JOURNAL OF VIROLOGY, Jan. 1985, p. 198–204
0022-538X/85/010198-07$02.00/0

Vol. 53, No. 1

Detection of Immunologically Cross-Reacting Capsid Protein of Alphaviruses on the Surfaces of Infected L929 Cells

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Received 16 July 1984/Accepted 18 September 1984

Hyperimmune, but not normal immune, monospecific antiserum made to capsid protein of Sindbis virus (SIN) was found to cause cytolysis equally well of both SIN- and Semliki Forest virus-infected L929 cells in antibody-dependent, complement-mediated cytotoxicity assays. The cell surface reactivity of the hyperimmune antiserum was also demonstrated by solid-phase radioimmune assays with unfixed infected cells or infected cells fixed with low concentrations of glutaraldehyde (0.025%) before reactivity with antisera. Higher concentrations of glutaraldehyde lowered the sensitivity of detection. Purified SIN capsid protein specifically inhibited antibody-dependent, complement-mediated cytotoxicity by the monospecific anti-capsid protein serum on SIN- and Semliki Forest virus-infected target cells. That hyperimmune anti-SIN serum also cross-reacts with capsid protein on the surface of Semliki Forest virus-infected cells was suggested by the fact that capsid protein inhibited cross-cytolysis in the antibody-dependent, complement-mediated cytotoxicity assay. The latter antiserum was collected after repeated injections of purified virions over a 9-month period. The results suggest that hyperimmune monospecific antisera made to SIN capsid protein or hyperimmune antisera to SIN or Semliki Forest virions detect homologous and cross-reacting capsid protein determinants on the surface of infected cells.

Sindbis virus (SIN) and Semliki Forest virus (SF) are members of two different subgroups among the alphaviruses, which are small enveloped RNA viruses of the family *Togaviridae* (41). During the maturation of these viruses, a subgenomic species of intracellular viral RNA (molecular weight, 1.6×10^6; sedimentation coefficient, 26S) is the mRNA for the structural proteins, which include a basic capsid protein (C) and three major glycoproteins, namely, E1, E2, and E3 (39, 49). The virus structural proteins are translated sequentially, using one initiation site near the 5′ terminus of the 26S RNA (8, 9, 11, 19), and proteolytic cleavages occur at several sites on the polypeptide that is being synthesized. The genes are translated on membrane-bound polyribosomes (47) in the order 5′C-E3-E2-6K-E1-3′ (14, 15, 33, 37). The first cleavage by a chymotrypsin-like protease releases the capsid protein from the polysome, and the remaining portion of the nascent polypeptide becomes inserted into the rough endoplasmic reticulum (33). The nascent polypeptide is glycosylated at several sites as synthesis and transport proceed (16, 47). A second cotranslational cleavage releases the precursor viral envelope protein, PE2, from the other envelope protein, E1 (46), and the latter is inserted into the cell membrane where it is externally exposed (40). The final proteolytic cleavage converts the PE2 glycoprotein to E2 and E3, with E3 being released into the culture medium with SIN, but retained in the SF virion (17).

There is evidence that the capsid protein combines with the 42S RNA and the nucleocapsid migrates through the cytoplasm to the plasma membrane where it associates with the glycoproteins E1 and PE2; proteolytic activity then cleaves PE2 to E2 and E3 (2). It is clear that E1 and E2 can be detected on the outer surface of the cell by the lactoperoxidase-radioiodination technique, but although PE2 cannot be iodinated, the high degree of glycosylation and its ease of isolation from the plasma membrane suggest that it has become integrated into the membrane before cleavage (36). Indeed, a PE2-capsid complex appears to be associated with the plasma membrane (36, 40).

We present evidence here that hyperimmune monospecific antiserum made to capsid protein of SIN causes cytolysis equally well of both SIN- and SF-infected L929 cells in antibody-dependent, complement-mediated cytolysis (ADCMC) assays. The cell surface reactivity of this antiserum can also be demonstrated by solid-phase radioimmune assay (RIA). In addition, we show that purified SIN-capsid protein specifically inhibits the ADCMC of SIN- and SF-infected target cells induced by the monospecific antiserum. On the basis of our results and current molecular knowledge of alphaviruses, we discuss a few alternative morphogenetic explanations that are based on the appearance on the outer surface of the plasma membrane of nucleocapsid or epitopes of it or epitopes that are related to it.

