LYSIS OF ONCORNAVIRUSES BY HUMAN SERUM
Isolation of the Viral Complement (C1) Receptor and Identification as p15E*

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Human serum inactivates RNA tumor viruses as Welsh and co-workers (1–4) have demonstrated. The mechanism of inactivation was shown to be complement-mediated viral lysis, not requiring virion-specific antibodies. All C-type RNA viruses were lysed by sera from primates but not from rabbits, mice, guinea pigs, or goats. Cooper et al. (4) showed that integrity of the classical complement sequence was an absolute requirement for lysis and that, in absence of specific antibody, C1q suberved the virus recognition function. The antibody independent nature of this virolytic reaction suggests that complement plays an important role in natural or nonspecific defense against C-type RNA viruses. We have therefore explored the envelope proteins of Moloney leukemia virus as to their capacity to activate complement, and have identified a single, low molecular weight surface protein as the viral receptor and activator of C1.

Materials and Methods

Chemicals and Reagents. Trypsin, soy bean trypsin inhibitor, chymotrypsinogen, RNase, cytochrome c, phospholipase A, sodium barbital; Tris, and cyanogen bromide were obtained from Sigma Chemical Co. (St. Louis, Mo.). Ampholines (Servalyt T4-9) were purchased from Accurate Chemical & Scientific Corp. (Hicksville, N. Y.), Pevikon C-890 from Mercer Consolidated Corp. (Yonkers, N. Y.), and 32P-Na and 131I-Na from New England Nuclear (Boston, Mass.).

Complement Components and Complement Reagents. Fresh human serum (Community Blood and Plasma, San Diego, Calif.) and fresh guinea pig serum frozen at −70°C were used as a source of complement. Zymosan (Nutritional Biochemicals Corp., Cleveland, Ohio) was boiled for 1 h at a concentration of 100 mg/ml and stored suspended in saline. Human sera were depleted of both C1q and C7 by immunoadsorption. Factor B-deficient serum was provided by Dr. R. Schreiber and C3a by Dr. T. Hugli both at Scripps Clinic. C7 was isolated as described earlier (5), and C1q was isolated by the method of Yonemasu and Stroud (6).

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1 Complement components are identified according to WHO recommendations (1968).
2 E. R. Podack, W. P. Kolb, and H. J. Müller-Eberhard. Manuscript in preparation.

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Viruses, Viral Proteins, and Antisera. Moloney (M-MuLV), Rauscher (R-MuLV), AKR (AKR-MuLV), and NZB xenotropic (X-MuLV) murine leukemia viruses (prepared by Electro Nucleonics Inc., Bethesda, Md.) were supplied by the Office of Program Resources and Logistics, Viral Oncology, National Cancer Institute. Antisera against these viruses and against the major viral proteins gp70, p30, p12, and p10 (prepared by Huntington Research Center, Baltimore, Md.) were also furnished by this office. Rat leukemia virus strain BN-p454-9 (Ra-LV) was a gift from Dr. F. C. Jensen, Scripps Clinic, gp70 and p30 from R-MuLV and AKR-MuLV were isolated by the method of Strand and August (7).

Complement Activation Assays. Activation of human complement was measured by conversion of C1 to C1 (8) and the formation of C5b-9, the end product of both complement pathways. In the latter test, to be described in detail elsewhere, C7-depleted human serum reconstituted with purified 125I-C7 (C7 reagent) formed a stable radiolabeled C5b-9 complex upon activation. To test for complement activation, 100 μl of C7 reagent was added to 50 μl of the test sample and incubated for 45 min at 37°C. 100 μl of the reaction mixture was then layered on a 50-μl cushion of 15% sucrose in a 175-μl nitrocellulose centrifuge tube and centrifuged for 90 min at 160,000 gmax in an air-driven ultracentrifuge (Airfuge, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). After the supernate was withdrawn into tissue paper, the total radioactivity of the pellet was determined. For each test, C7 reagent containing zymosan as a complement activator was used as a standard for maximal activation. In this sample, usually 35% of the C7 radioactivity was recovered in the form of labeled C5b-9.

