

LYSIS OF RNA TUMOR VIRUSES BY HUMAN SERUM:
DIRECT ANTIBODY-INDEPENDENT TRIGGERING OF THE
CLASSICAL COMPLEMENT PATHWAY*

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Type C RNA tumor viral (oncornavirus) genetic information, viral proteins, and intact virions have been detected in malignant tissues from many species of animals including cats, chickens, and mice by biochemical, immunological, and ultrastructural techniques (1-3). Hallmarks of the presence of RNA tumor virus genomes have also been found in human malignant tissues. These indicators include cellular nucleic acid sequences homologous to those of oncornavirus RNA (4, 5), RNA-dependent DNA polymerase (reverse transcriptase) functionally (6, 7) and antigenically (8) related to oncornavirus transcriptase, intracellular proteins reactive with antisera against oncornaviral proteins (9-12), and intracellular virus-like particles (13). Oncornaviral genetic information or proteins have also been found in normal cells from a variety of species (1), and some evidence suggests that this is also true of human cells (9, 14). C-type particles have been found in normal human placentas (15). Furthermore, oncornaviruses can productively infect human cells (16, 17) and under certain conditions transform such cells (18). These observations tend to suggest a major role for C-type oncornaviruses in human leukemias. However, viremia is not a characteristic feature of human leukemia in contrast to leukemias of other animals (1, 2). Although oncornaviruses can be antigenic in man (19), antibodies to viral antigens have only infrequently been found in the sera of patients with leukemias (20-22), yet circulating antibody is a common feature of leukemias in other animals (1, 21, 23, 24). Recently oncornaviruses have been isolated from cultures of human leukemic tissues in several laboratories (25-27), but the long history of earlier failures (13, 28) indicates that expression of intact oncornaviruses is not a prominent feature of human leukemias. Taken together, these studies indicate that some oncornaviral genetic information is present in human leukemic and perhaps normal cells but this information is not reflected in the production of oncornaviral antigens or particles. In seeking an explanation for this apparent failure of extracellular expression, we found that normal human serum, but not

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chicken, guinea pig, rabbit, rat, or most murine sera, had the ability to inactivate a number of oncornaviruses from avian, feline, murine, and simian sources as measured either by plaque or focus reduction assays (29, 30). The mechanism of inactivation was by viral lysis since the internal enzyme, RNA-dependent DNA polymerase (RDDP)¹ and also viral RNA were released after incubation of oncornaviruses with primate but not with nonprimate sera. Inactivation was complement (C)-dependent as heated human sera and C2- or C4-deficient human sera did not produce viral lysis. We were unable to find a role for antibody in this process (29, 30).

We report here an analysis of the mechanism of action of the C system in the inactivation process. These studies show that the C1q subunit of the first human C component subserves a specific recognition function which enables the C system to act as a natural defense mechanism against oncornavirus infection and replication in man. Human C1q attaches directly to the oncornavirus envelope in the absence of immunoglobulin. Binding of C1 via C1q in this manner leads to activation of C1 and thus of the classical C pathway, accompanied by deposition of C components on the viral surface and lysis on completion of the C sequence.

Materials and Methods

Chemicals and Reagents. Sodium barbital and Tris (Sigma Chemical Co., St. Louis, Mo.) were used to prepare buffers. DNase I and RNase were purchased from Worthington Biochemical Corp., Freehold, N. J.

C, Components, and C Reagents. Fresh serum frozen in portions at -70°C was employed as a source of C. Human C1q was isolated from fresh serum by the method of Yonemasu and Stroud (31) and homogeneity verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Preparations of C1q employed in this study showed no IgG, IgA, or IgM when tested with appropriate antisera (Behring Diagnostics, American Hoechst Corp., Somerville, N. J.) in Ouchterlony analyses. Proenzyme C1r (32), proenzyme C1s (33), C2 (34), C4 (35), and factor B (36) were isolated from fresh human serum by using the published methods. The cellular intermediates, EAC1, EAC14, and EAC1423 were prepared with human components as previously described (34, 37).

