Mechanism of Antibody-Independent Activation of the First Component of Complement (C1) on Retrovirus Membranes†

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ABSTRACT: Murine leukemia viruses activate human C1 in the absence of specific antibody. Such activation requires the binding of C1 to the viral surface through *two* subcomponents, C1q and C1s. This conclusion is based on the following results. (1) Isolated human C1q and C1s bind the same membrane protein on virions. (2) Binding of one subcomponent is independent of the other. (3) Only dimeric C1s binds, whereas monomeric C1s, prepared by dissociation with ethylenediaminetetraacetate (EDTA), has no affinity for the virus. (4) The activated C1s dimer, $C1\bar{s}$, does not attach to the virus. (5) Saturation of C1s binding sites on the viral surface does not prevent binding of macromolecular C1, but such bound C1 is not activated. (6) No exchange occurs between C1s bound to the viral membrane and C1s contained in C1, which in turn is attached via C1q to the same virus. Therefore, activation occurs only when both C1q and C1s in the same C1 complex are in contact with the viral activator. Human C1r has no affinity for the virus nor does guinea pig C1s. The latter result explains why guinea pig serum does not function in antibody-independent virolysis.

Complement is now recognized as the principal humoral defense system against bacterial and viral infections (Müller-Eberhard, 1977). The destruction of such pathogens by complement first requires the attachment of specific antibodies to the pathogen, after which the first component of complement (C1)[1] binds to the Fc portion of IgG and is activated. C1 in serum is a Ca^{2+}-dependent complex consisting of three distinct proteins, C1q, C1r, and C1s (Lepow et al., 1963). C1q is the binding site for antigen–antibody complexes in C1; C1r and C1s are zymogens that during activation convert to serine esterases by peptide bond cleavage. Ziccardi & Cooper (1976a) suggested that binding induces a conformational change in C1q that leads to autoactivation of C1r, whereupon $C1\bar{r}$ cleaves C1s to yield $C1\bar{s}$. A more detailed account of C1 activation was recently presented by Porter & Reid (1978).

In contrast to the antibody-dependent activation of C1, C1 sometimes can be activated without antibody. For example, Cooper et al. (1976) have shown convincingly that murine leukemia viruses (oncornaviruses or retroviruses) can directly activate human C1 ($C1_{hu}$). Such activation by retroviruses is species dependent in that only primate complement is activated and lyses such viruses; sera from phylogenetically lower animals, such as guinea pigs or mice, are not virolytic (Welsh et al., 1976). Interestingly, guinea pig C1 ($C1_{gp}$) can bind to viruses, and the lack of virolysis by guinea pig serum results from the failure of bound $C1_{gp}$ to become activated. C1s has been identified as the nonfunctional component in $C1_{gp}$ because replacement with $C1s_{hu}$ results in activation and virolysis, whereas incorporation of $C1s_{gp}$ into $C1_{hu}$ abrogated the activation (Bartholomew & Esser, 1978). These studies strongly suggest that the $C1s_{hu}$ subcomponent controls viral activation of C1. In the present study, we demonstrate that the binding of both C1s and C1q directly to the viral membrane is requisite for activation of the virolytic sequence.

Materials and Methods

Buffers. Veronal-buffered saline (VBS) at either physiologic ionic strength (150 mM NaCl and 5 mM sodium barbital, pH 7.3) or at half-physiologic ionic strength (75 mM NaCl and 2.5 mM sodium barbital, pH 7.3) was used throughout this work. Where indicated, Ca^{2+} and Mg^{2+} were added to 0.15 and 0.5 mM, respectively, and EDTA to 10 mM.

Polyacrylamide Gel Electrophoresis. Samples were denatured at 100 °C by mixing with equal volumes of 8 M deionized urea, 2% sodium dodecyl sulfate ($NaDodSO_4$), and, when reducing conditions were desired, 2% β-mercaptoethanol. Electrophoresis was performed according to the method of Weber & Osborn (1969) with 10-cm gels of 7.5% polyacrylamide containing 0.1% $NaDodSO_4$.

Sera and Serum Proteins. Fresh human sera (Community Blood and Plasma, San Diego, CA) and fresh guinea pig sera frozen at −70 °C were used as sources of complement. C1q (Yonemasu & Stroud, 1971) and C1s (Valet & Cooper, 1974) were isolated from human sera as described and from guinea pig sera by the same procedures with slight modifications. $C1r_{hu}$, purified as described (Ziccardi & Cooper, 1976b), was a gift of Dr. R. Ziccardi (Scripps Clinic). These proteins were homogeneous according to $NaDodSO_4$–polyacrylamide gel electrophoresis. Functionally pure $C1r_{gp}$ was isolated following an affinity method developed for $C1r_{hu}$ by R. Ziccardi (personal communication). In brief, the euglobulin precipitate from 5 mL of fresh guinea pig serum was dissolved in VBS containing 20 mM EDTA to dissociate $C1_{gp}$. The sample was then interacted overnight at 4 °C with excess immobilized $C1s_{gp}$ (200 μg of C1s/mL of packed Sepharose 4-B) in the presence of 50 mM $CaCl_2$. Under these conditions, a portion of $C1r_{gp}$ reassociates with the immobilized $C1s_{gp}$. After we washed away unbound protein with Ca^{2+}-VBS, $C1r_{gp}$ was

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[1] Abbreviations used: complement components are identified according to World Health Organization recommendations (1968); subscripts hu and gp denote human and guinea pig, respectively; MLV, Moloney murine leukemia virus; agg-IgG, aggregated immunoglobulin G; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; VBS, Veronal-buffered saline; Dnp, dinitrophenyl.

