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Infectious Virus-Antibody Complexes of Sindbis Virus

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Infectious virus-antibody complexes were formed when Sindbis virus was reacted with antibodies raised against purified viral envelope glycoproteins E1 and E2 as well as against preparations of intact virus. Results from rate zonal centrifugation in sucrose gradients of the complex formed with anti-E1 sera showed this complex to be about the same size as virions. A test of virus neutralization, based on direct plaque assay, by antibodies raised in rabbits and mice given virus in complete Freund adjuvant indicated the presence of antibodies able to complex but not neutralize virus. Conditions were found in which most of the virus was complexed and protected from neutralization, suggesting that these sera may contain a mixed population of antiviral antibodies with different specificities and different avidities.

Antibodies raised against viral surface proteins are able in general to neutralize the infectious particle and inhibit viral replication (see 1 and 4 for general reviews). It has been observed, however, that a population of anti-virus antibodies often contains immunoglobulins that can bind to virions and form soluble infectious virus-antibody complexes. Complexes of this type are found in certain viral diseases in which the infected animal remains in a state of chronic viremia. It has been suggested that appearance of this persistent viremia is related to the presence of infectious virus-antibody complexes (16); thus it is of considerable interest to investigate the nature of those viral determinants that give rise to "complexing" antibodies.

In this report we describe studies of infectious virus-antibody complexes with Sindbis virus, an enveloped ribonucleic acid virus of the family *Togaviridae* that contains two major glycoproteins, E1 and E2, on its surface (20). Antibodies raised against the virus neutralized the virus and also formed infectious complexes (13). Antisera have been obtained from rabbits and mice injected with purified preparations of the E1 and E2 viral envelope glycoproteins (2). The E1 protein possessed the hemagglutinating activity of the virus, and antisera to E1, but not E2, inhibited hemagglutination. Antisera to both E1 and E2 formed radioimmune precipitates with labeled virions at comparable high dilutions of sera, but the antisera showed relatively little cross-reactivity to the isolated glycoproteins. Both anti-E1 and anti-E2 sera were poor in neutralizing virus when compared with antisera raised against virions, but anti-E2 sera had higher titers for neutralization than did anti-E1 sera. We have tested these antisera to determine whether they contained antibodies that could form infectious complexes and whether one of the particular viral antigens was more effective in eliciting these antibodies.

MATERIALS AND METHODS

Virus and antisera. Our standard preparation of Sindbis virus was a sample obtained from B. Burge (Worcester Foundation, Shrewsbury, Mass.). This was plaque purified and passaged on chicken embryo fibroblasts (CEF). Plaque assays were performed on these cells according to the procedures of Pfefferkorn and Hunter (17). Antiserum against whole Sindbis virus was raised in rabbits (13). Rabbit antisera against the E1 and E2 glycoproteins and mouse anti-Sindbis serum were kindly provided by J. Dalrymple (U. S. Army Medical Research, Walter Reed Hospital, Washington, D.C.). A description of the properties of these antisera has been published (2). Goat anti-rabbit and anti-mouse immunoglobulin G (IgG) were obtained from Gateway Immunosera, Cahokia, Ill.

Assays for virus neutralization and formation of virus-antibody complex. Appropriate dilutions of antisera were made in phosphate-buffered saline with 1% fetal calf serum (FCS). These were mixed with an equal volume of Sindbis virus in the same diluent and incubated at 37°C for 60 min. For the detection of virus-antibody complex, goat anti-rabbit or anti-mouse IgG was added in an amount at least four times more concentrated than the antiserum dilution used, usually a 1:100 dilution. After a further incubation of 1 h at 37°C, the samples were kept at 4°C before titering on CEF.