Human serum genetically deficient in C2 was obtained from patient T. H. (38) and C8-deficient human serum from an individual homozygous for C8 deficiency (39). Fresh human serum was depleted of C4 or factor B by addition of 1×10^{-2} M EDTA and passage through an agarose column containing the IgG fraction of antiserum to the appropriate protein coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). After separation, sera were concentrated to their original volume and dialyzed against veronal-buffered NaCl containing 1.5×10^{-4} M calcium and 5×10^{-4} M magnesium and stored in aliquots at -70°C . For some studies factor B was depleted by heating at 50°C for 20 min. The absence of each respective protein was verified by Ouchterlony analyses. Measurements of functional activity of the depleted component showed less than 1% of the original activity. The C2-, C4-, and C8-depleted sera employed in this study could be restored to full CH_{50} (dilution of normal serum giving 50% lysis of sheep erythrocytes sensitized with IgM rabbit antiserum) activity by addition of the missing component in highly purified form, usually together with C1q. These sera possessed an intact alternative

¹ *Abbreviations used in this paper:* CH_{50} , the dilution of normal serum giving 50% lysis of EA; DTT, dithiothreitol; EA, sheep erythrocytes sensitized with IgM rabbit antiserum; MLV, Moloney leukemia virus; MuLV, murine leukemia virus; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PRLV, primate Rauscher leukemia virus; RaLV, rat leukemia virus; RDDP, RNA-dependent DNA polymerase; RLV, Rauscher leukemia virus; SDS, sodium dodecyl sulfate; STE, buffer containing 1×10^{-2} M Tris, 1×10^{-1} M NaCl, and 1×10^{-3} M EDTA, pH 7.5; TMP, deoxythymidine monophosphate; WHS, normal human serum.

pathway as evidenced by their ability to sustain C3 cleavage after the addition of inulin (39). The factor B-depleted serum used could be reconstituted by isolated factor B with regard to C3 cleavage after inulin addition; the classical pathway was essentially intact in these sera since the CH_{50} values were approximately 50% of normal.

Viruses and Labeling. Most of the viruses employed in this study and their cellular origins and methods of propagation have been described (30). In addition primate Rauscher leukemia virus (PRLV) propagated in rhesus embryonic monkey cells (40), Moloney leukemia virus (MLV) obtained from the NCI, and MLV isolated from SCRF 179 murine lymphoblasts (MLV-179S) and MLV from murine NIH-Swiss 3T3 fibroblasts (MLV-FAN) were used for some experiments. Xenotropic murine leukemia virus was isolated from testes of New Zealand Black mice by cocultivation with rabbit SIRC cells.

Viruses were obtained from filtered supernatant fluids by sedimentation through a layer of 15% sucrose (wt/vol) onto a cushion of 50% sucrose (wt/vol). Centrifugation was for 60 min at 25,000 rpm (105,000 *g*) in a Beckman SW 25 Rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). This step was repeated after which the virus was purified by isopycnic sedimentation in a 15–50% (wt/vol) sucrose density gradient. Centrifugation was for 16 h at 35,000 rpm (155,000 *g*) in a Beckman SW 41 Rotor in a 1×10^{-2} M Tris, 1×10^{-1} M NaCl, 1×10^{-3} M EDTA buffer, pH 7.5, (STE).

The RNA of rat leukemia virus was labeled by propagating infected Brown Norway rat fibroblasts in the presence of ^3H -uridine (10 $\mu\text{Ci/ml}$) for 21 h. Labeled virus was purified as described above. MLV was externally labeled with ^{125}I by incubation of 0.1 ml of purified virus with 0.5 mCi of carrier-free Na ^{125}I (50 mCi/ml; New England Nuclear, Boston, Mass.), 1 mg of human serum albumin, and 50 μg of lactoperoxidase (Calbiochem, San Diego, Calif.) in a total vol of 150 μl for 10 min at room temperature. During this time two additions of 25 μl of 0.03% H_2O_2 were made. The mixture was then layered over a 20–65% sucrose density gradient in 1×10^{-2} M Tris, 1×10^{-1} M NaCl buffer, pH 7.5, containing 10 mg/ml human serum albumin and centrifuged at 50,000 rpm for 60 min in a Beckman SW 50 rotor. Under these conditions the radiolabeled virus formed a discrete peak of radioactivity in the center of the gradient.

Sucrose Density Gradient Ultracentrifugation. C-mediated release of ^3H -RNA from MLV was examined by equilibrium sedimentation. MLV, internally labeled with ^3H -uridine, was reacted with an equal volume of serum or serum derivatives for 30 min at 37°C and then layered over a 15–50% (wt/vol) continuous sucrose density gradient in STE buffer and centrifuged for 16 h at 35,000 rpm in an SW 41 Rotor. The gradients were fractionated and the refractive index of each fraction was determined in a Bausch & Lomb refractometer (Bausch & Lomb Inc., Scientific Optical Products Div., Rochester, N. Y.). The radioactive counts were determined after the addition of Aquasol (New England Nuclear) containing acetic acid and water in a Beckman liquid scintillation system.

