

FORMATION AND BIOLOGIC ROLE OF POLYOMA  
VIRUS-ANTIBODY COMPLEXES

A CRITICAL ROLE FOR COMPLEMENT\*

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The ability of viruses to persist in the circulation and in tissues of man and animals for long periods of time is now well established. In many persistent infections, viral replication continues despite the production of circulating antibody(s) directed against viral antigens (1-6). Indeed, the major tissue injuries associated with such persistent viral infections are due to trapping of virus-antiviral antibody (V-Ab)<sup>1</sup> immune complexes along filtering membranes in glomeruli, arteries, and the choroid plexus with resulting glomerulonephritis, arteritis, and choroiditis, respectively (1, 2, 7).

Studies carried out on several persistent viral infections suggest that complement binds to V-Ab immune complexes *in vivo*. Evidence that virus in the serum complexes with host immunoglobulin (Ig) and complement (C) comes from experiments in which specific precipitation of either Ig or C from the serum removes significant infectivity, while precipitation of other serum proteins, such as albumin, removes none (3, 5, 6, 8, 9). Examination of glomeruli, arteries, or choroid plexus shows a progressive accumulation of Ig, C, and viral antigens in irregular deposits along the basement membrane of capillary walls (1, 2). In addition to demonstrating V-Ab-C immune complexes, these findings also indicate a failure of the immune surveillance mechanism to eliminate the infectious virus.

In the present study, we have investigated the role of antibody and C, in both the formation of V-Ab-C complexes and in the neutralization of polyoma virus. This virus is potentially oncogenic, able to cause persistent viral infection, and has been associated with immune complex formation (10). We have found that

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<sup>1</sup> *Abbreviations used in this paper:* BSA, bovine serum albumin; CPE, cytopathogenicity; PFU, plaque-forming units; V-Ab, virus-antiviral antibody.

C markedly enhances the aggregation of V-Ab complexes, and thereby plays a significant role in neutralization of this nonenveloped DNA virus. This is accomplished by C3-dependent cross-linking of viral particles with resulting reduction in the net number of infectious aggregates. This new action of C3 and the presence of C3b on the viral surface has important implications for the fate of polyoma virus and perhaps other viruses *in vivo*.

### *Materials and Methods*

#### *Purification and Titration of Virus.*—

*Polyoma virus:* Small plaque type polyoma virus passed through mouse kidneys was obtained from Dr. R. Dulbecco, Salk Institute, La Jolla, Calif. Virus pools were made as described by Winocour and virus was purified by modification of Crawford's method (11). Briefly, kidneys were taken from 14-day old Ha/ICR or SWR/J mice infected 10 days earlier with  $1 \times 10^6$  plaque-forming U (PFU) of virus, minced, trypsinized, and placed in culture. When cytopathogenicity (CPE) was noted 6–8 days later, the supernate from the culture was collected, cleared of debris by low speed centrifugation (15 min at 700 g), and pelleted by ultracentrifugation (4 h at 80,000 g in a Beckman L2-65 ultracentrifuge [Beckman Instruments, Inc., Fullerton, Calif.]). The pellet was suspended in 50 mM Tris-HCl buffer, pH 8, sonicated, and sequentially banded twice by equilibrium density gradient centrifugation in cesium chloride (24 h at 114,000 g). The lower band, which contains "full" polyoma particles, was collected and freed of cesium chloride by sedimenting the virus (4 h at 80,000 g). The pellet was resuspended in 50 mM Tris-HCl, pH 8, containing 0.1% bovine serum albumin and frozen at  $-70^\circ\text{C}$  until use. The hemagglutinating ability of polyoma virus was determined with 0.6% guinea pig red blood cells and its plaque-forming ability by CPE for Ha/ICR or SWR/J mouse embryo fibroblasts. An average preparation had  $2-8 \times 10^6$  hemagglutination U (HAU)/ml and a plaque-forming titer of  $2-8 \times 10^{11}$ /ml. The relationship between hemagglutinating U and particles was used to calculate the number of virus particles present (11). The accuracy of this method was verified in preliminary experiments in which both virus particles were counted (12) and hemagglutinating U determined.

*Preparation and Purification of Antiviral Antibodies.*—Antibodies to polyoma virus were made by injecting purified virus in incomplete Freund's adjuvant directly into popliteal lymph nodes of 3–4 kg rabbits. Rabbits were injected subcutaneously with virus in incomplete Freund's adjuvant every 28 days. Bleedings were done 7 days after each inoculation and antisera obtained were absorbed with noninfected tissue culture cells and media. Hamster antibody to polyoma virus was supplied by Dr. Kenneth Takemoto, NIAID, NIH, Bethesda, Md. Rabbit and hamster antibody to polyoma virus contained from  $2-9 \times 10^4$  hemagglutination inhibition U/ml and at a dilution of  $2 \times 10^{-3}$  the antibody inhibited 50% of polyoma virus PFU. Using  $^{125}\text{I}$ -labeled polyoma virus (see below) as an immunoabsorbant, we determined that 0.7% of  $^{131}\text{I}$ -labeled hamster IgG and 0.5% of  $^{131}\text{I}$ -labeled rabbit IgG was antibody to polyoma virus.

Antisera were heated at  $56^\circ\text{C}$  for 30 min. After initial fractionation with saturated ammonium sulfate, the IgG fraction was obtained by chromatography on DEAE-cellulose columns, 0.0175 M sodium phosphate, pH 7.4, for rabbit and 0.01 M, pH 8, for hamster, respectively. The effluent was collected, concentrated to 10 mg protein/ml, and shown to contain only IgG by immunoelectrophoresis and Ouchterlony analyses.