Labeling of virus and analysis of Sindbis virus-antibody complex. A monolayer of CEF cells ap-

proaching confluency in a 75-cm² Falcon T flask was infected with Sindbis at a multiplicity of infection of 100 in 2 ml of modified Eagle medium (MEM) with 3% FCS. After 1 h at 37°C, an additional 8 ml of MEM containing 3% FCS was added and the infection was allowed to proceed for 4 h. The medium was removed, and 50 μCi of [^{35}S]methionine (333 Ci/mmol; New England Nuclear Corp.) in 2 ml of MEM minus amino acids was added. After 1 h, 7 ml of MEM with 3% FCS was added. Medium was harvested at 18 h and centrifuged at 500 × *g* for 10 min. Virus was purified from the medium by isopycnic centrifugation (18). Samples from the gradient that contained labeled virus were dialyzed against 0.05 M tris(hydroxymethyl)aminomethane, pH 7.5, 0.1 M NaCl, 1 mM ethylenediaminetetraacetic acid (TNE).

Specific or control rabbit antiserum (100 μl) was added to 1.0-ml samples containing 10^{10} virions. After incubation at 37°C for 60 min, the samples were cooled to 4°C, layered on top of a 15-ml linear gradient of 15 to 30% sucrose containing 0.2% FCS, and centrifuged in a Spinco SW27 at 25,000 rpm for 60 min at 4°C. Fractions of 0.5 ml were collected, and aliquots were removed for determination of radioactivity in a Packard scintillation counter and virus titer on CEF. Another sample from each fraction was treated with 25 μl of goat anti-rabbit IgG, incubated at 4°C for 10 min, diluted, and titered.

RESULTS

Neutralization and complex formation between Sindbis virus and antibodies raised against viral components. The antibodies raised against Sindbis virus and the separated viral glycoproteins have been shown by Dalrymple et al. (2) to respond differently in their ability to neutralize viral infectivity and to inhibit hemagglutination of goose erythrocytes. We have tested the different antisera for the presence of antibodies that can form infectious complexes. Antiserum from rabbits given

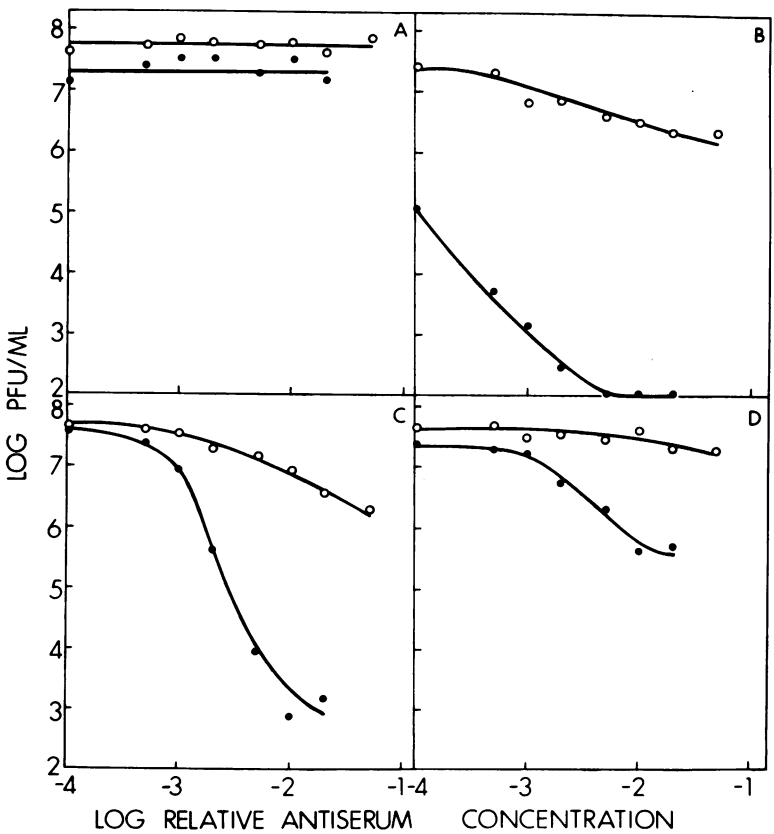

FIG. 1. *Infectious virus-antibody complex formation with rabbit antisera. Samples of virus were incubated with antisera from rabbits not immunized with viral antigens (A) and immunized with intact virus (B), glycoprotein E-1 (C), and glycoprotein E-2 (D). After incubation at 37°C for 60 min, a portion was further treated with goat anti-rabbit IgG at concentrations at least four times that of the rabbit serum, but usually at a 10^{-2} dilution. Incubation was at 37°C for 60 min, and samples were diluted for plaquing. Symbols: (○) Rabbit antisera alone; (●) rabbit antisera plus goat anti-rabbit IgG.*