The interaction of ^{125}I -MLV with serum or serum derivatives was examined by rate zonal sucrose density gradient sedimentation. Equal volumes of ^{125}I -MLV and serum or derivatives were incubated for 30 min at 37°C after which the mixtures were layered over 20–65% (wt/vol) sucrose density gradients formed in a 5×10^{-2} M Tris, 1×10^{-1} M NaCl buffer, pH 7.5. Centrifugation lasted for 60 min at 50,000 rpm in a Beckman SW 50.1 Rotor. Afterward the gradients were fractionated, and radioactivity was determined in a Beckman Biogamma II spectrometer. In some experiments a pool of fractions comprising the peak of radioactivity was incubated with the IgG fraction of antiserum to C4 for 30 min at 37°C and re-examined in a second identical rate zonal sucrose density gradient separation.

Binding of C1q to MLV was examined by incubating ^{125}I -C1q (approximately 1 μg) with MLV for 30 min at 37°C in a 5×10^{-2} M Tris, 1×10^{-1} M NaCl buffer, pH 7.5, adjusted to 7 mmho/cm at 22°C by addition of sucrose containing barbital buffer, pH 7.5. The mixtures were then layered over 20–65% (wt/vol) sucrose density gradients and processed as described above.

C1q Deviation Test. This test was performed as previously described (41) with certain modifications in the buffer to compensate for the lack of serum. 10 μl of ^{125}I -C1q (1 μg) were incubated with varying amounts of MLV in a total vol of 0.25 ml in a barbital-buffered, NaCl, and sucrose containing buffer, pH 7.5, having a conductance of 9 mmho/cm (41). After incubation for 20 min at room temperature, 0.2 ml of the same buffer containing 4×10^8 sheep erythrocytes (E) coated with rabbit IgG anti-sheep E (EA) were added and incubation continued for 10 min at room temperature. 200- μl samples were taken in duplicate from each reaction mixture and layered over a 40%

sucrose cushion in a Beckman microfuge tube. After centrifugation in a Beckman Microfuge for 5 min, the tubes were clamped and sectioned, and the radioactivity present in the pellet and supernate determined separately. Results were expressed as the percent of radioactivity bound to EA.

Radiolabeling of Proenzyme C1s. Isolated C1s was radiolabeled by the chloramine-T method (42).

RDDP Release Assay. Fresh serum or serum derivatives were mixed with an equal volume of virus and incubated for 30 minutes at 37°C. An equal volume of an assay mixture was then added and incubation continued for 30 minutes at 37°C. The RDDP assay mixture contained the following ingredients: 1.5 mM MgCl₂; 0.05 M Tris buffer, pH 8.1; 20 mM dithiothreitol (DTT); 100 μM deoxyadenosine 5'-triphosphate; 100 μM deoxycytidine 5'-triphosphate; 100 μM deoxyguanosine 5'-triphosphate; 10 μg/ml poly(rA)-oligo(dT)₁₂₋₁₈' (P-L Biochemicals, Inc., Milwaukee, Wis.); and 100 μCi/ml (2.5 mM) [methyl-³H]thymidine 5'-triphosphate (New England Nuclear, sp act 50 Ci/mmol). Nonidet P-40 (NP-40) (Shell Chemical Co., New York) (0.03-0.1% final concentration) was added to the virus in place of serum for maximum lysis, and a requirement for the poly(rA)-oligo(dT) template was assessed during the assays by running duplicate reactions in the absence of that synthetic polymer. After the incubation, polymerized radioactivity (³H-deoxythymidine monophosphate [TMP]) was quantitated by spotting the samples on DEAE-cellulose filters and washing the filters with 5% Na₂HPO₄, water, ethanol, and ether. Samples were counted in Aquasol using a Beckman liquid scintillation system.

PAGE. Analyses were performed in 7% polyacrylamide gels containing 0.1% SDS in phosphate buffer, pH 7.25, by using a modification (32) of the method of Weber and Osborn (43). One volume of reaction mixture was mixed with one volume of phosphate buffer containing 8.5 M urea, 2% SDS, and 1.4 × 10⁻² M DTT. After boiling for 5 min, an equal volume of 50% glycerol in water saturated with bromophenol blue was added and 25-μl samples were applied to 5 × 80 mm gel columns and electrophoresis carried out for 3 h at 8 mA/gel in a Hoefer gel electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) (32). The gels were then sectioned at 2-mm intervals and the segments analyzed for radioactivity in a Beckman Biogamma II Spectrometer.

Measurement of CH₅₀ and C Component Hemolytic Activity. Fresh human serum was incubated with an equal volume, usually 0.2 ml, of various concentrations of isolated MLV for 60 min at 37°C. Subsequently, residual C activity was determined by CH₅₀ titration (44) and residual C1 (32), C2 (34), C3 (45), C4 (36), C5 (45), C8 (46), and C9 (46) activities were quantitated by effective molecular titration according to the published methods. Consumption of more than 15% was considered to be significant.