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eluted with EDTA–VBS, concentrated by pressure dialysis, and dialyzed against Ca^{2+}-VBS.

Oxidized C2 was prepared as published (Polley & Müller-Eberhard, 1968). Antiserum against human C1s was raised in goats and made monospecific by appropriate adsorptions. Normal goat serum was used as the source of nonspecific goat IgG. Rabbit antiserum against goat IgG was donated by Dr. H. Spiegelberg (Scripps Clinic). Human IgG was aggregated (agg-IgG) at a concentration of at least 10 mg/mL by heating at 63 °C for 30 min. Particulate material was removed by centrifugation, and the soluble aggregates were used as a standard for maximal C1 activation. Sheep erythrocytes bearing specific antibody and C4 (EAC4) were prepared with purified reagents as described earlier (Cooper & Müller-Eberhard, 1968).

Virus, C1s-Coated Virus, and Viral p15(E). Moloney murine leukemia virus (MLV) prepared by Electro Nucleonics Inc., Bethesda, MD, was supplied by the Office of Program Resources and Logistics, Viral Oncology, National Cancer Institute. Routinely, virus was pelleted in an air-driven ultracentrifuge (Airfuge, Beckman Instruments, Spinco Division, Palo Alto, CA) at $160000g_{max}$ for 10 min and resuspended in the appropriate buffer prior to each experiment.

MLV was incubated with excess $C1s_{hu}$ (1 μg of $C1s_{hu}/\mu$g of virus) in half-physiologic ionic strength buffer at 30 °C for 60 min, and this C1s-coated virus was then sedimented in the Airfuge and resuspended in the same buffer. All virus samples were stored frozen at a concentration of approximately 1 mg/mL until used. From detergent-disrupted MLV, p15(E) was isolated by methods described earlier (Bartholomew et al., 1978).

Radiolabeled Proteins. Proteins (10–100 μg) were radioiodinated either by the chloramine T method (McConahey & Dixon, 1966) or by the immobilized lactoperoxidase method (David & Reisfeld, 1974). Radiolabeled products were separated from unincorporated iodine by sieving through a 0.7 × 10 cm column of Sephadex G-25 superfine (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with VBS containing 0.2 mg/mL ovalbumin. Specific activities of the radiolabeled products were between 0.5 and 2 μCi/μg of protein.

Radiolabeled C1 Assemblies. The preparation of macromolecular C1 complexes in serum containing radiolabeled proenzyme C1s and isolation of these complexes on sucrose density gradients have been presented (Bartholomew & Esser, 1977). These methods were extended to include the preparation of hybrid C1 assemblies with $C1_{gp}$ subcomponents (Bartholomew & Esser, 1978), and now C1 reagents have been prepared in the same way from human and guinea pig sera that contain radiolabeled C1r.

Measurement of C1s and C1r Activation. Activation of radiolabeled C1s or C1r in each C1 assembly was measured by monitoring the conversion of the proenzyme form to its activated counterpart by $NaDodSO_4$–polyacrylamide gel electrophoresis under reducing conditions (Bartholomew & Esser, 1977; Cooper & Ziccardi, 1977). With agg-IgG, routinely 80 to 90% of the radiolabeled subcomponent could be converted to its activated form, compared to 10 to 15% conversion in the absence of activators.

Binding of C1 and C1 Subcomponents to MLV. Mixtures of MLV (10–100 μg) and the desired radiolabeled C1 subcomponent(s) were incubated at 30 °C for 30–60 min in 100–200 μL of VBS at half-physiologic ionic strength. Binding of the subcomponents to the virion was then monitored in one of two ways. (1) Samples layered on 20–65% linear sucrose density gradients prepared with the same buffer were centrifuged in a Beckman SW 50.1 rotor at $280000g_{max}$ for 60 min. Under these conditions, virions and bound C1 subcomponents migrated halfway into the gradients. (2) Samples were ultracentrifuged in the Airfuge, and then the amount of radioactivity in each viral sediment was determined. To study displacement of bound subcomponents from virions, an additional incubation with a large excess of unlabeled components was carried out at 4 °C overnight before the binding analysis. The amounts of C1 binding to MLV or C1s-coated MLV were determined by measuring residual C1 activity with EAC4 cells as described (Augener et al., 1971). Binding of radiolabeled C1 was also assessed by measuring subsequent activation of the bound complexes either directly or after transfer to agg-IgG.

Preparation of Monomeric and Dimeric C1s. Monomers of C1s were prepared from dimeric C1s by treatment with 10 mM EDTA (Valet & Cooper, 1974). Molecular weights were determined by gel filtration on a 1.5 × 90 cm column of Ultragel AcA 34 (LKB, Rockville, MD), as described by Andrews (1965). The following proteins (Combithek, Boehringer-Mannheim) were used as standards: catalase (mol wt 240000), aldolase (158000), bovine serum albumin (67000), and ovalbumin (45000). For preparation of dimeric C1s, the column was equilibrated with Ca^{2+}-VBS, and for monomeric C1s the column was equilibrated with EDTA–VBS, both at half-physiologic ionic strength. Peak fractions were collected and used directly for binding assays. Free metal ions were removed from dimeric C1s by gel filtration on Sephadex G-25 equilibrated with VBS that had been freed of metal ions by treatment with a slurry of Chelex 100 (Bio-Rad Laboratories, Richmond, CA). Gel filtration on Ultragel AcA 34 substantiated that C1s remained a dimer under these conditions.

