Activation of Homologous Complement Via the Alternative Complement Pathway by Quail Cells Transformed by Rous Sarcoma Virus

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ABSTRACT. After incubation of the cells with fresh quail serum, deposition of the third component of complement (C3) was demonstrated on the cell surface of various quail cell lines transformed by Rous sarcoma virus (RSV) as well as on that of primary quail embryo (QE) cells transformed by RSV. The C3 deposition occurred irrespective of virus production. On the other hand, the C3-deposition was not observed on two quail cell lines transformed by a chemical carcinogen, QE cells infected with avian leukosis virus or normal QE cells. Moreover, QE cells infected with a temperature-sensitive mutant of RSV activated the complement at 37°C but not at 41°C. Since the progeny virus was generated even at 41°C, viral molecules on the cell surface may not play an essential role for the activation. The activation of complement was blocked by EDTA but not by EGTA-Mg⁺⁺. Therefore, the complement activation on the transformed cells appears to be mediated via the alternative complement pathway (ACP). Similar results were obtained with the complement consumption test; the residual cytolytic activity of fresh quail serum via ACP was markedly reduced by pre-incubation of the serum with transformed cells.—**KEY WORDS:** ACP, activation, complement, quail, transform.

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Autochthonous tumors induced by Rous sarcoma virus (RSV) in Japanese quails have been extensively investigated by the microcytotoxicity assay as a model to analyze the host responses to viral tumors, and the involvement of both cell-mediated immunity and humoral factors has been demonstrated [6]. In addition to these immunological responses, we observed spontaneous lysis of a RSV-induced quail tumor cell line (QT) by the incubation with fresh quail serum and found that this cytotoxicity was mediated by antibody-independent activation of complement via the alternative pathway [22].

To obtain direct proof for the complement activation by the alternative pathway, we examined in this study the deposition of the third component of quail complement (C3) on the surfaces of RSV-transformed cells incubated with fresh quail serum by using monospecific antibody to quail C3 [8] which was obtained in our previous study on the identification of quail C3 and became to enable this kind of study.

MATERIALS AND METHODS

Cells: The QT cells are transformed, non-producing cells derived from a quail tumor induced by the Schmidt-Ruppin strain of RSV (SR-RSV). The QERC-31N [21] and QERC-31F [23] are virus-producing cells derived from quail embryo (QE) cells transformed by SR-RSV and by avian erythroblastosis virus, respectively. The PERY cells are virus-producing transformed cells derived from peritoneal exudate cells infected with SR-RSV. The MCQ1-4 and MCQ3-5 cells were established from quail tumors induced by 3-methylcholanthrene (MCA) [21]. The Rous (-) cells are non-producing transformed cells derived from QE cells infected with the gp 85-defective Bryan strain of RSV [5],

and were supplied by Dr. R. Friis.

The primary culture of QE cells was prepared in plastic dishes (Falcon #3003) from 7-day-old embryos by using Dulbecco's modified Eagle's minimum essential medium (DEMEM) supplemented with 10% tryptose phosphate broth (TPB) and 5% newborn calf serum (NCS) as a growth medium.

Viruses: The SR-RSV was propagated in QE cells. The stock virus had an infectivity titer of 1.2×10^5 focus forming units (FFU)/ml when assayed in QE cells. The subgroup A avian leukosis virus (RAV1) was propagated in QE cells. Infectivity titer of the stock virus was 2×10^5 infectious units (IU)/ml when assayed in QE cells by the interference test with RSV. Ts-68 strain of RSV, which was isolated from SR-RSV grown in the presence of 5-fluorouracil and is a temperature sensitive mutant in transforming capacity, was supplied by late Dr. S. Kawai, Institute of Medical Science, the University of Tokyo [10]. The infectivity titer of the stock virus was 1×10^7 FFU/ml when assayed at a permissive temperature in chicken embryo cells. The QE cells were infected with these viruses and subcultured for more than three passages before use.