Measurement of C1 Activation. Proenzyme C1 was reconstituted by 10 μg each of highly purified, immunoglobulin-free human C1q, human proenzyme C1r, and ¹²⁵I-human proenzyme C1s in a total vol of 60 μl in a 5 × 10⁻² M Tris, 1 × 10⁻¹ M NaCl buffer, pH 7.5 containing 5 × 10⁻³ M CaCl₂. After formation of C1 for 10 min at 22°C, 5-μl samples of the reaction mixture were incubated with 15-30 μg of MLV in a vol of 25 μl. Incubation continued for 30 min at 30°C, after which SDS, urea, and DTT were added and electrophoresis was performed as described above. Controls always included C1r and C1s incubated together with the various virus preparations in the absence of C1q. Also included in each run were 5-μl samples of C1 incubated with 25 μl of buffer and with 25 μl of buffer containing 30 μg of aggregated human IgG (63°C, 15 min).

Results

C Requirements for Viral Lysis. Previously, we had found that fresh human serum released RDDP from multiple oncornaviruses including feline leukemia virus, MLV, and RLV (29). However, heated human serum, C2-deficient and C4-depleted human sera were unable to release RDDP from these viruses (29). Identical results have since been obtained with other oncornaviruses, including AKR murine leukemia virus (MuLV), rat leukemia virus (RaLV), and the xenotropic MuLV. Data for the latter virus are shown in Table I, which also indicates that completion of the C reaction sequence is probably required since C8-depleted serum did not mediate lysis.

TABLE I
Human C Requirement for RDDP release from Xenotropic MuLV

Virus treatment	³ H-TMP incorporated
	<i>pmol</i>
Fresh serum	2.17
Heated serum	0.04
C2-deficient serum	0.10
C4-depleted serum	0.02
C8-deficient serum	0.17
Control (NP-40)	3.73

The ability of fresh serum to release RDDP from oncornaviruses was reduced or eliminated by removal of factor B, an essential component of the alternative pathway. Serum depleted of factor B released from 15 to 54% of the amount of enzyme released by fresh human serum in various studies. Higher release was obtained with immunochemical depletion than with heat depletion of factor B. For example, a serum immunochemically depleted of factor B released sufficient RDDP from feline leukemia virus to incorporate 0.83 pmol of ³H-TMP while serum depleted by heating released no RDDP. These values compare with 1.55 pmol for fresh human serum.

It was considered important to also examine depleted serum after reconstitution since the depletion process may also reduce the levels of other limiting components. However, our measurements of RDDP release by reconstituted sera were erratic and usually only partial reconstitution of ability to release RDDP was found despite the fact that in parallel studies the same reconstituted sera released large amounts of RNA from the viruses. For this reason we used only RNA release to measure the effectiveness of reconstitution. Fig. 1 shows reconstitution by C4 of the ability of serum immunochemically depleted of C4 to release ³H-uridine from RaLV. The virus-associated radioactivity appeared as a single symmetrical peak located at a density of 1.14 g/cm³ after incubation of RaLV with C4-depleted human serum (center panel). After reconstitution with physiological amounts of C4, the serum effectively released ³H-uridine (lower panel). Although not shown, the virus was also concurrently incubated with buffer, heated human serum, fresh feline serum, or fresh guinea pig serum in the same experiment. In each instance the peak of radioactivity was found between 1.14 and 1.15 g/cm³ in a symmetrical distribution essentially identical to that found in the center panel of Fig. 1. Also in the same experiment, serum depleted of factor B by heating released 24% of the ³H-uridine from RaLV while 75% release was observed after reconstitution with physiological amounts of factor B. The above studies indicate that, whereas integrity of the classical pathway is an absolute requirement for lysis of oncornaviruses by human serum, factor B of the alternative pathway is also needed for maximal lysis.

As anticipated from the above results, oncornaviruses efficiently activated the C system as shown by a reduction in C activity of fresh serum after incubation with oncornaviruses as shown in Table II. There was, however, no correlation between the amount of protein added and the extent of C consumption by

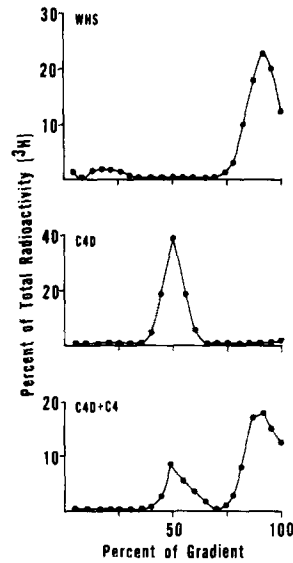


FIG. 1. Equilibrium sucrose density gradient of RaLV exposed to WHS serum (upper panel), C4-depleted (C4D) human serum (middle panel), and C4D human serum reconstituted with C4 (lower panel). RaLV, internally labeled with ^3H -uridine, was reacted with serum, depleted serum, or reconstituted depleted serum, and analyzed by sucrose density gradient ultracentrifugation as described in the Materials and Methods. The direction of sedimentation is to the left. The essential role of C4 for viral lysis is evident.