Formation of p15(E)–C1s Immune Complexes. Trace radioiodinated p15(E) and differentially labeled excess $C1s_{hu}$ were incubated at half-physiologic ionic strength for 2 h at 30 °C. Excess goat anti-$C1s_{hu}$ antiserum was added to bind $C1s_{hu}$ during a 2-h incubation at 25 °C, and the goat antibodies were then precipitated overnight with a 50-fold excess of rabbit anti-goat IgG. The immune precipitates were washed several times with saline and then were analyzed for radioactivity.

Results

C1r Activation by Retroviruses. We have shown previously (Bartholomew & Esser, 1978) that the difference in virolytic capacity between human and guinea pig complement resulted from the absence of C1s activation in guinea pig serum. Because incorporation of $C1s_{hu}$ into $C1_{gp}$ to form the hybrid complex $C1q_{gp}$–$C1r_{gp}$–$C1s_{hu}$ resulted in activation of that hybrid complex and rendered such fortified guinea pig serum virolytic, we decided to determine whether activation of C1s depends upon modulation of its own enzymatic activator $C1\bar{r}$ or whether C1s itself provides a second binding site for the viral activator on the C1 molecule. C1 assemblies containing radioiodinated $C1r_{hu}$ (e.g., $C1q_{hu}$–[^{125}I]$C1r_{hu}$–$C1s_{hu}$ and $C1q_{gp}$–[^{125}I]$C1r_{hu}$–$C1s_{gp}$) were prepared, and [^{125}I]$C1r_{hu}$ in both types of assemblies predominates in the proenzyme form (Figure 1, A and B). Incubation with agg-IgG produces $C1\bar{r}$ in both complexes (Figures 1, C and D). Incubation with MLV efficiently converts $C1r_{hu}$ only when it is present in human C1 (Figure 1E). Little, if any, conversion can be detected when $C1r_{hu}$ is in a C1 hybrid with the C1q and C1s subcomponents from guinea pig (Figure 1F). Similar experiments were performed with a complex consisting of [^{125}I]$C1r_{gp}$ reassembled with $C1q_{hu}$ and $C1s_{hu}$, and, as expected, $C1r_{gp}$ was activated by MLV in this C1 hybrid (data not shown). These results, exactly paralleling those obtained

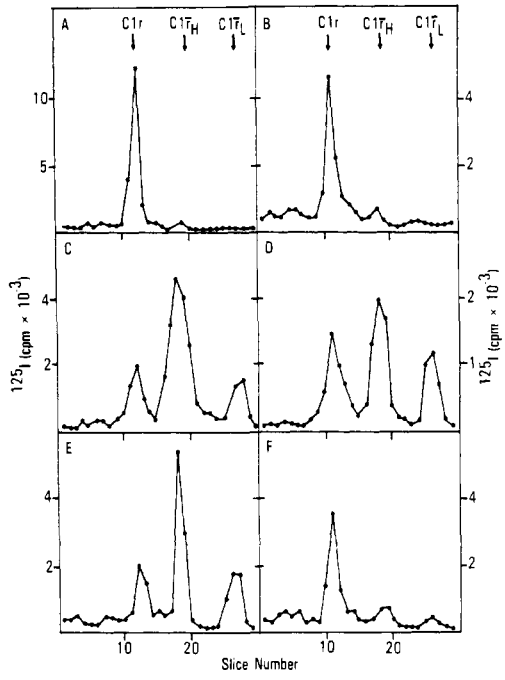

FIGURE 1: $C1r_{hu}$ activation in different C1 assemblies. Radiolabeled C1 (50 μL) formed with [125I]$C1r_{hu}$ in human serum (left panels) and in guinea pig serum (right panels) was analyzed by NaDodSO4–polyacrylamide gel electrophoresis under reducing conditions for C1r activation after a 45-min incubation at 37 °C with either buffer (A and B), 50 μg of agg-IgG (C and D), or 50 μg of MLV (E and F). C1r denotes the native subcomponent of C1 and $C1\bar{r}_H$ and $C1\bar{r}_L$ denote the heavy and light chains of the C1r̄ molecule which are produced by cleavage of C1r upon activation.

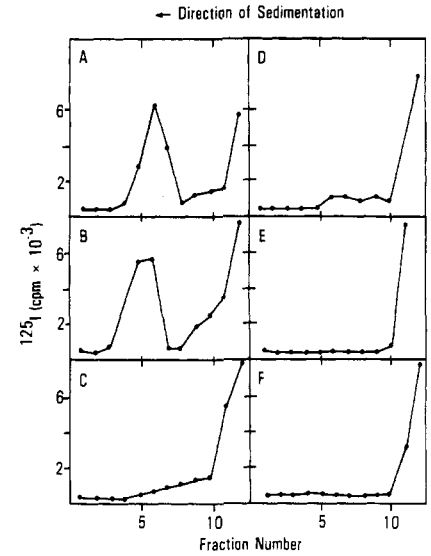

FIGURE 2: Binding of C1 subcomponents to MLV. The following virus samples were incubated for 60 min at 30 °C and half-physiologic ionic strength and then centrifuged in linear 20–65% sucrose density gradients (prepared in half-physiologic ionic strength buffer): (A) [125I]MLV; (B) [125I]$C1s_{hu}$ + MLV; (C) [125I]$C1s_{gp}$ + MLV; (D) [125I]$C1s_{hu}$ + trypsinized MLV; (E) [125I]$C1\bar{s}_{hu}$ + MLV; (F) [125I]$C1r_{hu}$ + MLV.