MATERIALS AND METHODS

Viruses and virus purification. SIN AR339 and SF viruses were grown in primary chicken embryo fibroblasts (CEF) at a multiplicity of infection (MOI) of 0.01. Virus was purified as described previously (18, 48) by low-speed clarification ($5,000 \times g$), polyethylene glycol precipitation (10% in 0.5 M NaCl), and isopycnic density centrifugation for 16.5 h at 25,000 rpm in a Beckman 27.1 rotor through a 20 to 60% linear sucrose gradient in TNE (0.01 M Tris, 0.05 M NaCl, 5 mM EDTA, pH 7.4). The virus-containing band was collected, pelleted at 25,000 rpm for 2 h, and suspended in TNE for storage at −70°C. The protein content was determined by the method of Lowry et al. (30).

Production of hyperimmune antisera to alphaviruses. Immunization of rabbits with alphaviruses has been previously described (18). Briefly, 250 μg of purified SIN or purified SF

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(approximately 10^{10} PFU) was emulsified in complete Freund adjuvant and injected into the gastrocnemius muscle. Secondary immunizations with 250 μg of purified virus were given intraperitoneally in incomplete adjuvant 1 week later. Additional immunizations over a period of 9 months were given intravenously with 250 μg of purified virus alone. Antisera contained homologous virus neutralizing titers of $>10^5$ and nondetectable heterologous virus neutralizing titers (<1:5) when assayed by plaque reduction on CEF monolayers. These antisera were shown to be cross-reactive by ADCMC of virus-infected L929 and CEF cells (18, 48).

Monospecific and hyperimmune antisera to SIN capsid protein. Rabbit hyperimmune antiserum to SIN nucleocapsid protein was a gift of C. M. Rice, California Institute of Technology, Pasadena. The purification of viral structural proteins (3) and the production of the hyperimmune antiserum to capsid protein have been described previously (35).

ADCMC. The ADCMC assay for microtiter plates has been described previously in detail (48, 49). Briefly, dilutions of rabbit hyperimmune serum or monospecific antisera were made in McCoy 5A medium and mixed with an equal volume of a 1:7 dilution of rabbit complement (Low-Tox-M rabbit complement; Cedarlane, Accurate Chemical Corp., Hicksville, N.Y.). The antiserum-complement mixtures were applied to 2×10^4 virus-infected or uninfected mouse L929 cells (MOI of 20) that had been previously labeled with $Na_2{}^{51}CrO_4$ (New England Nuclear Corp., Boston, Mass.). Samples of the supernatant fluids of triplicate assays were analyzed for ^{51}Cr release in a Beckman 4000 gamma spectrometer. Controls consisted of wells that received medium alone, antiserum alone, a 1:14 dilution of complement, or 3% Triton X-100 (total releasable counts). The percent specific release was calculated as:

$$\frac{\text{(cpm released by Ab + C)} - \text{(cpm released by C)}}{\text{(cpm released by Triton X-100)} - \text{(cpm released by C)}} \times 100$$

where cpm is counts per minute, Ab is antiserum, and C is complement.

Preparation of nucleocapsid protein for blocking ADCMC. Purified virus suspensions were made in TNE buffer supplemented with 2% Triton X-100 and incubated for 1 h at room temperature. The suspension was centrifuged at 25,000 rpm in an SW27 rotor. The pellet containing nucleocapsid was suspended in TNE, and Bio-Beads SM-2 (Bio-Rad Laboratories, Richmond, Calif.) were added to remove the Triton X-100 (22). These preparations contained only capsid protein as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis followed by Coomassie brilliant blue (R-250) staining or by immunoblotting with hyperimmune anti-SIN serum.