oncornaviruses. C consumption was not caused by the presence of contaminating viral or cellular RNA or DNA in the virus preparations, since C consumption by MLV was unaffected by the presence of high concentrations of RNase, DNase, or both enzymes. Measurement of residual C component levels after incubation of the MLV preparation obtained from the NCI with normal serum (89% CH_{50} depletion) showed a pattern of consumption typical of classical pathway activation with marked depletion of C2 and C4 activities (Fig. 2).

Deposition of C Proteins on the Viral Surface. The following approach was used to demonstrate the presence of C4 on the viral surface after incubation of MLV with whole human serum. MLV, surface labeled with ^{125}I , was incubated with normal human serum (WHS) or C4-depleted human serum and then separated from unbound serum constituents by rate zonal sucrose density gradient ultracentrifugation. A pool of fractions from the radioactive peak from the gradient representing ^{125}I -virus and bound serum proteins was then incubated with the IgG fraction of an antiserum to C4 after which the mixtures were sedimented in a second set of rate zonal sucrose density gradients. The sedimentation rate of MLV previously incubated with serum was increased further after incubation with the IgG fraction of antiserum to C4 as shown in Fig. 3, whereas the sedimentation rate of ^{125}I -MLV comparably incubated with C4-depleted serum was not increased after incubation with anti-C4. These studies thus demonstrate the presence of C4 on the viral surface.

Antibody-Independent Attachment of C1q to MLV. The above studies, which showed classical pathway involvement, when coupled with our earlier work indicating a lack of requirement for antibody, strongly suggest that MLV

TABLE II
Human C Consumption by Oncornaviruses

Viral preparation	Concentration	C consumption*
	mg/ml	%
RaLV	1.01	30
PRLV	0.36	36
RLV	0.36	36
MLV (FAN)	0.76	62
MLV (179S)	2.68	70
MLV (NCI)	1.25	89
MLV (NCI) + DNase‡	1.25	81
MLV (NCI) + RNase‡	1.25	82
MLV (NCI) + DNase/RNase‡	1.25	82

* C consumption was assessed after 30 min of incubation at 37°C of a mixture of equal volumes of virus and human serum.

‡ The DNase and RNase final concentrations in the reaction mixtures with virus and human serum were 1.7 mg/ml and 2.3 mg/ml, respectively.

directly activated the classical pathway. This possibility was explored in the following experiments. Two approaches were used to assess the ability of isolated MLV to interact with immunoglobulin-free C1q. First, in the C1q deviation test illustrated in Fig. 4, MLV inhibited the binding of C1q to EA in a dose related manner, a finding which strongly suggests binding of C1q to MLV. 50% inhibition occurred with 1 μ g of purified MLV, a value which corresponds to approximately 4,700 molecules of C1q per virion. Second, mixtures of 125 I-C1q and MLV were sedimented in rate zonal sucrose density gradients to examine the possibility of binding of C1q to MLV. These studies clearly documented the direct attachment of C1q to MLV. It was possible to select ratios of reactants to maximize binding of C1q to MLV as shown in Fig. 5. In this study, virtually all of the C1q in the 125 I-C1q-MLV mixture bound to MLV as it sedimented at a rate identical to that of 125 I-MLV which was sedimented in a separate tube. In the absence of MLV, C1q was found at the top of the gradient (Fig. 5).

Antibody-Independent Activation of C1 by MLV. The following experiments were designed to show that the interaction of C1q with MLV led to activation of macromolecular C1 and thus was responsible for activation of the classical C pathway. These studies employed precursor C1 reconstituted from highly purified immunoglobulin-free human C1q, isolated proenzyme human C1r, and purified 125 I-labeled proenzyme human C1s. After incubation of reconstituted C1 with MLV the following were added: SDS, urea, and reducing agent. The mixtures were analyzed by SDS-PAGE, sectioned, and analyzed for radioactivity. Proenzyme C1s is known to consist of a single polypeptide chain with a mol wt of 87,000 daltons. After activation, which is accomplished by C1r, C1s is cleaved into two fragments of 59,000 and 28,000 daltons. As depicted in Fig. 6, which represents a typical experiment, the mol wt of 125 I-C1s was reduced from 89,000 to 59,000 daltons after incubation with aggregated IgG or with MLV, thus documenting activation. As shown in the controls included in each run (Fig. 6) activation required the presence of the C1q subunit and thus was not explainable by a direct effect of the MLV preparation on C1r or C1s. Three preparations