*Complement.*—Individual guinea pig, rabbit, or human sera were used as a C source. For some studies, C4-deficient guinea pig or C6-deficient rabbit sera were employed. Sera were collected, divided into small aliquots, and stored at  $-70^\circ\text{C}$  until use. In those experiments in which the action of C was studied, sera were diluted in barbital saline with gelatin, calcium chloride, and magnesium chloride (13).

C components C1, C2, C3, and C4 were isolated from human serum as reported (14–17). Human C2 was used exclusively in the oxidized form (18). C component titrations were per-

formed after incubation of 50 $\lambda$  of polyoma virus (usually containing 3–4  $\times 10^9$  virus particles) with 50  $\mu$ l of antipolyoma antibody and 100  $\mu$ l of fresh serum for 60 min at 37°C. The methodology employed has been published (19). Controls without virus and without virus and antibody were included and simultaneously titered.

*Labeling of Virus and Antibody.*—To label virus with  $^{125}\text{I}$  we used a modification of the chloramine-T technique (20). Briefly, 2–3 mCi of  $^{125}\text{I}$  and 5  $\mu$ g of chloramine-T were added to 8–12  $\mu$ g of purified virus (approximately  $2 \times 10^{11}$  full particles) suspended in 0.5 ml of 50 mM Tris, pH 7.2, containing 0.1% bovine serum albumin (BSA), for 2 min at 4°C. The reaction was stopped by adding 5  $\mu$ g of sodium metabisulfite. After iodination, virus was diluted in C buffer and centrifuged at 105,000 g for 2 h to remove nonviral-associated free iodine. The pellet was resuspended in 50 mM Tris, pH 7.2, containing 0.1% BSA. The uptake of  $^{125}\text{I}$  was 2–8%.

The nucleic acid of polyoma virus was labeled with [ $^3\text{H}$ ] or [ $^{14}\text{C}$ ]thymidine and virus was purified as previously reported (11). Over 95% of [ $^{125}\text{I}$ ], [ $^3\text{H}$ ], or [ $^{14}\text{C}$ ]polyoma virus was precipitable with TCA.

To insure that  $^{125}\text{I}$ ,  $^3\text{H}$  or  $^{14}\text{C}$  labels were on the virion, a portion of radiolabeled virus was first incubated with specific rabbit or hamster antibody to polyoma virus for 30 min at 37°C and then with antibody to rabbit or hamster IgG, in excess, for 30 min at 37°C. The mixture was spun at 1,300 g for 20 min at 4°C, after which the supernate and precipitate were collected and analyzed for radioactivity. In other experiments labeled virus was rebanded either on a cesium chloride (11) or a sucrose density gradient and the relationship of virus density to radioactivity in the gradient was plotted.

*Formation and Centrifugation of V-Ab and V-Ab-C Complexes.*—All reagents were diluted in C buffer. Virus was incubated with antiviral antibody for 10 min at 37°C with gentle agitation in a water bath. In some experiments, fresh or heated (56°C for 30 min) guinea pig, rabbit, or human serum was added to the V-Ab complex. In other experiments, highly purified individual C components were added to the V-Ab complex. In the experiments in which complement components were added to V-Ab mixtures, an attempt was made to duplicate the prevailing concentrations in 50  $\mu$ l of human serum. Approximately 10  $\mu$ g of C1q,  $8 \times 10^{11}$  effective molecules of C1,  $7 \times 10^9$  effective molecules of oxidized C2, 20  $\mu$ g of C4, and 70  $\mu$ g of C3 were used. When C1 and C4, or C1, C4, and C2 or C1, C4, C2, and C3 were employed, each component except for C1 was added to the V-Ab complex. C1 was then added and the mixture incubated in a vol of 0.25 ml at 37°C for 20 min. The various samples of virus, V-Ab or V-Ab-C were then layered on 5–20% (wt/vol) linear sucrose gradients (50 mM Tris, 0.15 M sodium chloride, pH 8) which were formed over 0.3 ml of 65% sucrose. The gradients were centrifuged at 4°C at 114,000 g for 18 min in a 50.1 rotor in a Beckman L2-65 ultracentrifuge (Beckman Instruments, Inc.).

*Radioactive Analysis.*—After centrifugation, fractions were collected from gradient tubes and assayed for radioactivity and refractive index.  $^{125}\text{I}$  samples were counted in a Baird Atomic gamma well counter (Baird Atomic, Inc., Bedford, Mass.). In some experiments fractions were precipitated with 10% TCA in the presence of 0.1 mg BSA before counting. Fractions containing either  $^3\text{H}$  or  $^{14}\text{C}$  were collected directly into 15 ml of aquasol containing water (75 ml/liter) and glacial acetic acid (50 ml/liter). Alternatively, the fractions were treated with 10% TCA and the precipitates collected on gs/A filters. The filters were washed three times with 5% TCA, dried under an infrared lamp, placed in a vial containing 8 ml of aquasol, and counted in a Beckman LS-230 spectrometer (Beckman Instruments, Inc.).

*Sizing of V-Ab and V-Ab-C Complexes.*—Markers used to size the V-Ab and V-Ab-C complexes were radiolabeled polyoma virus (242S), rabies virus (550S)<sup>2</sup> and bacteriophages<sup>3</sup>

<sup>2</sup> Rabies virus, HEP strain, was kindly supplied by T. Wiktor, Wistar Institute, Philadelphia, Pa.