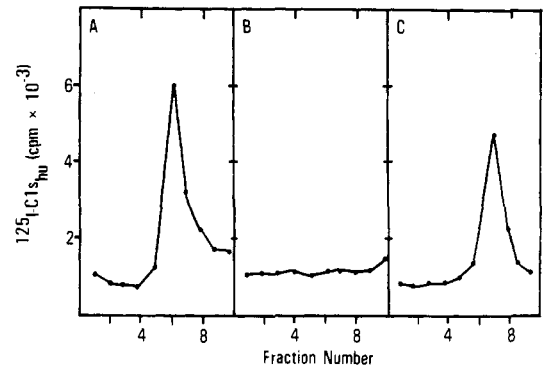

FIGURE 3: Displacement of bound $C1s_{hu}$ from MLV. Three 25-μg samples of MLV were incubated in 200 μL of half ionic strength buffer with [125I]$C1s_{hu}$ for 60 min to bind $C1s_{hu}$ to the virion. Samples were then incubated overnight at 4 °C with (A) buffer, (B) 50 μg of $C1s_{hu}$, and (C) 50 μg of $C1s_{gp}$ and analyzed by sucrose density gradient ultracentrifugation.

earlier with C1 hybrids containing $C1s_{hu}$ (Bartholomew & Esser, 1978), confirm that C1r activation occurs only when $C1s_{hu}$ is present in the C1 assembly.

Because the studies on C1r and C1s activation seemed to rule out any dysfunction of C1r, we tested for binding of subcomponents to the viral surface. Evidence that $C1q_{hu}$ and $C1q_{gp}$, as well as the C1 assemblies from both species, bind to virions has been presented previously (Bartholomew & Esser, 1978). In similar binding experiments, MLV was incubated at 30 °C with purified 125I-labeled C1s in VBS at half-physiologic ionic strength in the presence of divalent metal ions. This mixture was then subjected to rate zonal ultracentrifugation in 20–65% sucrose density gradients. Under the conditions chosen for centrifugation, intact virions sediment halfway into the gradient (Figure 2A), whereas free proteins remain on top. As is evident from Figure 2B, a portion of [125I]$C1s_{hu}$ comigrated with the virions into the gradient. In contrast, hemolytically active [125I]$C1s_{gp}$ remained on top of the gradient (Figure 2C), indicating that the binding of $C1s_{hu}$ is specific. Trypsinization of the virus under conditions that leave the virus intact, but remove the C1 receptor (Bartholomew et al., 1978), abrogates $C1s_{hu}$ binding (Figure 2D). Cleavage of C1s to form its enzymatically active form $C1\bar{s}$ destroys the binding capacity (Figure 2E). Additionally, the $C1r_{hu}$ subcomponent does not have viral affinity (Figure 2F). The bound [125I]$C1s_{hu}$ can be displaced by unlabeled $C1s_{hu}$, but not by $C1s_{gp}$ (Figure 3). However, in the absence of free $C1s_{hu}$, the association between bound $C1s_{hu}$ and virus is relatively stable. After three consecutive washings with half-physiologic ionic strength buffer, the viral pellet still contains significant amounts of bound $C1s_{hu}$ (data not shown). Binding of $C1s_{hu}$ to virus is influenced by the ionic strength of the buffer solution and is most efficient at approximately half-physiologic strength and less efficient at higher ionic strength (Table I).

Earlier studies by Valet & Cooper (1974) established that C1s is present in C1 and free in solution as a noncovalently bonded dimer held together by Ca ions; exposure to EDTA dissociates the dimer into monomers. But in subsequent studies, Sim et al. (1977) reported that $C1s_{hu}$ is monomeric with an approximate molecular weight of 83 000 regardless of exposure to 5 mM EDTA. Under the conditions specified in the Materials and Methods section, we found that [125I]-$C1s_{hu}$ in the presence of 10 mM EDTA eluted from the Ultragel AcA 34 column with an apparent mol wt of 91 000. In the absence of EDTA and independent of Ca^{2+} or other free metal ions in the buffer, [125I]$C1s_{hu}$ had an apparent mol wt of 160 000–180 000. These results are in agreement with those published by Okamura et al. (1973), Valet & Cooper (1974), and Campbell et al. (1979), all of which indicate that in the absence of EDTA C1s is dimeric.

Thus, it was of interest to know whether binding of C1s to viral membranes was a property of only the dimeric form or of both forms. As shown in Table I, in the presence of 10 mM EDTA, monomeric [125I]$C1s_{hu}$ does not bind to the virus. Removal of EDTA by dialysis against metal ion free buffer

Table I: Effect of Ionic Strength and Metal Ions on C1q and C1s Binding to MLV[a]

subcomponent[b] (^{125}I labeled)	MLV (10 μg)	buffer (5 mM barbital, pH 7.3)	radioact. in sediment (%)
$C1s_{hu}$	−	+150 mM NaCl	9
$C1s_{hu}$	+	+30 mM NaCl	38
$C1s_{hu}$	+	+60 mM NaCl	39
$C1s_{hu}$	+	+90 mM NaCl	40
$C1s_{hu}$	+	+120 mM NaCl	32
$C1s_{hu}$	+	+150 mM NaCl	21
$C1s_{hu}$	+	+300 mM NaCl	7
$C1s_{hu}$	+	+75 mM NaCl + Ca^{2+}/Mg^{2+}	38
$C1s_{hu}$	+	+75 mM NaCl − metal ions[c]	37
$C1s_{hu}$	+	+75 mM NaCl + 10 mM EDTA	9
$C1q_{hu}$	−	+75 mM NaCl + Ca^{2+}/Mg^{2+}	30
$C1q_{hu}$	+	+75 mM NaCl + Ca^{2+}/Mg^{2+}	87
$C1q_{hu}$	+	+75 mM NaCl + 10 mM EDTA	86