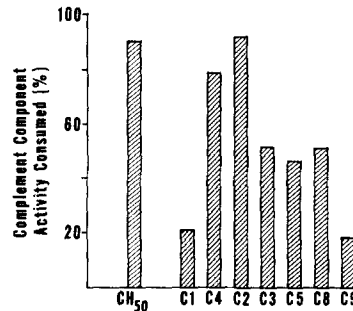


FIG. 2. Pattern of consumption of C activity in normal serum after incubation with MLV. WHS was incubated with MLV (NCI) after which the remaining CH₅₀ and C component levels were determined. The percentage of consumption was determined by subtraction from the values obtained from the control of normal serum incubated with buffer. The pattern of consumption is typical of classical pathway activation.

of MLV examined in this manner in 15 experiments were able to activate reconstituted C1.

Discussion

In our earlier studies we found that fresh human serum, but not serum from a number of other species could lyse and thus inactivate oncornaviruses from several diverse species. Lysis required an intact source of C. The detailed analysis of the role of the human C system in this reaction presented here indicates that the classical C pathway is required for lysis since neither C2- nor C4-deficient sera were able to mediate lysis. Lytic ability was restored on addition of the missing component (Fig. 1). The marked consumption of C2 and C4 in serum treated with MLV also documents primary activation of the classical pathway (Fig. 2). Since the alternative or properdin pathway, which also possesses cytolytic activity, was shown to be functional in the C2- and C4-deficient sera, it might be inferred that the oncornaviruses examined do not effectively activate this pathway. However, we also regularly observed a reduction, and on occasion a complete loss, in ability to lyse MLV after depletion of factor B from the serum. These findings suggest that factor B and presumably the C3b-dependent feedback system are required for full expression of the cytolytic potential of the serum. The feedback system, which requires factor B, factor D, and C3b and which becomes operative after generation of C3b, leads to additional formation of C3b and activation of late-reacting C components. In this context, the system may be visualized as amplifying an initial relatively minor degree of C activation and thus rendering it biologically significant.

Classical pathway activation by oncornaviruses results from direct interaction of the virus with C1q which leads in turn to activation of C1r and C1s, and thus of the C1 molecule. These conclusions stem from studies in which isolated MLV bound highly purified human C1q (Figs. 4 and 5). Both the C1q deviation test and sucrose density gradient ultracentrifugation studies demonstrated binding of MLV to C1q mixtures. Although both types of studies indicated that saturation occurred between 5,000 and 8,000 molecules of C1q per virion, these levels must be considered as approximate because of uncertainty as to the exact concentration of viral particles in the MLV preparations.

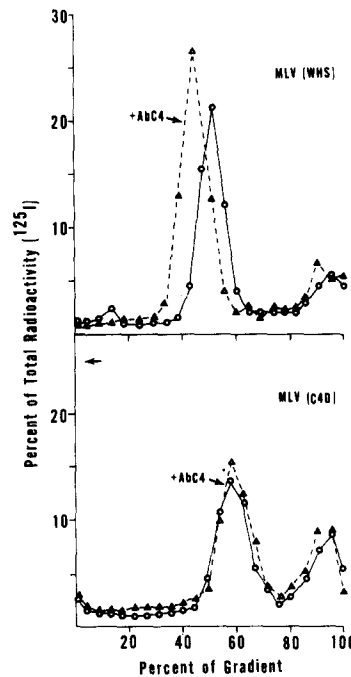


FIG. 3. Rate zonal sucrose density gradient ultracentrifugation of MLV (surface labeled with ^{125}I first incubated with WHS (upper panel) or C4-depleted (C4D) human serum (lower panel) and then with anti-C4 MLV. Solid lines denote the position of ^{125}I -MLV previously incubated with normal (upper panel) or C4-depleted (lower panel) sera while the dashed lines indicate the position of the comparable samples incubated with anti-C4. The ability of anti-C4 to increase the sedimentation rate of MLV previously incubated with serum documents the presence of C4 on the virus surface.

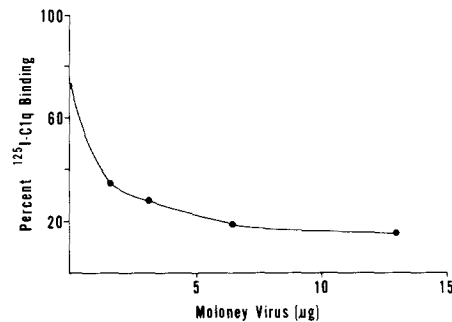


FIG. 4. Reactivity of C1q with MLV as demonstrated by the C1q deviation test. MLV was incubated with ^{125}I -C1q after which EA were added. The percent of ^{125}I -C1q binding to EA in the absence of MLV was 74% of the total added, as shown on the ordinate. The progressive inhibition by MLV of C1q binding to EA documents C1q binding to MLV.