<sup>3</sup> The assays were kindly done by Dr. Junetsu Ito, Department of Microbiology, Scripps Clinic and Research Foundation, La Jolla, Calif.

$\phi$ -15 (262S), and T7 (453S) (21, 22). The position of phages in the various gradients was confirmed by plaquing gradient fractions on susceptible bacterial cultures.<sup>3</sup>

*Electron Microscope Studies.*—Mixtures of virus, V-Ab and V-Ab-C were resuspended in 50 $\lambda$  of distilled water and mixed immediately with 50 $\lambda$  of 3% phosphotungstic acid, pH 6. A drop of the mixture was placed on an electron microscope grid and excess fluid removed with absorbent paper; the grid was observed immediately in the electron microscope.

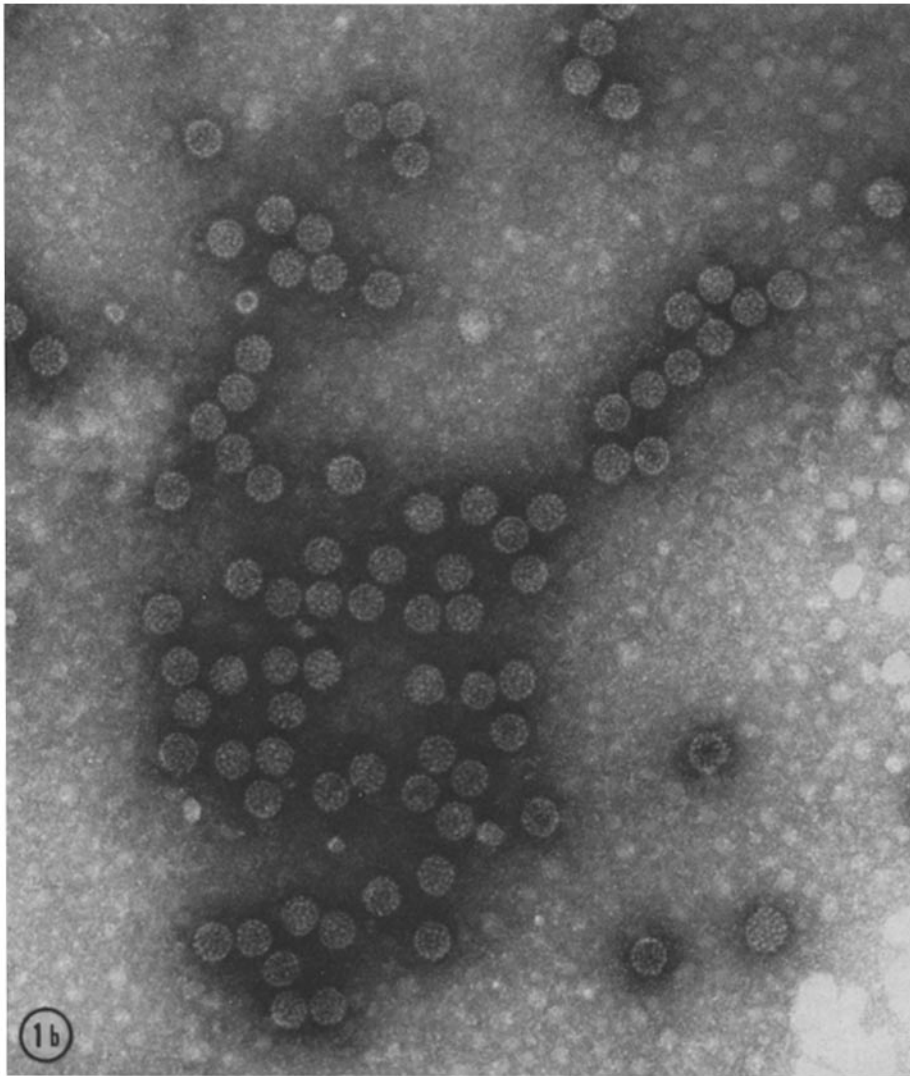
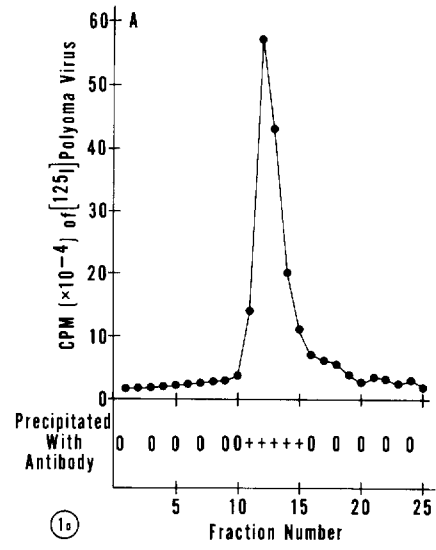
*Virus Neutralization Studies.*—The antibody and C neutralization test was done in vitro by use of standard plaque assay techniques (23). In all instances, virus was first incubated with antibody for 5 min at room temperature and then with C or C components for 30 min at 37°C with gentle agitation. Control mixtures were incubated with C buffer for the same period of time under similar conditions.

## RESULTS

*Effects of Radioactive Labeling on Polyoma Virus.*—After surface labeling with iodine, polyoma virus sedimented in cesium chloride equilibrium density gradients as a single sharp peak with its characteristic buoyant density of 1.32 g/ml (Fig. 1 *a*) (11). In sucrose density gradients, labeled polyoma sedimented as a 242S peak of radioactivity (Fig. 2). Electron microscope studies showed no gross morphological changes in virus structure (Fig. 1 *b*). Over 95% of the radioactivity was precipitable if labeled virus was first incubated with hamster antibody to polyoma virus and then with rabbit antibody to hamster IgG. Polyoma virus was not precipitated under these conditions if first mixed with hamster antibody to SV40 virus. Although labeled polyoma virus appeared physically intact as indicated above, infectivity studies showed a loss of from  $1-5 \times 10^2$  PFU/ml after labeling with iodine. Loss of infectivity was not reduced by changes in the conditions employed for labeling or through use of lactoperoxidase.