To determine the blocking or inhibitory effect of capsid protein on ADCMC, viral proteins were reacted with antisera before complement was added to the assay. Monolayers were infected at an MOI of 20 and incubated for 11 h. The antisera were diluted in McCoy medium to a concentration that would give 90 to 95% of maximal ^{51}Cr release in an ADCMC assay. Various concentrations of capsid protein, diluted in TNE, were added to an antiserum dilution, and the mixtures were incubated for 30 min at room temperature. Complement was then added in a final concentration of 1:30.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed on 0.75-cm-thick slab gels of 11 or 12.3% acrylamide by a modification of the method of Laemmli (28) as described by Smith and Brown (40) for use with an *N,N'*-diallyltartardiamide (DATD) cross-linking reagent (acrylamide/DATD ratio, 30:1.6). Stacking gels were 3% acrylamide photopolymerized with riboflavin as described by Powell and Courtney (32). Samples were disrupted by boiling for 2 min in an equal volume of 2% SDS, 2% 5 M urea, and 2% beta-mercaptoethanol in 150 mM Tris (pH 8.8) and electrophoresed in a discontinuous system at 15 to 20 mA per gel for approximately 2.5 h, when the bromphenol blue tracking dye reached the bottom of the gel. A protein mixture was obtained from Amersham Corp. (Chicago, Ill.) as molecular weight markers (14,300 molecular weight [14.3K] to 200K).

Electroblot (immunoblotting). The electroblot method was performed as described by Towbin et al. (42) and Burnette (7), except that electrophoretic transfer was carried out at 15 V/cm for 2 to 2.5 h at 4°C. Briefly, filter papers, polyethylene sheets, and nitrocellulose papers (Schleicher & Schuell Co., Keene, N.H.) were wetted in transfer buffer (20 mM Tris, 150 mM glycine, plus 20% reagent- grade methanol). Immediately after electrophoresis, the SDS-gel was placed in direct contact with the nitrocellulose, and these were sandwiched between the filter papers and the polyethylene sheets. These were placed in a Bio-Rad electroblot unit filled with cold transfer buffer such that the nitrocellulose was oriented toward the anode. Electrophoretic transfer of the proteins from the gel to the nitrocellulose sheet was accomplished by using a Hoeffer power supply (model TE-15; Transhor, San Francisco, Calif.).

The nitrocellulose sheets were immersed in 3% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) in 10 mM Tris-hydrochloride with 0.9% NaCl (pH 7.4) for 60 min at room temperature. Transfers were washed 10 times in distilled water. Antiserum was diluted in 0.05% Nonidet P-40 in NETG buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-hydrochloride [pH 7.4], 0.25% gelatin type III [225 bloom, Sigma]) and added to the transfers for 2 h at room temperature with shaking. The sheets were then washed 10 times in distilled water. Staphylococcal protein A (Zymed Laboratories, Burlingame, Calif.) was labeled with ^{125}I ($Na^{125}I$; Amersham) by the chloramine T method (24). ^{125}I-labeled staphylococcal protein A was diluted to 10^6 cpm/ml in 0.05% Nonidet P-40 in NETG, and 10 ml was incubated with each 10- by 14-cm transfer for 2 h at room temperature with shaking. The sheets were washed 10 times in NETG containing 0.5% Triton X-100 and 0.1% SDS, washed in distilled water, and air dried. Transfers were autoradiographed on Kodak X-OMat AR X-ray film at room temperature or at −70°C with intensifying screens.

RIA. A solid-phase RIA was adapted to detect binding activity of antibody to cell surface viral antigens. Microtiter plates (96 wells, Linbro) were seeded with 2×10^4 L929 cells and incubated for at least 14 h before infection (approximately one doubling time) at 37°C. The monolayers were then infected at an MOI of 20. After a period of incubation, the monolayers were washed in phosphate-buffered saline (PBS) containing 0.01 M sucrose and fixed by one of the following three methods: (i) incubation for 1 h at 4°C with PBS containing the designated concentration of glutaraldehyde; (ii) incubation with technical grade methanol for 10 min at room temperature; (iii) preincubation with heat-inactivated antiserum diluted in McCoy medium for 1 h at 37°C followed by a wash with PBS (0.01 M sucrose) and fixation with 0.025% (vol/vol) glutaraldehyde. We found that 0.025% is the lowest concentration of glutaraldehyde that will maintain the solid phase of the RIA.