Several examples of substances able to interact with and precipitate C1q but which do not lead to activation of the C system have been reported. We felt it important to rule out this type of interaction by demonstrating that the interaction of C1q with MLV led to C activation. The test system developed for this purpose involved activation of proenzyme macromolecular C1 reconstituted from highly purified immunoglobulin-free human C1q and isolated proenzyme

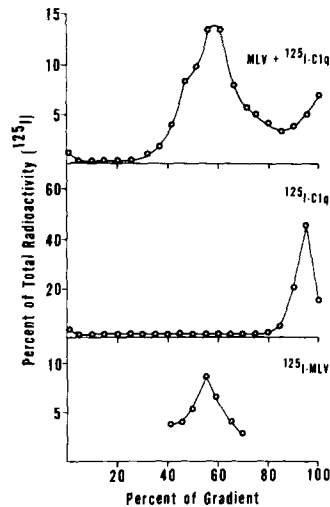


FIG. 5. Rate zonal sucrose density gradient ultracentrifugation of an MLV-C1q mixture (upper panel), ^{125}I -C1q alone (middle panel), and ^{125}I -MLV alone (lower panel). ^{125}I -C1q previously incubated with MLV (upper panel) sediments at the same rate as ^{125}I -MLV alone (lower panel), documenting the direct binding of C1q to MLV.

C1r and C1s. Activation of C1s in C1, which is accomplished by C1r, leads to cleavage of the single 89,000 dalton polypeptide chain of proenzyme C1s into 59,000 and 28,000 dalton subunits. Three different preparations of MLV were found to consistently activate reconstituted C1 when examined in this test system (Fig. 6). Control studies showed that MLV produced no activation on omission of C1q, a finding which not only demonstrates the C1q requirement but also rules out a contaminating cellular enzyme present in the MLV preparation as responsible for C1 activation. It is quite probable that the activation of C1 by MLV shown in the isolated system fully explains the activation of the classical C pathway on addition of MLV to serum since the concentrations of C1q, C1r, and C1s employed in the C1 activation test duplicate, except for a twofold reduction in C1q, the relationship between these components in serum. In addition MLV activates serum C in a concentration range similar to that employed in the C1 activation test when the ratio of MLV to C1 is considered.

Antibody is clearly not responsible for binding of C1q by MLV since C1q preparations lacking detectable immunoglobulin bound completely to MLV and triggered C1 activation (Fig. 5). These findings thus confirm our earlier contention based on multiple indirect lines of evidence that oncornaviral lysis by human serum does not require antibody (29, 30). Therefore, in this system, the C1q molecule fulfills the functional role of antibody since it subserves the specific recognition function normally characteristic of the antibody molecule. A direct recognition role for C1q, or antibody-independent activation of the classical pathway has also been demonstrated for several other diverse systems. These include cardiac mitochondrial membranes (47), C-reactive protein-C polysaccharide complexes (48), polynucleotides and nucleic acids (49, 50), lipid A of lipopolysaccharide (51), cholesterol-containing liposomes (52), and heparin-protamine complexes (53). Curiously, at least two of the above systems, i.e., mitochondrial membranes and C-reactive protein-C polysaccharide complexes are