*Effect of Antibody on Polyoma Virus.*—Mixtures of <sup>125</sup>I-labeled polyoma virus and varying amounts of <sup>131</sup>I-labeled IgG fraction of hamster antipolyoma antibody were analyzed by sucrose gradient ultracentrifugation. As depicted in Fig. 3, increasing amounts of antibody shifted the sedimentation rate of [<sup>125</sup>I]-polyoma virus in increments, and <sup>131</sup>I-labeled antibody cosedimented with the virus. In comparison to markers sedimented in parallel with the V-Ab mixtures, the sedimentation rate of polyoma virus was enhanced from 242S to 260S with the least amount of antibody employed. We knew the specific radioactivity of the IgG fraction of antipolyoma virus antibody and thus were able to determine the number of molecules of antibody bound to polyoma virus from the number of <sup>131</sup>I counts cosedimenting with the virus. By comparison of these values with the number of polyoma virions (HAU titer of [<sup>125</sup>I]virus) present in the reaction mixtures, we were able to calculate that the 242S–260S sedimentation rate enhancement was accomplished by 2 antibody molecules/virion (Table I). An

FIG. 1. Panel *a*, cesium chloride equilibrium density gradient of <sup>125</sup>I-labeled polyoma virus. The radioactive peak had a buoyant density of 1.32 g/ml and, as shown in the lower portion, was precipitable with antibody to polyoma virus. Panel *b*, electron microscope examination of <sup>125</sup>I-radiolabeled polyoma virus.



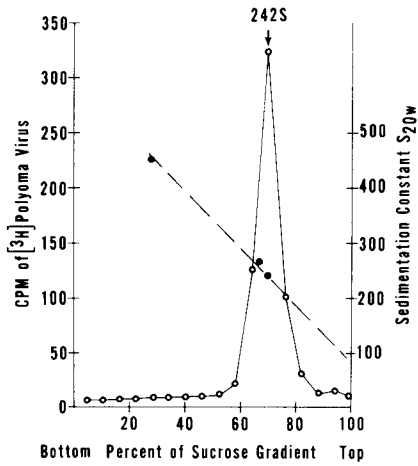


FIG. 2. Sucrose gradient ultracentrifugal analysis of  $[^3\text{H}]$ thymidine-labeled polyoma virus. The straight line connecting the marker proteins gives a sedimentation rate of 242S for polyoma virus.

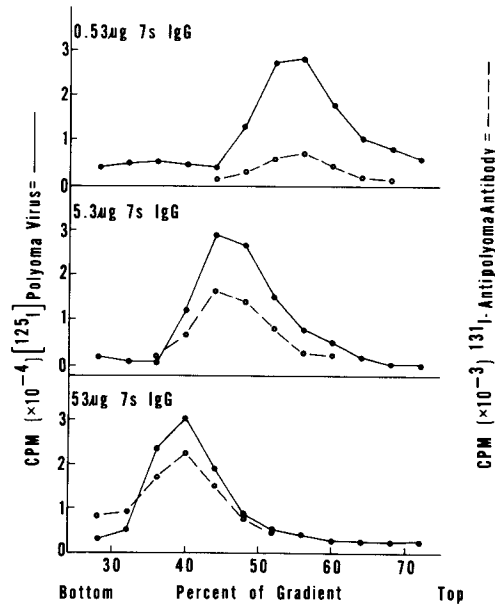


FIG. 3. Sucrose gradient ultracentrifugal analyses of  $^{125}\text{I}$ -labeled polyoma virus incubated with varying amounts of  $^{131}\text{I}$ -labeled antipolyoma antibody. In each panel a portion of the  $^{131}\text{I}$ -labeled antibody cosedimented with the labeled polyoma virus. The sedimentation rates of the complexes by comparison with marker proteins were 260S, 350S and 410S for the experiments shown in the upper, middle, and lower panels, respectively. Calculations from the amount of IgG cosedimenting with the virus and the specific radioactivity permitted a determination of the number of antibody molecules per virus particle as shown in Table I.

TABLE I  
Formation and Characterization of Various V-Ab Complexes

Gradient description		Molecules of specific antibody bound	Ratio Ab/V	S rate §
Virus*	Antibody †			
	$\mu\text{g}$			
$3.2 \times 10^9$	0.00	0		242
$3.2 \times 10^9$	0.53	$7.0 \times 10^9$	2.18/1	260
$3.2 \times 10^9$	5.30	$30.6 \times 10^9$	9.57/1	350
$3.2 \times 10^9$	53.00	$70.4 \times 10^9$	20.2/1	410

\* Number of virus particles calculated from the known number of HAU (320 HAU of  $^{125}\text{I}$  placed in each gradient tube); 1 HAU is equivalent to  $10^7$  virus particles. HAU determined after iodination of virus.

† Sp act of antibody used was  $3.19 \times 10^5$  cpm/ $\mu\text{g}$  protein.