After fixation, the monolayers were washed and then incubated overnight at 4°C with 3% bovine serum albu-

min–PBS containing 0.05% Tween 20, 0.05% NaN_3, and 0.01 M glycine. Fixed monolayers untreated with antibody were incubated at 37°C for 1 to 1.5 h with antiserum diluted with 1% bovine serum albumin–PBS containing 0.05% NaN_3, 0.01 M glycine, and 0.05% Tween 20. After appropriate incubations, all wells were washed in PBS and incubated for 1 h with 10^5 cpm of ^{125}I-labeled staphylococcal protein A diluted in 1% bovine serum albumin–PBS containing 0.05% Tween 20, 0.05% NaN_3, and 0.01 M glycine. Greater than 90% of the added counts were precipitable with trichloroacetic acid (43).

RESULTS

Reactivity of anti-capsid serum in ADCMC. Although nucleocapsid antigen is generally thought to be absent from the outer surface of infected cell membranes, the availability of hyperimmune anti-capsid protein serum allowed us to test for its presence in the ADCMC reaction. The antiserum was prepared to isolated capsid protein from purified SIN (3, 5). The antiserum reacted to high titer with both SIN- and SF-infected cells at 11 h postinfection (Fig. 1). Higher levels of cytolysis are indicated with SF-infected cells compared with homologous SIN-infected cells in this experiment; however, this was not consistently reproducible in other experiments.

The kinetics of the appearance of viral antigens on SIN-infected cells was determined by using hyperimmune rabbit anti-SIN and anti-SF sera. This was compared with the cytolysis caused by the monospecific anti-capsid protein serum (Fig. 2). Reactivity of anti-SIN serum was evident (30% cytolysis) by 6 h after infection and appeared to reach near-maximal levels (>70%) by about 9 h. However, significant cross-cytolysis of SIN-infected cells by anti-SF serum was not found until 9 h. Similarly, anti-capsid protein serum caused significant cytolysis (10%) at 9 h. The appearance of antigen reactive with anti-capsid protein serum lagged slightly behind the reactivity of anti-SF serum in cross-cytolysis.

The kinetic appearance of viral antigens on SF-infected cells is shown in Fig. 3. Similar levels of cytolysis were observed at 7.5 h and thereafter by both anti-SF and anti-SIN sera. As with SIN-infected cells, the appearance of antigen reactive with anti-capsid protein serum lagged behind the reactivity of anti-SIN (cross-cytolysis) and anti-SF (homologous) sera.

These results suggest that SIN antigens appear earlier on plasma membranes of L929 cells than do SF antigens, although this may reflect an increased potency of the anti-SIN over the anti-SF serum. SF-infected cells do tend to round up and loosen from the monolayer about 2 h earlier than SIN-infected cells at the same MOI. In addition, SF-infected cells show a granular appearance earlier than SIN-infected cells.

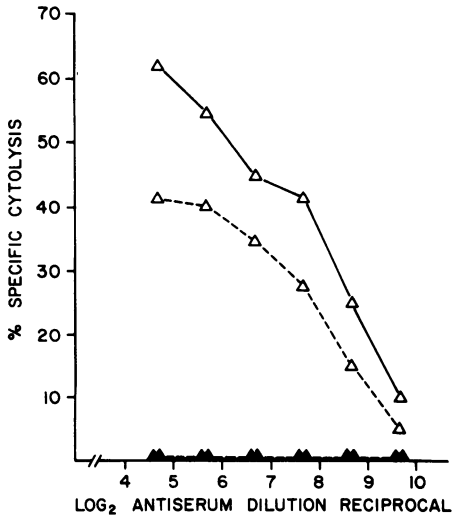

FIG. 1. ADCMC assay with hyperimmune, monospecific anti-capsid protein serum on L929 cells infected for 11 h with SIN (△—△) or SF (△- -△) or with ordinary immune anti-capsid protein serum on cells infected with SIN (▲—▲) or SF (▲- -▲).

