo was verified by growth of Sendai virus-treated tumour cells in guinea pigs depleted of complement by administration of cobra venom factor [13].

Since VV infection of murine tumour cells made them so immunogenic perhaps VV might also make human tumour cells immunogenic. Some preliminary trials to treat cancer patients with VV-infected tumour cell vaccines have indicated that the vaccines may be able to induce an immunological response to the tumours in some instances [1]. We anticipated that VV might have increased the immunogenicity of tumour cell vaccine by inducing the capacity to make human melanoma cells reactive to homologous human complement. Advantageously, the present results demonstrated that VV and UV-VV can make C24 human melanoma cells reactive to homologous complement. The same result was also obtained in experiments with Molt 4F cells, a cultured T cell leukaemia cell line (data not shown) indicating that the phenomenon described here is not particular to C24 cells.

Acknowledgements. We thank Mr. M. Mihara and Miss I. Sakakibara for their help in FACS analysis. This study was supported by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, from the Ministry of Health and Welfare, Japan, from The Naito Foundation, and from the Mochida Memorial Foundation.

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Received September 29, 1986/Accepted March 9, 1987