Growth curve of Ts-68 virus in QE cells: Ts-68 virus was inoculated to QE cells in a plastic dish at a multiplicity of infection (moi) of about 10. After 1 hr of adsorption at 37°C or 41°C, the cultures were washed five times with prewarmed medium and incubated further with fresh medium at 37°C or 41°C. At various times after infection, culture supernatants were collected and replaced with prewarmed fresh medium. The titer of focus-forming virus in the supernatant was determined at 37°C using chicken embryo cells.

Fresh quail serum (FQS): Twenty quails at 5 to 10 weeks of age were bled by heart puncture. The serum was

separated in ice bath, pooled and stored at -80°C .

Immunofluorescence technique: The deposition of C3 on the cell surface was examined by an indirect immunofluorescence technique using anti-quail C3 rabbit serum [8] and anti-rabbit IgG sheep IgG conjugated with fluorescein isothiocyanate (Cappel Lab. Inc., PA). Both sera were used after two times of absorptions with QE cells at 37°C for 30 min each. The cells grown on glass slides were air-dried, fixed in acetone at -20°C for 20 min and incubated in fresh quail serum with or without 10 mM ethylenediamine tetraacetate (EGTA) containing 2 mM MgCl_2 at 37°C for 2 hr, and then incubated at 37°C for 30 min with 1/50 diluted anti-quail C3 serum. After washing well in phosphate buffered saline (PBS), they were stained at 37°C for 30 min with 1/30 diluted FITC-labeled anti-rabbit IgG sheep IgG.

Consumption assay of complement via the classical and alternative pathways: The procedures were as described previously [9]. Briefly, complement activity via the classical pathway (CCP) was assayed by using rabbit red blood cells (RRBC) sensitized with quail serum containing natural hemolysin to RRBC. Two-fold dilutions of test serum in GVB⁺⁺ were mixed with a half volume of 0.5% sensitized RRBC suspension, incubated at 37°C for 1 hr, and reciprocals of the highest dilution giving 50% hemolysis were taken as complement titers via the CCP.

Complement activity via the ACP was assayed by using the QT cells [22]. Two-fold dilutions of test serum in PBS containing 10 mM EGTA plus 2 mM Mg^{++} were added to five wells of microtiter plates (Falcon, #3034) each containing approximately 500 QT cells. After incubation at 37°C for 1 hr, reciprocals of the highest dilution of serum that caused lysis of 50% of the target cells were taken as complement titer via the alternative pathway (ACP).

Treatment of transformed cells bearing C3b with Nonidet P40 or methylamine: The QE cells transformed by Ts-68 virus were grown on glass slides, air dried and fixed in acetone at -20°C for 20 min. Then the cells were treated with 1% Nonidet P 40 in PBS at 37°C for 2 hr or with 50 mM methylamine-HCl (Wako Pure Chemicals, Tokyo) in GVB⁺⁺ at 37°C for 1 hr. These samples were washed well and examined by immunofluorescence assay.

RESULTS

Deposition of C3 on quail transformed cells: As summarized in Table 1, the four RSV-transformed cell lines, the QERC-31N, PERY, QT and Rous (-), and one avian erythroblastosis virus-transformed cell line, the QERC-31F, had C3 deposition (Fig. 1). Thus, the complement was thought to be activated by these virus-induced tumor cells regardless of virus production, type of transforming viruses, or origin of transformed cells. The C3 deposition was not demonstrated on the two lines of tumor cells induced by methylcholanthrene.

The passage of these cell lines through many generations suggests the possibility of selecting a specific type of cell that can activate the complement. Therefore, we tested the complement activation by primary QE cells and those infected with RAV1 or SR-RSV. C3 deposition was found on RSV-transformed QE cells but not on RAV1-infected cells or on normal QE cells (data not shown).

C3 activation by Ts-68 virus-transformed cells: To confirm whether the C3 deposition on the cell surface is associated with transformation, we tested the complement

Fig. 1. C3 deposition on QT cells detected by the indirect immunofluorescence (IF) technique. After fixation in acetone, cells were treated with FQS (left) or in FQS with 10 mM EDTA (right) and stained by indirect IF technique using anti-quail C3 serum and anti-rabbit IgG sheep IgG conjugated with fluorescein isothiocyanate.