[a] Radioiodinated subcomponents (10^5 cpm) were incubated with or without MLV for 1 h at 30 °C in 150 μL of barbital buffer containing the indicated additions. The samples were then centrifuged for 10 min at 160000g_{max} in the Airfuge, and the sedimented radioactivity was determined. [b] Subcomponents were equilibrated with the desired buffer by gel filtration on Sephadex G25. [c] Buffer was freed of metal ions by exhaustive treatment with Chelex 100.

causes aggregation of $[^{125}I]C1s_{hu}$ precluding binding tests of monomeric $[^{125}I]C1s_{hu}$ to MLV in the absence of EDTA. In contrast to the monomeric form, dimeric $[^{125}I]C1s_{hu}$ binds to MLV even in the absence of free metal ions. Similar binding studies showed that $C1q_{hu}$ binds to MLV independently of EDTA.

Both $C1q_{hu}$ and $C1s_{hu}$ bind to viral surfaces; however, whether they bind independently of each other was not previously determined. Therefore, increasing amounts of differentially labeled $C1q_{hu}$ and $C1s_{hu}$ were incubated separately or simultaneously with virus in VBS at half-physiologic ionic strength. The binding curves for each subcomponent and the concentration of subcomponents required to saturate specific sites on the virus were not influenced by the presence of the other subcomponent (Figure 4). Thus, binding of $C1s_{hu}$ to virus is independent of $C1q_{hu}$ binding and vice versa. In agreement with this conclusion, we found that unlabeled $C1q_{hu}$ displaced $[^{131}I]C1q$ without significantly removing $[^{125}I]C1s$, and, in similar fashion, unlabeled $C1s_{hu}$ exchanged with $[^{125}I]C1s_{hu}$ without affecting bound $[^{131}I]C1q$ (data not shown).

Effect of C1s Binding on the Ability of Retroviruses to Activate C1. If binding of $C1s_{hu}$ to the virion is a necessary part of C1 activation by virus, then presaturation of all binding sites with $C1s_{hu}$ would suppress the ability of that virion to activate subsequently offered macromolecular C1. This proposed inhibition does occur; the top two panels of Figure 5 (A, B) show background activation of $C1_{hu}$ and activation induced by MLV. C1 activation was incomplete because limiting amounts of virus were chosen to assure high sensitivity. MLV preincubated with excess purified $C1s_{hu}$ was unable to activate $[^{125}I]C1_{hu}$ (Figure 5C), yielding values equivalent to background C1 activation. However, virus preincubated with the same amount of purified $C1s_{gp}$, which does not bind to MLV, activated C1 as efficiently as untreated virus (Figure 5D).

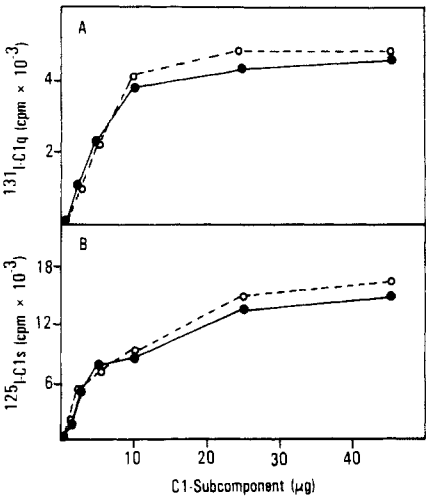

FIGURE 4: Binding of human C1 subcomponents C1q and C1s to MLV. MLV (40 μg) in a final volume of 150 μL of half-physiologic ionic strength buffer was incubated individually (●) or simultaneously (○) for 1 h at 30 °C with increasing concentrations of C1q (A) and/or C1s (B) containing trace amounts of $[^{131}I]C1q$ or $[^{125}I]C1s$, respectively. After centrifugation in an air-driven ultracentrifuge, the viral pellets were analyzed for bound radioactivity. Binding curves for the individual subcomponents are identical in the absence (●) or presence (○) of equal amounts of the other subcomponent.

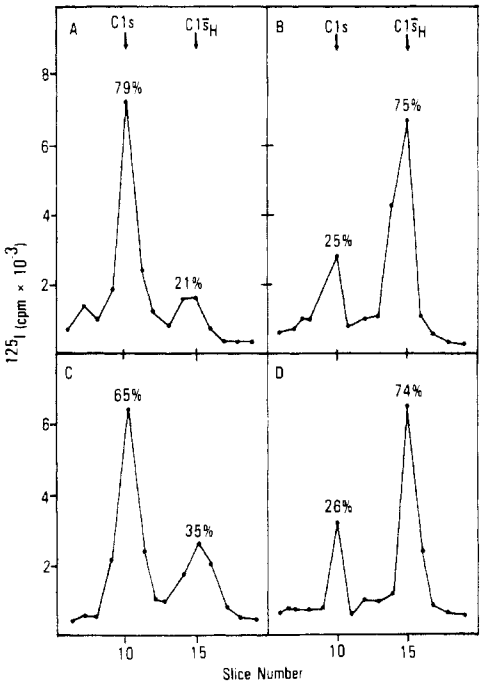

FIGURE 5: Activation of C1 by C1s-coated MLV. Radioactive C1 reagent (50 μL) was incubated for 45 min at 37 °C in a final volume of 100 μL with (A) buffer; (B) 25 μg of MLV; (C) 25 μg of MLV preincubated with $C1s_{hu}$; and (D) 25 μg of MLV preincubated with $C1s_{gp}$. Conversion of C1s to C1s̄ was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis; C1s denotes the native subcomponent of C1 and C1s̄$_H$ denotes the heavy chain of the C1s̄ molecule which is produced by the cleavage of C1s upon activation. The light chain of C1s̄ is not significantly radioiodinated in the procedure used here. The percentage of each component is indicated above the respective peaks.