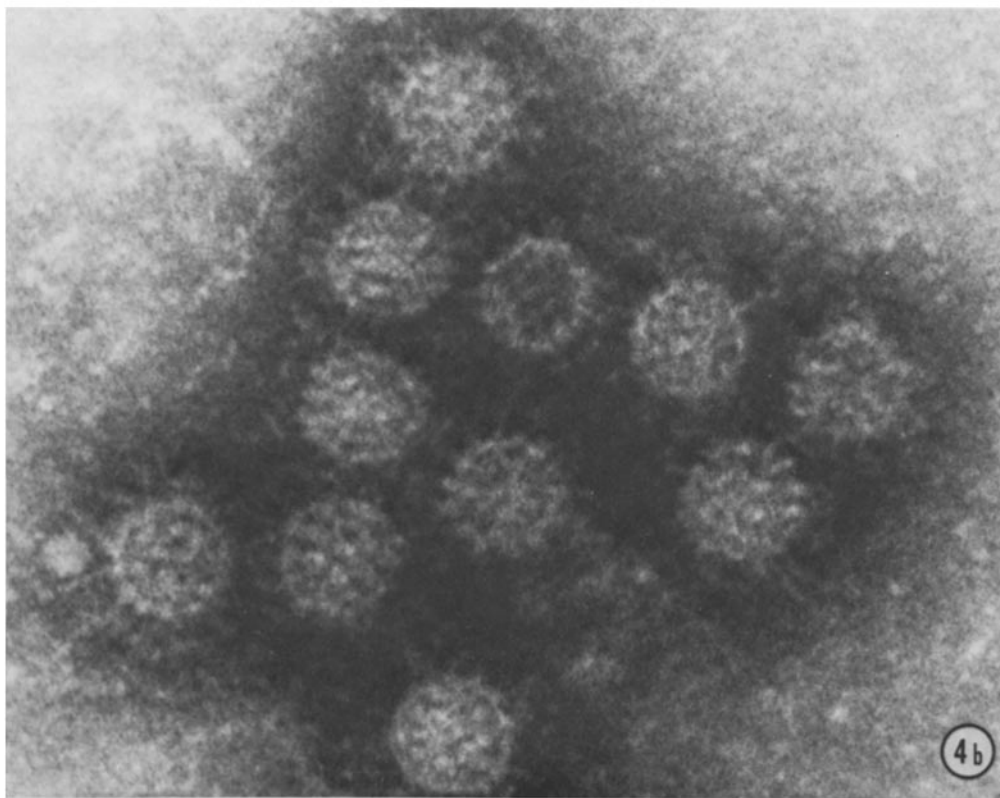
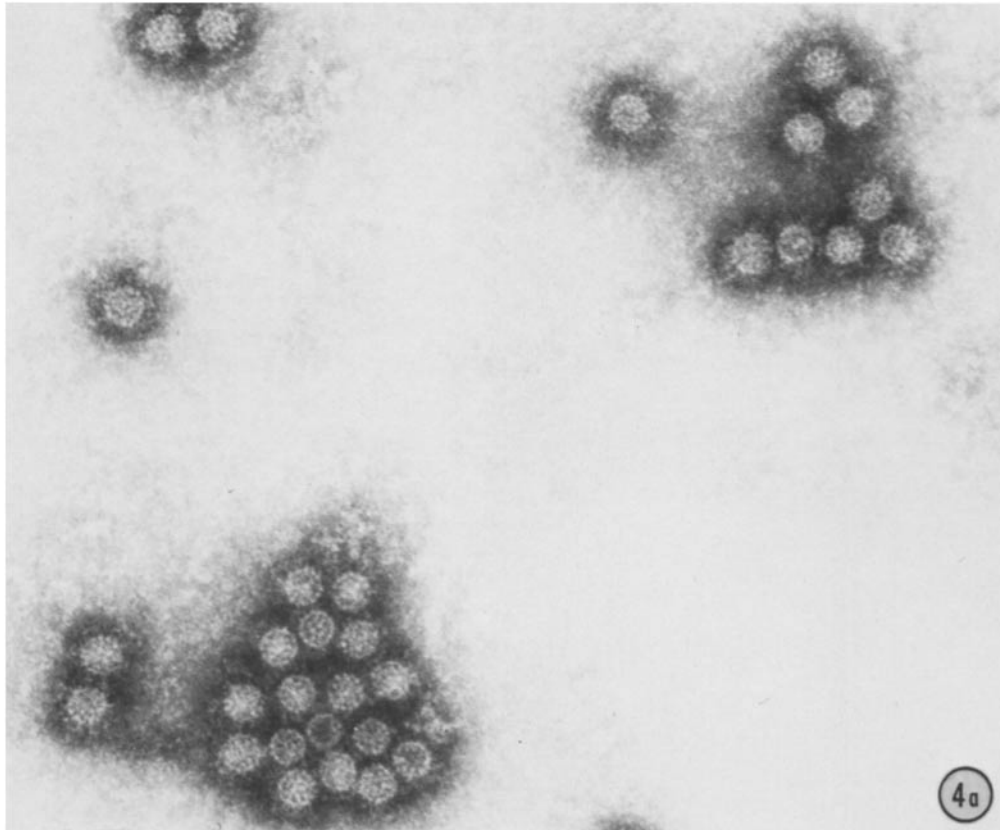
§ S rate was determined by use of the following markers: polyoma virus, rabies virus,  $\phi$ -15, and T7 bacteriophages.

additional 10-fold increment in antibody increased the number of bound antibody molecules per virion 5-fold, to 10, and shifted the sedimentation rate to 350S. A further doubling of the antibody molecules per virion and an increase in the S rate to 410S was accomplished by an additional 10-fold increase in antibody.