whole virus in Freund adjuvant was able to neutralize about 90% of the infectious virus when used alone, but the addition of a preparation of goat anti-rabbit IgG lowered infectious titers by seveal logs (Fig. 1B). This is presumably due to reaction of anti-IgG with the infectious virus-antibody complexes present (7). When tested with high titers of virus, the antiserum from rabbits injected with the E1 glycoprotein was also effective in direct neutralization and contained antibodies that could form infectious complexes with most of the virions present (Fig. 1C). Anti-E2 serum was less effective in directly neutralizing our strain of virus, but infectious complexes were formed (Fig. 1D). At concentrations of anti-E1 and anti-E2 that cause an equal amount of neutralization, 2×10^{-3} and 2×10^{-2}, respectively, infectious complexes were formed which led in both cases to a 2-log drop in infectivity when goat anti-rabbit IgG was added. However, at a concentration of 10^{-2}, the anti-E1 serum contained a titer of antibodies sufficient to complex with nearly all the virus.

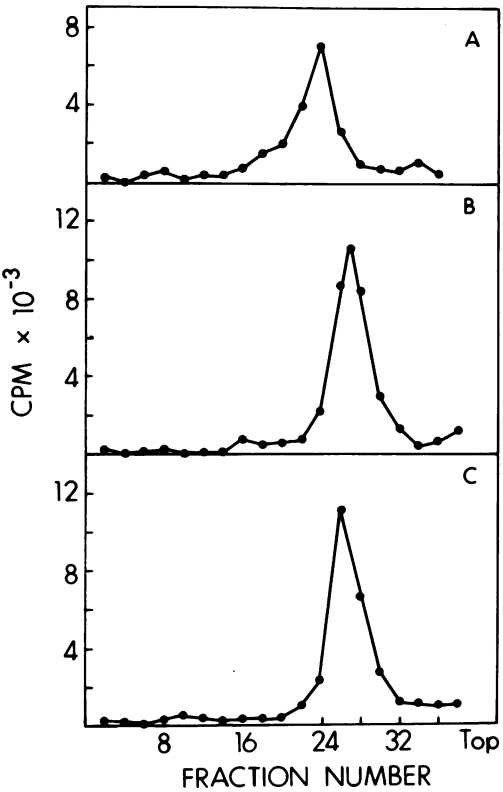

FIG. 2. *Rate zonal centrifugation in sucrose gradients of Sindbis virus-antibody complexes. Samples of ^{35}S-labeled virus were incubated with rabbit antisera against glycoprotein E-1 (A), glycoprotein E-2 (B), or nonimmunized control serum (C). See text for experimental details. Samples from the fractions were analyzed for ^{35}S cpm by scintillation counting, and other samples were tested for viral infectivity (see Table 1).*

Isolation of infectious complexes containing anti-E1 and anti-E2 antibodies. A direct test for the formation of an infectious virus-antibody complex was made by examining the profile of ^{35}S-labeled virions that had been reacted with antisera and then subsequently centrifuged in a sucrose gradient. Anti-E1, anti-E2, and nonimmune rabbit serum were allowed to incubate with virus, and samples were analyzed after rate zonal centrifugation in a sucrose gradient. Fractions from the gradient were assayed for radioactivity and titered after incubation in the presence or absence of goat anti-rabbit IgG. The profiles of ^{35}S radioactivity indicated that the virus from preparations treated with anti-E1 and anti-E2 antibodies has sedimentation properties similar to those of untreated virus (Fig. 2), although virus preincubated with anti-E1 sedimented slightly faster than untreated virus (Fig. 2A). About 97% of the infectious virus recovered from the peak of the gradient containing virus pretreated with anti-E1 serum apparently was in the form of a