MLV preincubated with increasing amounts of free $C1s_{hu}$ lost its ability to activate $[^{125}I]C1_{hu}$ in a dose-dependent fashion (data not shown).

The inability of C1s-coated virus to activate subsequently offered C1 could simply be caused by a lack of C1 binding, although the C1q binding experiments just described argue against such an explanation. But C1q binding may not be

Table II: Activation of C1 by MLV and C1s-Coated MLV[a]

sample (25 μL)	physiologic ISB[b] (% max C1s conversion)	half-physiologic ISB[b] (% max C1s conversion)
buffer	20	13
50 μg of agg-IgG	100	92
50 μg of MLV	100	100
50 μg of C1s-coated MLV	26	18
50 μg of C1s-coated MLV + 50 μg of agg-IgG[c]	100	55

[a] Indicated samples were incubated for 30 min at 37 °C with each of the two C1 reagents, and activation was monitored by $NaDodSO_4$-polyacrylamide gel electrophoresis. Activation with MLV was set at 100% activation. [b] ISB, ionic strength buffer. [^{125}I]C1s-containing C1 reagents were isolated in 10–30% sucrose density gradients made in the indicated buffer. [c] Aggregated IgG was added 15 min after incubation was started.

identical with C1 binding, and for this reason we tested directly for C1 attachment to C1s-coated virus. These assays take advantage of the fact that C1 interactions are strong at half-physiologic ionic strength but that C1 can be transferred readily from one entity to another at physiologic ionic strength. In a procedure developed by Augener et al. (1971), a known amount of C1 is first incubated with the test sample at half-physiologic ionic strength, and the remaining unbound C1 is then assayed by hemolysis of EAC4 cells. By using this assay we found that 35 μg of untreated virus and 80 μg of $C1s_{hu}$-coated virus were necessary to bind 63% of the offered C1. Although these data demonstrate C1 binding to $C1s_{hu}$-coated virus, it is unclear whether the observed difference in binding reflects a true difference in affinity between treated and untreated virus. The small amounts of $C1\bar{s}$ present in the $C1s_{hu}$ sample used to coat the virus raised background lysis of EAC4 cells, making it difficult to quantitate the exact amount of $C1s_{hu}$-coated virus required for C1 binding. For this reason, radiolabeled C1 was used in a more direct assay to measure binding as well as activation of C1.

C1 reagents containing [^{125}I]C1s were prepared in Ca^{2+}-VBS at both physiologic and half-physiologic ionic strength. Both [^{125}I]C1 preparations were activated by agg-IgG and MLV, whereas neither was activated by C1s-coated virus. When C1s-coated virus was incubated with either C1 reagent at 37 °C and agg-IgG was added later, the C1 reagent in half-physiologic ionic strength buffer was only partially activated (Table II).

That both C1 preparations actually bound to C1s-coated MLV could be verified directly by transfer to agg-IgG. In these experiments, C1s-coated virus was first incubated at 4 °C with C1 reagent at physiologic ionic strength, divided into two portions, and then sedimented. One sediment was resuspended in physiologic buffer and the other in half-physiologic ionic strength buffer. To each sample 50 μg of agg-IgG was added, and, after a 30-min incubation at 4 °C, the agg-IgG was sedimented in a microfuge at *g* forces insufficient to pellet the virus. Analysis of the sediments indicated that almost 80% of the C1 radioactivity became associated with agg-IgG under physiologic ionic strength conditions, compared to less than 25% under half-physiologic ionic strength. These results, when considered collectively, demonstrate that C1s-coated MLV retains binding sites for C1, but that such bound C1 is not activated.

Binding of $C1s_{hu}$ to the Purified Viral Activator. The foregoing studies were performed with intact virus, but our prior experiments show that the isolated viral C1 activator, a membrane protein of approximately 15 000 daltons termed p15(E), also binds C1q and activates human C1 (Bartholomew et al., 1978). In light of our current findings, one would predict that $C1s_{hu}$ should bind to purified p15(E). Association of $C1s_{hu}$ with p15(E) was demonstrated by formation of $C1s_{hu}$ immune precipitates containing [^{125}I]p15(E). In these experiments, increasing amounts of radioactive p15(E) were incubated sequentially with $C1s_{hu}$, goat anti-$C1s_{hu}$, and rabbit anti-goat IgG to form immune precipitates. After extensive washings, almost 50% of the viral activator was recovered in dose-dependent fashion in the precipitates (Figure 6). At most, 12% of [^{125}I]p15(E) was trapped in the precipitates either when $C1s_{hu}$ was omitted or when goat anti-$C1s_{hu}$ was replaced with nonspecific goat IgG.