Table 1. Activation of C3 by quail tumor cell lines

cell lines	transforming agent	origin	virus production	C3 deposition ^{c)}
QERC-31N	RSV	<i>in vitro</i> QE cells	+	+
PERY	RSV	<i>in vitro</i> peritoneal exudate cells	+	+
QT	RSV	<i>in vitro</i> QE cells	-	+
Rous (-)	defective RSV	<i>in vitro</i> QE cells	-	+
QERC-31F	AEV ^{a)}	<i>in vitro</i> QE cells	+	+
MCQ1-4	MCA ^{b)}	<i>in vivo</i>	-	-
MCQ3-5	MCA	<i>in vivo</i>	-	±

a) avian erythroblastosis virus.

b) methylcholanthrene.

c) C3 deposition was examined by the immunofluorescence test.

activation by QE cells infected with Ts-68 virus. Ts-68 virus was isolated from SR-RSV, and was demonstrated to be temperature-sensitive in morphological transformation but not in virus production in chicken embryo cells [10].

QE cells were fully transformed at 37°C at 3 days after infection with Ts-68 virus showing round and refractile morphology. When the transformed cells were incubated at 41°C for 24 hr, they exhibited a normal flat form. By reducing the temperature from 41°C to 37°C, these cells again showed transformation.

Multiplication of Ts-68 virus in QE cells at 37°C and at 41°C occurred at a similar level yielding about the same titer of virus (Fig. 2). By the indirect immunofluorescence assay using anti-RAV1 rabbit serum, the viral antigens were detected on the QE cell surface at both 37°C and 41°C, 10 days after infection with three passages (data not shown).

The C3 deposition was found on QE cells transformed by Ts-68 virus at 37°C, but not on the cells with a normal phenotype at 41°C (Fig. 3). These results indicate that the C3 deposition on the cell surface is associated with morphological transformation.

Fig. 2. Growth of Ts-68 virus in QE cells at 37°C (●----●) or 41°C (○----○). Virus was inoculated to QE cells at a multiplicity of infection of 10. At various times after infection, culture fluids were collected and replaced with prewarmed fresh medium. The titer of focus-forming virus in the culture fluid was assayed at 37°C in chicken embryo cells.

Analysis of complement activation pathway: EDTA and EGTA, which chelate divalent cations, were used to investigate dependency of the C3 deposition on Ca^{++} and Mg^{++} ions. EDTA at a concentration of 10 mM completely blocked the C3 deposition on the virus-transformed cells in Table 1 (data not shown). However, the 10 mM EGTA containing 2 mM MgCl_2 failed to block the deposition on any of these cell lines. These results indicate that the C3 deposition on these cells requires Mg^{++} ion but not Ca^{++} ion.

To investigate whether the complement activation is caused by the CCP or ACP, consumption of complement components of each pathway were measured after incubation of FQS with various numbers of QE cells, QT cells, MCQ3-5 cells, QE cells transformed by Ts-68 virus, or quail red blood cells (QRBC). As shown in Fig. 4, the QT cells and QE cells transformed by Ts-68 virus caused marked reduction of the complement activity via both the CCP and ACP; especially the activity via the latter was completely reduced after incubation with each 10^8 cells. Moderate reduction via the both pathways was caused by QE cells and MCQ3-5 cells. No reduction was observed by QRBC as the control. These results indicate that the deposition of C3 on these cells is caused by complement activation mainly via the ACP.

Removal of C3 deposited on transformed cells: To determine the binding nature of C3 to transformed cell surface, QE cells transformed by Ts-68 virus at 37°C were fixed, incubated with FQS and then treated with 1% Nonidet P 40 or 50 mM methylamine. The C3 deposited on the cell surface was not removed by treatment with Nonidet P 40, but was removed completely by treatment with methylamine (Fig. 5).

DISCUSSION

Using the anti-quail C3 antibody, the C3 deposition was detected by the indirect immunofluorescence technique after

Fig. 3. C3 deposition on QE cells infected with Ts-68 virus detected by indirect IF technique. C3 deposition was observed in QE cells transformed with Ts-68 virus at 37°C (left), but not in QE cells which showed a normal phenotype at 41°C (right).