TABLE 1. *Assay for infectious virus-antibody complex from sucrose gradient fractions*[a]

Sample		Amt of virus (PFU/ml x 10^6)[b]		Ratio of no anti-rabbit IgG/plus anti-rabbit IgG
Gradient	Fraction	No anti-rabbit IgG	Plus anti-rabbit IgG	
Sindbis + anti-E1 serum	20	1.2	0.04	30
	24	5.7	0.20	28
Sindbis + anti-E2 serum	26	4.2	1.3	3.2
	28	1.3	0.5	2.6
Sindbis + nonimmune serum	26	7.0	3.5	2.0
	28	3.2	3.2	1.0

[a] Infectivity was determined by assay on CEF monolayers. See Materials and Methods for experimental details.

[b] PFU, Plaque-forming units.

virus-antibody complex (Table 1). The amount of virus contained in an infectious complex after pretreatment with anti-E2 serum was too low to evaluate accurately by our method of analysis.

Biphasic characteristic of Sindbis virus neutralization by antibodies. The assay for neutralizing antibodies can be erroneous if high concentrations of virus are used and dilution is required before plaquing (11, 12, 22). In addition, small variations in virus titers are not detected at high virus concentrations. Therefore, we tested for neutralization and the presence of antibodies forming infectious complexes with titers of virus that could be directly plaqued without prior dilution. Under these conditions we observed a biphasic neutralization curve with antibodies from either rabbits or mice injected with Sindbis virus in complete Freund adjuvant (Fig. 3). At concentrations of antisera between 10^{-3} and 10^{-4}, most of the virus remained infectious, although at lower and higher dilutions there was a reduction of infectious particles. The non-neutralized virus at the 10^{-4} concentration of antiserum was in the form of an infectious antibody complex since addition of goat anti-rabbit or anti-mouse IgG completely blocked the viral infectivity (Fig. 3). Of particular interest was the observation that, at concentrations of antiserum less than that required for optimum formation of complex, there was an increase in virus neutralization. At the 10^{-5} dilution, more than 90% of the virus was neutralized by the anti-Sindbis serum without addition of anti-IgG. With very dilute antiserum there was the expected decrease in virions neutralized or complexed. It appears that the complexing antibodies can protect the virus from some of the neutralizing antibodies present in the serum.

This biphasic curve was also obtained when the initial virus titer was 3×10^7 plaque-forming units/ml and dilutions were made after reaction with antibody. When anti-E1 or anti-E2 was tested in the low or high virus titer assay, we did not find a biphasic curve. At low titers of virus (150 plaque-forming units/plate), anti-E1 and anti-E2 neutralized 50% of the virus at a relative concentration of 7×10^{-3}.

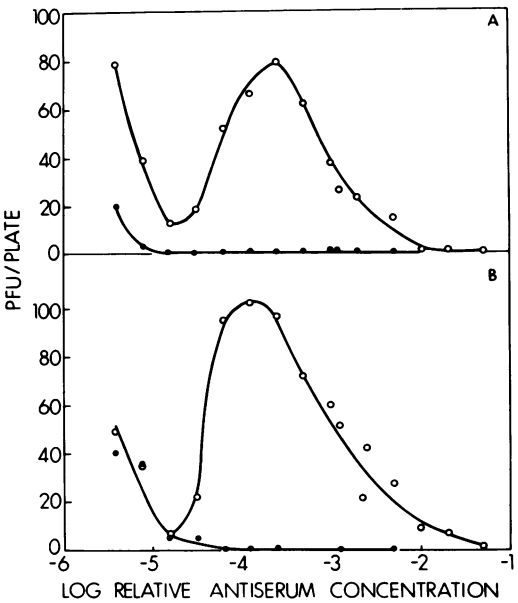

FIG. 3. *Biphasic neutralization and infectious complex formation of Sindbis virus. Reactions with antibody were carried out under conditions noted under Fig. 1, but samples were added to CEF monolayers without additional dilution, and goat anti-rabbit IgG was used at a 1:100 dilution. (A) Rabbit anti-Sindbis sera; (B) mouse anti-Sindbis serum. Symbols: (○) Antisera alone; (●) antisera plus goat anti-rabbit or anti-mouse IgG.*