Complement Consumption. Samples being tested for their ability to consume complement were incubated for 30 min at 37°C and then titrated for remaining activity by standard methods (9).

Radioiodination. Proteins and viruses were trace labeled with 125I or 131I by using the solid-phase lactoperoxidase procedure of David and Reisfeld (10). Bovine lactoperoxidase bound to Sepharose 4B was kindly provided by Dr. G. David, Scripps Clinic.

Sucrose Density Gradient Centrifugation. Samples were sedimented at 4°C in linear sucrose gradients (prepared in 10 mM Tris, 100 mM NaCl, 1 mM EDTA [TNE]) in an air-driven ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.). Times and gravity forces are noted in respective figure legends.

Electron Microscopy. Virus preparations were negatively stained with phosphotungstic acid without prior fixation and examined and photographed in a Hitachi 11E electron microscope (Hitachi Ltd., Tokyo, Japan) at 75 kV at a direct magnification of 36,000.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Electrophoresis was carried out in 10-cm long gels containing 0.1% SDS and 15% polyacrylamide according to the procedure of Weber and Osborne (11). After electrophoresis, bands were visualized by staining with Coomassie Brilliant Blue R-250, or sliced into equal segments and analyzed for radioactivity.

Hemagglutination Inhibition Assays. The C5b-9 content in samples from sucrose gradients was estimated from the degree of inhibition of agglutination of EAC1-7 by antiserum to C5b-9-specific neoantigens (12).

Digestion Studies. Protein digestions were carried out at a final trypsin concentration of 1% (weight/weight protein) whereas for viral digestions, 10% trypsin (weight/weight virus) was used. After incubating the mixtures for 1 h at 25°C, the reaction was terminated by adding excess soybean trypsin inhibitor. Digestion with cyanogen bromide was performed in 70% formic acid at room temperature for 24 h followed by lyophilization of the samples to dryness.

RDDP5 Release Assay. The assay, which measures incorporation of [3H]TMP into (dT)12-1~ (rA), catalyzed by the virion’s reverse transcriptase, was performed as described in reference 1.

Isoelectric Focusing. Isoelectric focusing (IEF) was carried out in a granulated support mixture (48 g Pevikon, 2.5 g Sephadex G-75) as described by Otavsky et al. (13) in the presence of 2% ampholines (pH range 4–9) and 0.2% Triton X-100. Usually 1–2 mg of virus was disrupted in 1% Triton X-100 in TNE at room temperature and, after centrifuging for 10 min at 160,000 g to
Formation of the C5b-9 Complex on the Viral Surface. The cytolytic entity formed by both activation pathways is the C5b-9 complex. Previous work (14) demonstrated that human serum containing $^{125}$I-C7 upon incubation with zymosan formed a soluble radiolabeled C5b-9 complex. C5b-9 formed by viral activation, unlike the complex formed by zymosan activation, is not free in solution. During rate zonal centrifugation the complex detected by hemagglutination inhibition assay sediments more slowly than free C5b-9 or intact virus. From its location in the sucrose density gradient the complex appears to be attached to lipid-rich viral membrane fragments produced during lysis (Fig. 1). The characteristic ultrastructural lesions associated with the membrane-bound C5b-9 complex as well as the disintegration process of the virus after complement attack can be seen in the electron micrographs shown in Fig. 2.

Virus Activates both Complement Pathways. In addition to classical pathway activation we found activation of the alternative pathway by the virus. When C1q-depleted serum was used, some C5b-9 formation was observed at the highest virus concentration (Fig. 3). Since incubation with aggregated IgG did not form C5b-9, complex formation did not result from residual C1 activation. Saturation of viral C1 receptors with purified C1q did not completely prevent lysis unless the serum was depleted of factor B. These results indicate that M-MuLV can activate both pathways but because of more efficient activation, the classical pathway predominates in the virolytic reaction.
FIG. 2. Electron microscopic appearance of Ra-LV strain BN-1454-9 before (A) and after (B) incubation with human serum. Virus preparations were negatively stained with phosphotungstic acid without prior fixation and examined in a Hitachi 11E electron microscope at a direct magnification of 36,000. Bar corresponds to 100 nm. Arrows indicate typical complement lesions.