Electron microscope examination confirmed that polyoma virus particles were cross-linked and agglutinated in the presence of antibody (Fig. 4 a). Normal hamster IgG did not lead to aggregation of polyoma virus. Under higher power, strands or "whiskers" measuring the reported size of IgG molecules (24, 25) were seen protruding from the surface of viral particles (Fig. 4 b). In experiments using an antibody concentration of 5.3  $\mu\text{g}$ , approximately 10–13 strands or whiskers were counted/virion, which was in good agreement with the estimation, by radioactivity measurement, of 10 molecules/virion (Table I). There was no evidence of disruption or fragmentation of virus particles in any electron microscope field.

In other studies the effect of antibody on polyoma virus infectivity was examined. Doses of antibody in the range of approximately 1–2 antibody molecules bound/virion, which are the amounts employed in most of the studies described below, did not lead to significant virus neutralization. When 10-fold higher doses of antibody were used, polyoma virus was neutralized in the absence of serum.

*Effect of Serum on Polyoma Virus-Antibody Complexes.*—Sucrose gradient ultracentrifugation studies were performed on V-Ab mixtures incubated with fresh serum. The amount of antiviral antibody in 0.53  $\mu\text{g}$  of hamster IgG was chosen for these studies. As shown in Fig. 5, serum increased the sedimentation rate of the V-Ab complex from approximately 260S to about 450S. In this study viral nucleic acid had been labeled with [ $^3\text{H}$ ]thymidine; entirely comparable





results were obtained with [ $^{125}\text{I}$ ]polyoma virus. The enhanced cross-linking produced by fresh serum was heat labile, because serum previously heated at  $56^\circ\text{C}$  for 30 min was unable to enhance the sedimentation of V-Ab complexes.

C did not lead to disruption of the outer viral coat with release of viral nucleic acid as shown by the lack of  $^3\text{H}$  counts near the top of the gradient after treatment of  $^3\text{H}$ -labeled polyoma virus with antibody and C (Fig. 5). This conclusion was further strengthened when  $^3\text{H}$ -labeled polyoma virus proved to be resistant to DNase. Electron microscopic examination of V-Ab-C mixtures also failed to show evidence of virus disruption. In addition, no ultrastructural lesions corresponding to those produced by C in membranes (26, 27) were seen.

Residual activity of the various C components in serum after incubation with V-Ab complexes was determined. As shown in Table II, polyoma virus-antibody complexes efficiently activated C. Consumption of all C components measured, including the terminal reacting components C8 and C9, was observed.

*Role of C Components in the Polyoma V-Ab-C Complex.*—To better understand the role of various C components in enhancing cross-linking of polyoma virus-antibody complexes, guinea pig serum deficient in C4 and rabbit serum deficient in C6 were incubated with radiolabeled virus and  $0.53\ \mu\text{g}$  of antibody. Rabbit serum deficient in C6 increased the sedimentation rate of the V-Ab complexes as well as did normal rabbit, human, or guinea pig serum. In contrast, guinea pig serum deficient in C4 was devoid of enhancing activity.

These studies indicated that early reacting C components were involved in cross-linking of polyoma virus-antibody complexes. Reaction mixtures of labeled V-Ab and various C components were prepared and ultracentrifuged in sucrose gradients. As demonstrated in Fig. 6, C1q markedly enhanced agglutination of V-Ab complexes. However, identical mixtures of labeled virus and antibody with C1 and C4 did not result in enhanced agglutination, and attempts with larger amounts of C1 and C4 were unsuccessful. As shown in Fig. 7, mixtures of polyoma virus and antibody with C1, C4, C2, and C3 also led to enhancement of the sedimentation rate of polyoma virus. In the absence of C2, the sedimentation rate was not enhanced over that observed in the presence of antibody alone, which indicated an essential role for activated C3 in enhancement of viral aggregation.

*Role of C and C Components in Viral Neutralization.*—Polyoma virus was first incubated with an amount of antibody which did not produce neutralization, and then this mixture was incubated with various combinations of serum or C components. As depicted in Table III, both whole serum and serum deficient in C6 were able to neutralize polyoma virus-antibody mixtures, while serum deficient in C4 was unable to do so. In correlation with the sucrose gradient ultra-

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FIG. 4. Electron microscope examination of polyoma virus incubated with  $5.3\ \mu\text{g}$  of the IgG fraction of antipolyoma antibody. Low power and high power views are shown. In the higher power, examination shows the existence of strands or "whiskers" protruding from the viral surface. These whiskers measure within the reported size of IgG molecules (24, 25).

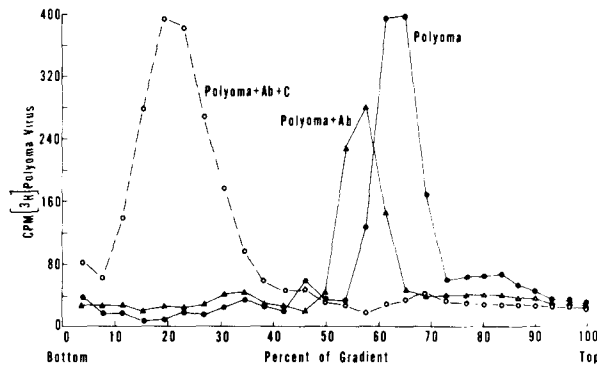


FIG. 5. Sucrose gradient ultracentrifugal analysis of mixtures of [<sup>3</sup>H]thymidine-labeled polyoma virus incubated with antibody or with antibody and serum. There is no evidence of viral lysis on treatment of polyoma virus with antibody and complement. The sedimentation rate of the polyoma virus-antibody-complement aggregate is approximately 450S.