DISCUSSION

The data presented here provide evidence that antibodies raised against Sindbis virus and its surface glycoproteins can react with virions to form complexes in which viral plaque-forming ability is not neutralized. Antisera from rabbits injected with purified preparations of either E1 or E2 glycoproteins contained these antibodies, and the antiserum raised against E1 glycoprotein was more effective in complex formation. This was shown by the enhanced effect on virus neutralization after addition of goat anti-rabbit IgG to virus pretreated with antiviral sera, as well as by an analysis of radioactive virus treated with anti-E1 and anti-E2 sera and purified by rate zonal centrifugation in sucrose gradients. Although other preparations of anti-E1 and anti-E2 sera should be tested, it is possible that one of the surface proteins of Sindbis may be a more potent immunogen and stimulate the formation of antibodies that can form infectious viral complexes. Different surface proteins of viruses have been found to elicit particular kinds of antibodies. With influenza virus, one of the virion surface proteins, the neuraminidase, was unable to induce neutralizing antibodies in contrast to the other viral glycoprotein, the hemagglutinin (21). Neuraminidase was, however, able to induce antibodies that could sensitize influenza to inactivation with antiglobulin (14). Friend murine leukemia virus has a surface protein, p15, which is reported to induce complexing, non-neutralizing antibodies (9) as

well as a glycoprotein, gp71, which induces neutralizing antibodies (8). We have recently shown that antibodies raised against a denatured form of the envelope glycoprotein, gp85, from Rous sarcoma virus are "complexing" antibodies (19).

Neither of the antisera from rabbits given isolated Sindbis virus glycoprotein was reported to be a good neutralizing serum (2). In contrast, the intact virus is a good immunogen in rabbits, and high titers of neutralizing antibodies are routinely made. In addition, these latter antisera appear to contain antibodies that can bind to virus without neutralizing the particle. Because of the multiple determinants present on virions, this result is not surprising, and the presence of non-neutralizing antibodies is a characteristic of antisera to many viruses (15). The surprising result noted here was the apparent ability of these non-neutralizing, complexing antibodies to block the neutralization of virus by other antibodies present in the serum. There may be alternative explanations for the biphasic character of the neutralization curve described in Fig. 3, but a reasonable interpretation is that the antiserum raised in mice or rabbits against Sindbis virus contains a high concentration of a low-avidity antibody that can neutralize virus. At high dilutions, this is the major anti-Sindbis antibody present. Also present in the serum, however, are lower amounts of antibodies with high avidities for nonsensitive sites on the viral surface. At certain concentrations of antisera and virus these antibodies can out-compete the low-avidity neutralizing antibodies and block the latter from attaching to neutralizing sites, possibly by changing the conformation of the viral surface or by steric hindrance. Finally, a third type of antibody, a neutralizing antibody in low concentrations but with very high avidity, could block the complexing antibodies. These three kinds of immunoglobulins could generate the curve described by Fig. 3. A similar biphasic curve was generated by radioimmune precipitation of Sindbis virus with heterologous, cross-reacting antiserum (3). The existence of multiple determinants and antibodies of varying avidity for different determinants seemed the most appropriate explanation for this latter result as well.

In the infected animal, Sindbis virus induces an immune response that effectively eliminates virus and produces neutralizing antibodies (6). A number of other viruses, however, maintain a chronic viremic state in the infected animal, and the immune response is aberrant. A characteristic of the latter type of infection is the presence of circulating infectious virus-antibody complexes. It has been proposed that this form of the virus and the aberrant immune response are essential features of persistent viral infection. The kind and proportion of antibodies formed may be host dependent, as has recently been shown with visna virus (10). These studies on the characteristics of antiviral antibodies may help to clarify important aspects of this problem of infectious virus-antibody complexes and chronic viral infections.

The possibility of using purified viral protein rather than entire virions for vaccination (5) gives added interest to the analysis of reactions elicited by antibodies to individual virion components.

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