FIG. 3. Activation of classical and alternative pathway of complement by M-MuLV. 100 μl of C7 reagent (●) and C1q-deficient C7 reagent (○) were incubated with the indicated number of virions. Virus counts of the undiluted sample had been determined electron microscopically by the supplier (Electro-Nucleonics Inc.).

Complement Activation by Disrupted Virus. Before purification of the viral complement receptor could be attempted it was necessary to show that disrupted virus retains the ability to activate the complement system. Since C5b-9 formation is insensitive to 1% Triton X-100 we used C5b-9 formation to test the ability of detergent disrupted MuLV to activate complement. Disrupted M-MuLV activated complement and the responsible material was recovered in the supernate after centrifugation at 160,000 g_{max} for 10 min.

Isolation of the Viral Complement Activator. Moloney leukemia virus was
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surface labeled with $^{131}$I by solid-phase lactoperoxidase and then banded in a 20–65% sucrose density gradient to separate intact virions from other labeled components. As shown by SDS-PAGE analysis, banded material completely lacked radioactivity in the internal p30 viral protein (Fig. 4), demonstrating that only components at or near the viral surface had become labeled. Two main peaks of radioactivity were apparent, one corresponding to the 68,000-dalton glycoprotein and the other to a protein of ~15,000 daltons.

To purify the viral receptor the surface labeled and disrupted virus was subjected to IEF in a Pevikon-Sephadex mixture in the presence of Triton X-100 (Fig. 5 A). Two peaks of radioactivity were detected. Analysis by SDS-PAGE identified the peak at pH 5.3 as a 68,000-dalton protein, and the peak at pH 7.5 as a low molecular weight protein. IEF of a sample of M-MuLV disrupted before labeling yielded additional peaks of radioactivity (data not shown). After removing Pevikon and ampholines, each fraction was tested for its ability to cause C5b-9 formation in C7 reagent. Only one discrete peak of activity was noted which coincided with the pH 7.5 peak of radioactivity (low molecular weight protein) (Fig. 5 B). Several such focusing experiments consistently yielded only one discrete band of activity, with a pI ranging from 7.4 to 7.8, always coinciding with the smaller radioactivity peak. Formation of C5b-9 was a result of activation of the classical pathway, because the protein at pH 7.5 showed strong affinity for Clq in sucrose density gradient centrifugation experiments (data not shown) and converted C1 to C1 very efficiently (Fig. 6), whether or not Triton X-100 was present in the assay. In contrast, activation of
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Fig. 5. Localization of complement-activating ability to a protein with pI 7.5. Surface-labeled, sucrose density-banded, virus was disrupted in 1% Triton X-100 and centrifuged at 160,000 g_{max} for 10 min. The supernate was then subjected to IEF in Pevikon/Sephadex G-75 containing 0.2% Triton X-100 and 2% ampholines (pH 4-9). After division of the bed into 30 equal segments, pH and radioactivity of each segment were determined (A). The carrier material was removed by filtration, and each fraction was neutralized and freed of ampholines by dialysis against PBS containing 0.1% Triton X-100. The ability of each fraction to form C5b-9 was then determined (B).