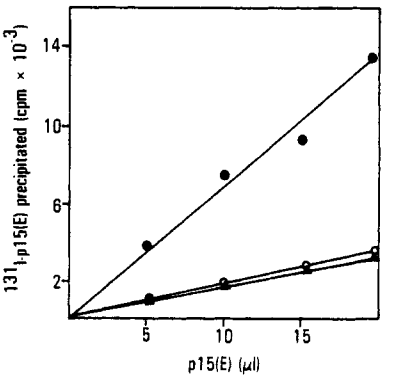

FIGURE 6: Binding of $C1s_{hu}$ to the viral activator p15(E). Increasing amounts of [^{131}I]p15(E) were incubated at 30 °C for 2 h at half-physiologic ionic strength with either buffer (○) or excess $C1s_{hu}$ containing a trace of [^{125}I]$C1s_{hu}$ (●). Goat anti-$C1s_{hu}$ (50 μg) was then added to form immune complexes, followed by rabbit anti-goat IgG to precipitate goat IgG. After overnight incubation, the precipitates were washed extensively and counted for radioactivity. About 85% of the [^{125}I]$C1s_{hu}$ was removed in the immune precipitates. When goat anti-$C1s_{hu}$ is replaced with nonspecific goat IgG, the amount of [^{131}I]p15(E) precipitated (▲) is similar to that found in buffer controls.

Discussion

The ability of retroviruses to activate human C1, but not guinea pig C1, provided an ideal model to study the mechanism of complement activation by these viruses. Two important facts concerning viral C1 activation resulted from the current work. First, macromolecular C1 must bind to virions through the well-established C1q binding site and a second site located on the C1s subcomponent. $C1s_{hu}$ possesses such a binding site for retroviruses, but $C1s_{gp}$ does not. This absence of a $C1s_{gp}$ binding site is the reason that guinea pig serum cannot lyse such viruses in the absence of specific antibody. However, addition of $C1s_{hu}$ to guinea pig serum enables activation and formation of $C1\bar{s}_{hu}$ as well as subsequent assembly of the membrane attack complex and virolysis (Bartholomew & Esser, 1978). This phenomenon clearly indicates that in this system C1s activation is a biologically significant event.

The second important aspect that emerged from this work is that C1s must bind to the virion in order for C1r activation to proceed. To be functional, C1s has to be present in the dimeric and proenzymatic form; neither the monomer in the presence of EDTA nor the activated dimer binds to virus. Bound C1s triggers C1r activation only together with bound C1q. This conclusion takes into account both our previous observation (Bartholomew & Esser, 1978) that hybrid C1 assemblies lacking $C1s_{hu}$ are not activated by MLV, despite the presence of $C1s_{hu}$ in neighboring complexes, and the finding

of Cooper et al. (1976) that stable C1r–C1s complexes are not activated by retroviruses in the absence of C1q. Earlier, Goers et al. (1977) noticed that an immune complex consisting of anti-Dnp IgG and mono-Dnp–polylysine bound C1r and C1s very efficiently. Furthermore, such binding was sufficient to convert proenzyme C1r–C1s complexes to the activated $C1\bar{r}$–$C1\bar{s}$ form, which led the authors to suggest that C1r and C1s binding might be involved in C1 activation. However, this particular activation mechanism, in which the antigen alone in the absence of specific antibody and without C1q binding converted C1r to $C1\bar{r}$ in the C1r–C1s complex, is different from the usual C1 activation process. As a matter of fact, these authors found that addition of C1q to the C1r–C1s complex to form C1 abolished direct activation by the antigen. In contrast, classical C1 activation, as well as antibody-independent activation by retroviruses, absolutely requires C1q binding, a fact that clearly distinguishes these processes from the C1r–C1s activation described by Goers et al. (1977).

Interestingly, C1s-coated virus binds macromolecular C1, but such bound C1 does not become activated. This result not only demonstrates that C1q and C1s must be present in the same C1 assembly for activation to occur but also demonstrates that no significant exchange takes place between C1s bound directly to virus and C1s in the C1 complex which is attached via the C1q binding site to the same virus. Nevertheless, free C1s exchanges readily with C1s in C1 when both molecules are free in solution (Bartholomew & Esser, 1977).

The fact that the isolated subcomponents C1q and C1s each bind to the viral surface independently raises interesting questions with respect to topography and the detailed mode of attachment. Consideration of our finding that even the isolated p15(E) requires binding of both C1q and C1s for activation demands that both subcomponents bind to identical or very similar acceptors on the viral surface. C1q is a molecule of 400 000 daltons and C1s is a dimer of 180 000 daltons, whereas p15(E) has a mass of only 15 000 daltons, part of which is buried in the membrane. The fact that the maximal amount of one subcomponent binding to virus is unchanged by saturation of all of the binding sites with the other subcomponent (see Figure 4) suggests that, at saturation, both subcomponents bind to only one p15(E) molecule. Such an assembly seems unlikely because of steric hindrance, although recent results by Stone & Nowinski (1980) reveal that p15(E) on the viral surface can bind two monoclonal IgG antibodies simultaneously and independently. Certainly further studies are required to solve this puzzle. One possible explanation may be provided by the fact that our studies with isolated p15(E)—a protein notorious for its insolubility in detergent-free buffer—were performed on aggregated material, but future experiments will utilize p15(E) reconstituted in liposomal membranes. Furthermore, we do not know whether C1q attaches itself to virus through the globular "heads" or through the collagen-like "stem". The first mode has been proposed for C1q binding to the Fc domain of IgG (Yasmeen et al., 1976), and the second has been hypothesized for the interaction of C1q with cellular receptors (Sobel & Bokisch, 1975). In addition, it is conceivable that there could be a second site or protein on the virion that is lost or damaged during purification of p15(E).