TABLE II  
*Consumption of C Components on Addition of Antiviral Antibody and Serum to Isolated Polyoma Virus\**

C component	Percent C component depleted
C1	75
C4	97
C2	97
C3	50
C5	60
C6, C7	> 50
C8	60
C9	67

\* These percentages represent consumption of C by virus and antibody as compared to samples from which virus and antibody were omitted. In the absence of antibody, average consumption of C by polyoma virus was: C1 (20%), C2 (36%), C3 (0%), C4 (55%), C5 (20%), C6, C7 (not determined), C8 (24%), and C9 (0%).

centrifugal studies, C1q but not C1 or C1 and C4; and C1, C4, C2, and C3 but not C1, C4, and C3 were able to neutralize polyoma virus-antibody complexes.

#### DISCUSSION

The specific immunologic factors which function in the host's defense against pathogenic viruses may include antibody, antibody and C, sensitized lymphocytes, and phagocytic elements. The roles of each and the mechanisms which facilitate neutralization and clearance of virus on one hand or permit viral persistence on the other are incompletely understood. Antibody may neutralize a virus by interfering with viral attachment, by aggregating viruses, or by stimulating engulfment by phagocytic elements. The protective role of antibody

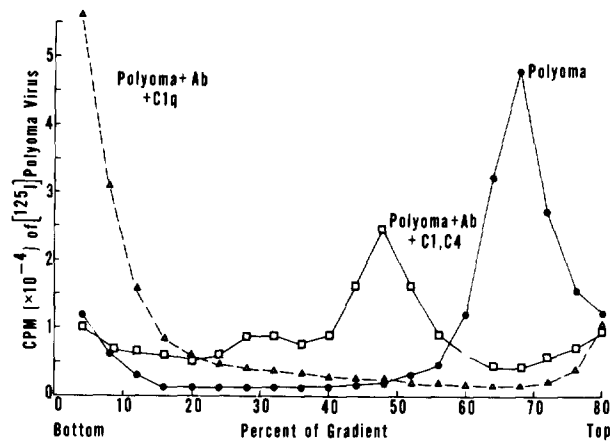


FIG. 6. Sucrose gradient ultracentrifugal analysis of  $^{125}\text{I}$ -labeled polyoma virus incubated with antibody and C1q or C1 and C4. C1q, but not C1 and C4, enhanced sedimentation of the virus-antibody complex.

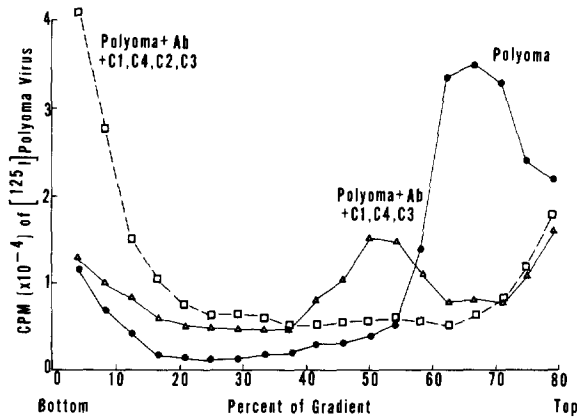


FIG. 7. Sucrose gradient ultracentrifugal analysis of  $^{125}\text{I}$ -labeled polyoma virus incubated with antibody and C1, C4, C2, and C3 or with C1, C4, and C3. Aggregation is dependent on activation of C3.

has been amply demonstrated in epidemiologic surveys of man and experimental studies in animals, as well as by use of viral vaccines, and passive administration of antibody. In these instances the role of the C system which may be activated by V-Ab immune complexes must be considered. In addition to possibly mediating viral lysis, the bound C proteins on the virion surface provide sites which may cause the complex to be handled differently in vivo. In particular, bound C components may enhance attachment of the V-Ab complex to phagocytic cells and to B lymphocytes. While this may facilitate virus destruction and perhaps formation of additional antibody, it may, with certain viruses, provide a means

TABLE III  
*Role of Various C Components in Enhanced Neutralization of V-Ab Complex\**

Reagents added to virus-antibody mixture	% Virus neutralization
Medium	0
Heated guinea pig serum	0
Normal guinea pig serum	86
C4-deficient guinea pig serum	0
Normal rabbit serum	73
C6-deficient rabbit serum	65
Normal human serum	80
Purified C1	0
Purified C1, C4	0
Purified C1, C4, C2	0
Purified C1, C4, C2, C3	84
Purified C1q	60

\* For the polyoma plaque and neutralization assay stock polyoma virus was diluted and mixed with a dilution of rabbit antibody to polyoma virus to give 50–55 PFU on confluent mouse embryo monolayers per  $60 \times 15$  mm Falcon petri dish. All mixtures were diluted in Eagle's basic medium and incubated at 37°C for 30 min; a final vol of 200 $\lambda$  was added to mid log Ha/ICR embryo cells.

for virus entry into cells and/or furnish an environment conducive to virus persistence or replication.

In the present study with polyoma virus, we have found that antibody may physically agglutinate virus particles and that this process was associated with the ability of antibody to neutralize polyoma virus infectivity. Further, the formation of V-Ab aggregates and the accompanying virus neutralization were significantly enhanced by C. Very limited amounts of antibody, only 2 molecules of antibody bound/virion, were able to activate C and so cause formation of large viral aggregates. In rate zonal centrifugation studies with mixtures of polyoma virus whose nucleic acid has been labeled with [ $^3$ H]thymidine, antibody, and C showed enhanced virus aggregation with radioactive counts being both restricted to the V-Ab-C complex location and resistant to DNase treatment. If viral lysis had occurred, radiolabeled nucleic acid would have appeared at the top of the gradient and [ $^3$ H]thymidine counts would have been degraded by DNase. The lack of viral lysis by antibody and C was further supported by electron microscope analyses.