Fig. 6. Conversion of C1 to C1 by the viral activator. The fraction at pH 7.5 of Fig. 5 was isolated, dialyzed and freed of Triton X-100 by passage through SM-2 Bio-Beads (Bio-Rad Laboratories, Richmond, Calif.). One-half of the sample (50 µl) was then incubated with 50 µl of C1 containing 125I-C1s for 30 min at 37°C in the absence (A) or in the presence (B) of 0.1% Triton X-100. Under these conditions activation of C1 results in proteolytic cleavage of C1s to form C1s. The labeled, heavy chain (C1s_{H}) of C1s can be identified by SDS-PAGE under reducing conditions. (The light chain of C1s is not susceptible to radiiodination; for further experimental details see reference 8). The extent of nonspecific C1s conversion by 0.1% Triton X-100 in buffer is shown in C.

The alternative pathway required intact virus, since Triton X-100-disrupted virus did not promote C5b-9 formation in C1q-depleted serum (not shown).

Despite the cogency of these results, the possibility remained that the complement-activating property resided in an unlabeled substance with a similar pI that was liberated during Triton X-100 disruption. This possibility
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FIG. 7. Characterization of purified viral complement receptor by SDS-PAGE. The dialyzed receptor, purified by IEF from surface-labeled M-MuLV, was labeled with 125I (•) and then mixed with 131I-labeled, disrupted M-MuLV (○). Electrophoresis was carried out in 15% polyacrylamide gels in the presence of 0.1% SDS under reducing conditions. Unfixed gels were sliced into 0.7-mm segments. Note the absence of contaminating proteins in the receptor preparation.

was precluded by the following results. Incubating surface-labeled virus with trypsin released >70% of the total radioactivity, without change in viral density or release of internal reverse transcriptase. This trypsin-digested, intact virus had completely lost its potential to form C5b-9 and to convert C1 to C1. Activity was not restored when the trypsin-treated virus was disrupted with 1% Triton X-100 showing that internal components could not initiate the complement sequence. When the isolated complement receptor was digested with trypsin its activity was likewise lost, yet incubation with RNAse, lipase, or phospholipase A had no effect.

That gp70 (the protein at pH 5.3) is not the viral complement receptor is supported further by the observations that (a) several gp70 isolated from different oncornaviruses by a different method (7) did not activate complement, (b) AKR-MuLV that was stripped of gp70 by repeated sucrose density centrifugation still activated complement, and (c) noninfectious Ra-LV strain BN-1454-9 that lacks gp70 (F. Jensen, personal communication) and the surface knobs characteristic for this glycoprotein (see Fig. 2A), can be lysed by human serum (2).

Identification of the Viral Complement Receptor as p15E. Isolated activator was labeled with 125I, mixed with 131I-labeled, detergent-disrupted virus, and the mixture was analyzed by SDS-PAGE. Fig. 7 indicates that the activator migrated together with the p15 complex and that no other proteins with different molecular weight but identical pl were present in this preparation. When analyses were performed on slab gels in the presence of molecular weight markers, the mobility of the receptor was consistently found to be similar to that of p15. Treatment of the receptor protein with cyanogen bromide cleaved the receptor (data not shown). These results strongly implicate p15E (15) as the viral complement receptor. In accordance with this conclusion we found that
internal M-MuLV p15 supplied by Dr. J. Ihle, Frederick Cancer Research Center, Bethesda, Md., did not form C5b-9 at concentrations as high as 2 µg, whereas a p15 preparation from Moloney murine sarcoma virus (M-MuSV) provided by Dr. G. Vande Woude, National Cancer Institute and recently characterized as a surface protein (16) did consume complement in human serum but not in guinea pig serum.

In addition to Moloney virus, we have also tested Rauscher-MuLV, AKR-MuLV, and a xenotropic MuLV derived from NZB mice, and obtained similar results.

Discussion

Activation of the classical pathway of complement is usually mediated by antigen-antibody complexes. In addition, several systems of classical pathway activation independent of antibodies have been observed (summarized in reference 4). In every case, including the lysis of oncornaviruses by human complement, the recognition function is provided by the C1q subcomponent of C1.