Thus far, our finding that successful C1 activation requires two C1 subcomponents interacting with the activator is unique. In the case of the antibody-dependent activation, interaction between C1s and immune complexes (Ziccardi & Cooper, 1976b) or agg-IgG (our unpublished observations) could not be demonstrated. Either the methods used to detect such interactions are not sensitive enough and are perhaps disruptive for such putative weak interactions or the necessity of C1s binding to the virion for C1 activation is an exception to the general rule. However, such a second C1 binding site may be a clue to understanding the influence of membrane dynamics on complement activation. Lipid vesicles carrying specific antibody bound to lipid haptens consumed complement better in the "fluid" than in the "solid" state, although binding of C1q to both types of vesicles is identical (reviewed by McConnell, 1978). Nevertheless, Esser et al. (1979) have demonstrated that lipid motion directly influenced C1 activation, thereby explaining the differences in complement fixation observed earlier, and, conceivably, the simultaneous binding of *two* C1 subunits to IgG can be modulated by the motion of the hapten to which the antibody is attached. In addition to viral C1 activation, there are several other nonimmunologic (antibody-independent) activators of C1 such as bacterial lipid A (Cooper & Morrison, 1978) and heart mitochondrial membranes (Giclas et al., 1979). It will be of interest to learn whether C1q and C1s bindings are required events in the mechanism of C1 activation by such nonimmunologic activators.

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Purification and Characterization of Androgen Binding Protein from the Rat Epididymis†

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ABSTRACT: Androgen binding protein (ABP) was purified from rat epididymides by sequential ammonium sulfate precipitation, affinity chromatography, gel filtration, and DEAE ion exchange chromatography. The column matrix formed by coupling dihydrotestosterone-17α-(hexanoic acid) to agarose via diisopropylamine was stable during the extensive washing required following application of crude tissue extracts to the affinity matrix. In addition, when used under the optimal conditions, the column produced a 1600-fold purification in a single step. Apparent homogeneity of the final product was shown by polyacrylamide gel electrophoresis, sedimentation equilibrium, and constant specific activity across the peak of the final chromatograph. The molecular weight determined by sedimentation equilibrium at pH 7.4 was 85000. By contrast, the molecular weight determined by sedimentation equilibrium in guanidine·HCl and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis was approximately one-half that of the native protein, suggesting that ABP is comprised of subunits.

The Sertoli cells of the seminiferous tubules from rat testes secrete an androgen binding protein (ABP),[1] which can be assayed by its ability to bind testosterone or dihydrotestosterone (DHT) with high affinity (for a review, see Hansson et al., 1975). After it is secreted into the lumen of the seminiferous tubule, ABP is transported along with the spermatozoa to the epididymis, where it is concentrated and degraded. Several investigators have demonstrated that ABP synthesis in and secretion by the Sertoli cells are stimulated by testosterone and follicle-stimulating hormone (FSH). ABP is thus one of the few specific markers for hormone action on this key cell of the seminiferous tubule. We, therefore, thought it pertinent to purify and characterize this protein, so that it would be available as a reagent for subsequent studies on the hormonal control of Sertoli cell function. In this report we present a method for isolating homogeneous ABP from rat epididymides in high yield and describe the physical–chemical properties of this protein.

Materials and Methods

General. Protein was measured by a modification (Bensadoun & Weinstein, 1976) of the method of Lowry et al. (1951) and by the dye binding method of Bradford (1976). Bovine serum albumin was used as a standard. The former procedure was used to monitor recovery and specific activity changes, while the latter was used to screen column fractions in order to locate binding activity.

1,2-5α-[^{3}H]Dihydrotestosterone ([^{3}H]DHT), 44 Ci/mmol, was obtained from New England Nuclear Corp. and purified on silica gel thin-layer plates developed in chloroform–methanol (25:2). Radioinert DHT was obtained from Sigma Chemical Co.

[^{3}H]DHT in aqueous solution was quantified by liquid scintillation spectrometry using a fluor (toluene–Triton X-100 (2:1) containing 4 g of PPO/L and 50 mg of POPOP/L) at 27% counting efficiency. [^{3}H]DHT in polyacrylamide gel slices was measured using toluene-based fluor (4 g of PPO/L and 50 mg of POPOP/L) at an efficiency of 37%. 3,3′-Diaminodipropylamine was purchased from Aldrich Chemical Co.; Sepharose and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide·HCl were obtained from Sigma Chemical Co. Frozen epididymides were obtained from Dr. A. Parlow (Harbor General Hospital, Torrance, CA).

Column fractions were concentrated at 4 °C with constant stirring in Amicon ultrafiltration cells (Models TCF10 and

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[1] Abbreviations used: ABP, androgen binding protein; DHT, 5α-dihydrotestosterone; $NaDodSO_4$, sodium dodecyl sulfate; TeBG, testosterone–estradiol binding globulin; BSA, bovine serum albumin; DHT-HA, 3-oxo-17β-hydroxy-5α-androstane-17α-(6-hexanoic acid); PAS, periodic acid Schiff; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; TEG buffer, 20 mM Tris, 2 mM EDTA, and 10% glycerol; TG buffer, 20 mM Tris and 10% glycerol; TDK buffer, 20 mM Tris, 10% dimethylformamide, and 1 M KCl.

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