COMPLEMENT LYSIS OF RNA TUMOR VIRUSES

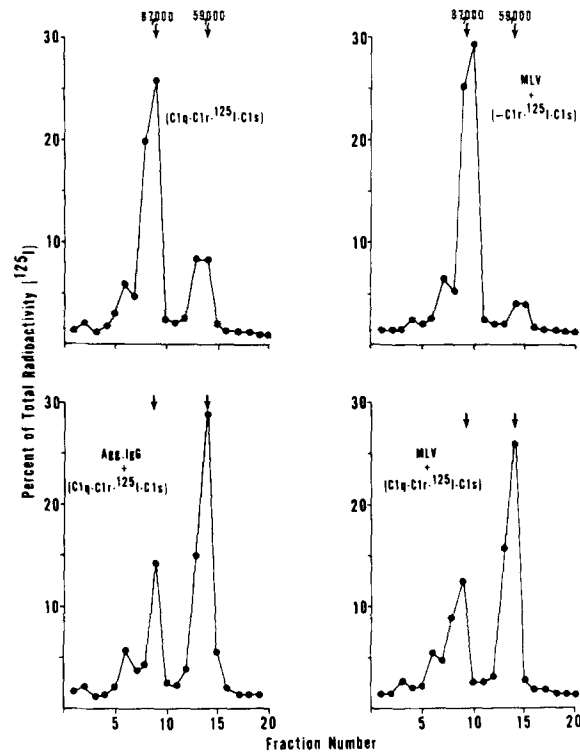


FIG. 6. SDS-PAGE analysis of C1 activation by MLV. C1 was reconstituted by C1q, proenzyme C1r, and ^{125}I -proenzyme C1s and incubated with $30\ \mu\text{g}$ of MLV (NCI) or $30\ \mu\text{g}$ of aggregated IgG. The mixtures were then subjected to SDS-PAGE analysis after which the gels were sectioned and radioactivity measured. The mixture of C1q, C1r, and C1s (upper left panel) indicates that the ^{125}I -C1s is primarily in the 87,000 dalton single chain proenzyme form. This mixture, which represents proenzyme C1, is capable of being activated by aggregated (Agg) IgG (lower left panel) or MLV (lower right panel) as shown by cleavage of the single 87,000 dalton polypeptide chain of C1s into a 59,000 dalton fragment (and also a 28,000 dalton fragment which is not radiolabeled). MLV does not possess the ability to activate C1 in the absence of C1q (upper right panel).

similar to the oncornaviral system in activating human but not guinea pig C. As is obvious from this diverse list of substances, multiple different mechanisms, in addition to the familiar interaction with the Fc region of immunoglobulins, may lead to C1q binding. These include charge attraction between C1q, one of the most cationic proteins of serum, and negatively charged molecules such as DNA, RNA, and acidic polynucleotides. C1q interaction with some of the substances listed above is difficult to explain on this basis and it is probable that other unknown types of interactions of C1q with proteins, carbohydrates, and perhaps lipids occur.

The structural features on the oncornaviral envelope responsible for C1 activation have not been addressed by this study. Several proteins have been found to be associated with the MuLV oncornaviral envelope including p12, p15, and gp70 (54). Any of these could potentially be involved in binding of C1q. Alternatively, it is possible that a polysaccharide or lipid constituent of the viral envelope acquired from the host cell membrane is responsible for C1q binding, a

not unreasonable possibility in view of C1q binding by certain polysaccharides and lipid-containing substances as noted above. In this context, it is of interest that cellular membranes of certain continuously growing lymphoid cells also directly activate C (55-57). Although alternative pathway activation is involved, in these instances, our recent studies indicate that continuously growing lymphoid cells also activate the classical pathway. Alternatively, direct activation by cells may be related to the surface expression of oncornaviruses in analogy to the direct activation reported here. In this regard, the ability to directly activate C was engendered in a cell line by oncornavirus infection (55).

The biological relevance of the reported results is not clear at the present time, although one might speculate that the ability of human serum to inactivate oncornaviruses represents a natural defense mechanism operative *in vivo* which limits horizontal spread of oncornaviruses and also prevents expression of intact oncornaviruses by cells which may have become infected or which bear oncornavirus genomes. This interpretation is in accord with the frequent occurrence of intact oncornaviruses in malignancies of many species including chickens, guinea pigs, rabbits, rats, and mice, whose C is unable to lyse oncornaviruses and with the rare expression of intact oncornaviruses in human malignancies. An apparent contradiction to this hypothesis is the occasional expression of oncornavirus during the course of certain malignancies in rhesus monkeys. Since normal rhesus serum lyses oncornaviruses (30), an evaluation of the C status of monkeys expressing oncornaviruses is particularly indicated. It will also be of considerable interest to determine whether the disappearance of oncornaviruses from normal human and rhesus monkey placentas during ontogeny (15) correlates with the development of cytolytic potential of the classical C system. Finally, a continuing evaluation of individuals, who have congenital or acquired deficiencies in components of the classical pathway, for oncornavirus expression, and development of malignancies is indicated in determining the *in vivo* relevance of these findings.

Summary

In earlier studies we found that human serum, but not serum from multiple other species, inactivated and lysed oncornaviruses from a number of diverse sources in the apparent absence of antibody. A detailed analysis of the role of the human complement (C) system in mediating this lytic process indicates that human C1q interacts directly, in the absence of immunoglobulin, with oncornaviruses. Binding of C1 via C1q in this manner leads to activation of C1r, C1s, and thus of the classical C pathway. Integrity of the classical pathway is an absolute requirement for lysis although activation of the alternative pathway considerably amplifies the amount of lysis obtained, possibly through involvement of the C3b-dependent feedback mechanism. Activation of C is accompanied by deposition of C components on the viral surface and lysis on completion of the C reaction sequence. Thus in this system, the C1q subunit of C1 subserves a specific recognition function normally associated with antibody. This ability of human serum to inactivate oncornaviruses may represent a natural defense mechanism operative *in vivo* which deters expression of intact oncornaviruses in human malignancies.

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