To elicit lysis, C1 must interact with the viral envelope. For this reason proteins, lipids, and carbohydrates might be involved in C1q recognition. Our studies indicate that a protein is necessary for activation. However, it is possible that the protein, which we isolated by IEF in the presence of detergent, is tightly associated with phospho- or glycolipids to form a proteolipid. Although such lipid moieties could provide the actual C1q-binding sites, several lines of evidence argue against this possibility: (a) lipase or phospholipase treatment did not abrogate activation; (b) a surface protein from M-MuSV, isolated by methods that would dissociate a proteolipid into its constituents (chromatography in 6 M guanidine hydrochloride and preparative SDS-PAGE), still showed activity, and (c) the lipid portion of oncornaviruses is derived from the host cell, yet all C-type RNA viruses were lysed regardless of the cell from which they were derived (2).

Identification of the C1 receptor as one of the known viral proteins is not straightforward. Thus far, five proteins have been unequivocally identified as viral gene products (17): the major glycoprotein, gp70, the core protein, p90, an internal phosphoprotein, p12, an RNA-binding protein, p10, and another internal protein, p15. Furthermore, based on studies of precursor proteins, there is good evidence for two other viral proteins in the R-MuLV system, p15E and p12E (18, 19). The former is derived from a precursor protein Pr2a + b by cleavage before virus maturation and the latter appears to be a degradation product of p15E in the intact virus (20). On SDS-PAGE, p15E is slightly slower and p12E slightly faster in mobility than the internal p15. In the M-MuSV system, Witte et al. (21) provided evidence that a surface protein of 14,000 daltons (on SDS-PAGE) with an isoelectric point of 4.4–4.6, is disulfide linked to gp70. Whether this protein corresponds to p15E or p12E of the R-MuLV system remains to be established. Much less is known about surface proteins and their precursors in M-MuLV, the virus predominantly used in this study. However, since viruses in the Friend-Moloney-Rauscher group are closely related (17), it seems safe to assume that the results obtained with one member of the group apply in general to all of them.
Our results of trypsin digestion of intact virus show unequivocally that the isolated C1 receptor on M-MuLV is a surface protein. The receptor has a pI of about 7.5 as determined by IEF and co-migrates with the p15 complex on SDS-PAGE, well separated from p12 and p10. Only p15E contains methionine while p15 does not (19), and thus, p15E should be susceptible to cyanogen bromide cleavage. Treatment of the isolated receptor with cyanogen bromide cleaves the protein. For these reasons we identify the receptor as p15E. It is quite possible that R-MuLV p12E, if it is derived from p15E, can also activate complement. Indeed we have observed that complement activation is not lost when the receptor is cleaved by aging. Whether precursor proteins of p15, such as p60 (22) or of p15E, such as Pr2a + b (20), can also activate complement is not known with certainty, although we have found that one p60 preparation from M-MuSV did consume complement (A. F. Esser and G. F. Vande Woude, unpublished observations). However, in the latter case complement consumption was not restricted to human serum, guinea pig serum was also depleted.

Summary

Moloney leukemia virus activated both the classical and alternative pathways of human complement. About 500,000 virions were required to detect activation of the classical pathway whereas 5,000 times as many virions were necessary to initiate the alternative pathway, indicating that in this system only the former is of biological significance. Disruption of the virus with Triton X-100 destroyed its ability to initiate the alternative pathway without affecting its ability to activate the classical pathway. After ultracentrifugation of disrupted virus the active component could be recovered in the supernate and was isolated by isoelectric focusing in granulated gels. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis and cyanogen bromide digestion studies revealed that the activity resided in a methionine-containing protein having a pI of 7.5 and a molecular weight of \( \approx 15,000 \) daltons. The purified protein interacts strongly with C1q and efficiently activates C1. RNase and lipolytic enzymes had no effect on the isolated protein but incubation with trypsin resulted in loss of activity. Enzymatic digestion studies of surface-labeled virus indicate that the active protein is a viral membrane protein. On the basis of these results it is concluded that the complement receptor of Moloney leukemia virus is the surface protein p15E.

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