These studies clearly demonstrate that C, in the presence of small amounts of antibody, enhances virus aggregation. This process has the effect of greatly reducing the net number of infectious particles and probably explains the augmented neutralization of infectivity observed in such mixtures. This is the first experimental verification of this C-dependent mechanism of virus neutralization. Although Wallis and Melnick earlier postulated C-associated aggregation as the mechanism for the neutralization of Herpes simplex virus (28), sub-

sequent data of Daniels and his associates (29) using C1 and C4 to enhance the neutralization of Herpes simplex virus-antibody complex indicated that C acts by contributing bulk to the viral surface or by some other mechanism not dependent on C3. Bound C4 or C3 were able to facilitate neutralization if large amounts were used. Further, using radiolabeled Herpes simplex virus, Notkins and his colleagues (30, 31) were unable to show any enhanced aggregation of the V-Ab complex with the addition of complement. Linscott et al. postulated a bulk-producing role for C in Newcastle disease virus neutralization, which required the first four C components (32). Finally, Berry and Almeida (33), using electron microscopy, noted that addition of antibody to avian bronchitis virus caused formation of large aggregates of virus particles and a  $300\text{\AA}$  halo around the virus. After addition of fresh serum to the virus-antibody mixture, no increase in virus antibody aggregation was noted, although the halo of bound protein more than doubled in diameter. Thus, this study also suggested C enhancement of neutralization by protein blanketing of the virion.

Another mechanism of C enhancement of viral neutralization is through disruption of the viral envelope or viral lysis. C-mediated viral lysis has been demonstrated with Gross leukemia (AKR) virus (34), Moloney leukemia virus (2), and equine arteritis virus (35) by use of sucrose gradient ultracentrifugation analysis of internally labeled virus reacted with antibody and C. Our electron microscope examination of Moloney leukemia virus reacted with antibody and C revealed marked distortion of viral architecture with a twofold increase in diameter, decreased radio-opacity, and presence of typical C-dependent ultrastructural lesions.<sup>4</sup> Similar ultrastructural lesions after C action have been shown on avian bronchitis, influenza, and rubella viruses (25).

Our studies indicate that C1q is able to aggregate virus with accompanying neutralization. Since C1q does not occur free in the circulation, it is unlikely that aggregation by C1q is biologically important. Native C1, which is a complex of C1q with C1r and C1s, did not possess this aggregating ability, in all probability because some Ig-binding sites on C1q are masked or rendered unreactive in the presence of C1r and C1s. Agglutination did not occur with sequential addition of C1 and C4, even in excess, or with C1, C4, and C2; however, enhanced aggregation and neutralization were evident on addition of C3 and C1, C4 and C2 to the V-Ab complex. This finding documents an essential role for C3 in facilitating cross-linking of polyoma virus-antibody complexes. Preliminary evidence indicates that polyoma virus possesses a receptor for C3b which binds C3b deposited on surfaces during activation of the C system.<sup>5</sup>

The complexes formed with isolated C1, C4, C2, and C3 added to polyoma virus-antibody complexes were somewhat larger than those generated by addition of whole serum. While the reason for this is not clear, it is possible that C3

<sup>4</sup> Cooper, N. R., M. J. Polley, and M. B. A. Oldstone. Manuscript in preparation.

<sup>5</sup> Oldstone, M. B. A., and N. R. Cooper. Unpublished observations.

inactivator present in normal serum degrades some bound C3b and thereby reduces the number of cross-links and the aggregate size. Other possible explanations are that the quantitative relationships of C1, C4, C2, and C3 present in serum may differ from those of the isolated components or other serum inhibitors, such as C1 inhibitor, may influence C activation. These conditions might well lead to reduced C3b binding.

The binding of C1, 4, 2, 3 to the V-Ab complex has important theoretical implications. Macrophages, monocytes, polymorphonuclear leukocytes, and B lymphocytes have receptors for C3b and these cells bind complexes bearing C3. This binding may be followed by phagocytosis of the V-Ab-C1, 4, 2, 3 complex. Monocytes and fixed phagocytes of the reticuloendothelial system may concentrate and handle V-Ab-C mixture in this way, while attachment to B lymphocytes may facilitate infection of this cell type. This mechanism may explain why cells with C3 receptors, i.e. B lymphocytes (36-38) and macrophages (39-42), usually carry virus during persistent infections. In studies to be reported later,<sup>6</sup> the importance of C3 in the clearance of V-Ab complexes was shown by delayed removal of polyoma virus-antibody complexes from the serum after *in vivo* depletion of C3 by cobra factor treatment.

#### SUMMARY

Interaction of polyoma virus, specific antibody, and complement has been studied. Firm evidence has been gathered that C1 through C3 and not C5 through C9 enhance neutralization of virus-antiviral antibody (V-Ab) complexes. C enhancement of neutralization occurs primarily by agglutination of V-Ab complexes and not by virion lysis or attachment of large protein molecules to the V-Ab complex. In this model, binding of C1, 4, 2, 3 to the V-Ab complex may explain why some viruses concentrate in or infect certain cells bearing C3 receptors such as B lymphocytes, macrophages, and monocytes.

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