Fig. 4. Consumption of serum complement activity by incubation with various cells. Residual complement activities via CCP (A) and ACP (B) of FQS were assayed after incubation with QT cells (●—●), MCQ3-5 (■—■), QE cells (○—○), QE cells transformed with Ts-68 virus (★—★) or QRBC (△—△). The titers were expressed as reciprocal of the highest serum dilution giving 50% hemolysis of sensitised RRBC via CCP (A), and QT cell lysis via ACP (B).

Fig. 5. Removal of C3 deposited on Ts-68 virus-transformed QE cell surface by treatment with methylamine. Cells were incubated with 1% Nonidet P40 at 37°C for 2 hr (left), or with 50 mM methylamine at 37°C for 1 hr (right).

incubation of the cells with FQS on the cell surface of primary QE cells transformed by RSV and by ts-mutant of RSV at permissive temperature as well as for all the five quail cell lines transformed by the avian retroviruses. However, the C3 deposition was not observed on the two chemically transformed cell lines, QE cells infected with RAV1 and those infected with the ts-mutant of RSV at non-permissive temperature. These findings indicate that the C3 deposition on the surface was associated with transformation by the avian retrovirus.

The antibody-independent activation of human complement leading to the lysis of the viruses [2, 3, 7, 15, 19, 20] was suggested for murine, feline and avian retroviruses. In these instances, virion serves as complement activator mainly via the CCP. In the present study, the C3 deposition was found on the surface of all retrovirus-transformed quail cells tested regardless of the production of progeny viruses. Moreover, the deposition of C3 was not detected on RAV1-infected QE cells which produce viruses but remained untransformed. QE cells infected with Ts-68 virus, which produces virus in QE cells at both 41°C and 37°C but has transforming capacity only at 37°C, failed to activate the complement when the cells had been cultivated at 41°C, while they activated quail complement when transformed morphologically at 37°C. These findings indicate that the complement-activating capacity is closely related to the morphological transformation and that the molecules responsible for the complement activation on RSV-transformed cells might not be viral components, but some molecular changes induced by transformation.

The C3 deposition on these RSV-transformed cells occurred in the presence of EGTA-Mg⁺⁺ but not in the presence of EDTA. The similar dependency on Mg⁺⁺ ion was found in the consumption of complement after the incubation with QT cells or QE cells transformed by Ts-68. This suggests that the complement activation is mediated via the ACP.

The consumption of the complement also indicates that the cascade of complement was induced, and that the deposition of C3 on RSV-transformed cells was due to complement activation but not to direct adsorption of C3 molecules to some complement receptors on the cell surface. The C3 fragments binding to the surface of the transformed QE cells was removed by treatment with methylamine, a nucleophilic reagent, but not by Nonidet P 40. Since Nonidet P 40 destroys the hydrophobic bond but not covalent bond and methylamine abrogates covalent bond, the binding form of C3 fragments to the cell membrane appears to be covalent bond. Since the binding of C3b to C3 receptors is mediated by a hydrophobic bond and that to the acceptor by a covalent bond, the C3 may bind to putative C3 acceptor. The result is compatible with the fact that fibroblasts lack C3 receptors in normal stage.

Significance of complement activation via the ACP in nonspecific immunity especially at an early stage of infection has been indicated in such infections as bacteria [18] and trypanosoma [11]. However, activation of the ACP in these studies was demonstrated by using sera derived

from heterologous animal species [4]. Except a few cases such as bovine respiratory syncytial virus-infected cells [12], homologous combinations of cells and sera have been reported only for transformed cells such as spontaneous tumors [17], human B cell lymphoma lines associated with Epstein-Barr virus [1, 13, 14, 16]. As these cell lines have C3 receptors, the complement activation via the ACP may be related to C3 receptors. Thus, the present system may provide a unique model to investigate significance in tumor immunity of homologous complement activation possibly involving C3b binding to C3 acceptor.

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