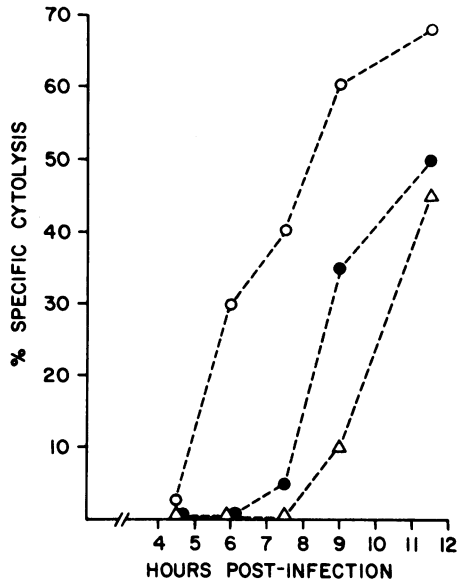

FIG. 2. ADCMC assays with hyperimmune anti-SIN (○, 1:250), anti-SF (●, 1:250), or anti-capsid protein (△, 1:25) sera on L929 cells infected with SIN as a function of time after infection.

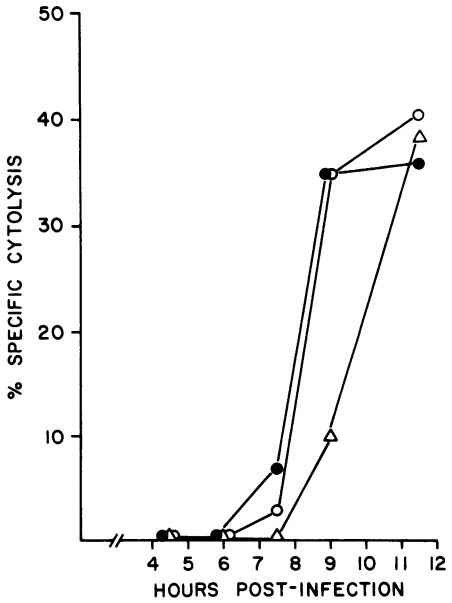

FIG. 3. ADCMC assays with hyperimmune anti-SIN (○, 1:250), anti-SF (●, 1:250), or anti-capsid protein (△, 1:25) sera on L929 cells infected with SF as a function of time after infection.

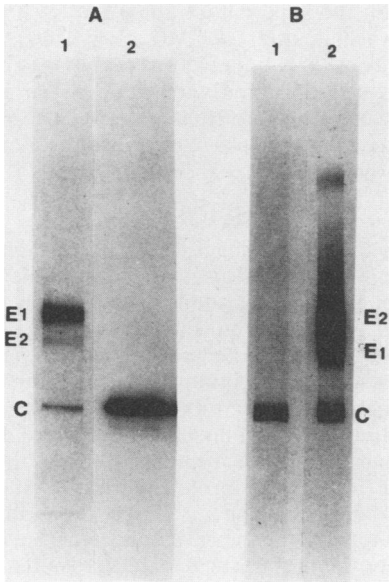

FIG. 4. Immunoblot of SIN or SF virion proteins with homologous hyperimmune antisera and hyperimmune anti-capsid protein serum after separation by SDS-polyacrylamide gel electrophoresis. A, Reactivity of SIN virion proteins with anti-SIN (lane 1) and anti-capsid protein (lane 2) sera. B, Reactivity of SF virion proteins with anti-SF (lane 2) and anti-capsid protein (lane 1) sera.

Specificity of the anti-capsid protein serum. Since the anti-capsid serum had been prepared in rabbits by hyperimmunization with SDS-treated nucleocapsids fractionated from purified SIN virions, it was important to ascertain its specificity. Purified SIN and SF virions were fractionated and electrophoresed on SDS-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred to nitrocellulose by the electroblotting technique and reacted first with anti-capsid protein serum or anti-virion sera and then with [^{125}I]SPA. The anti-capsid protein serum reacted only with capsid protein (Fig. 4).

Inhibition by capsid protein of ADCMC induced by anti-capsid protein serum. To show further the specificity of the monospecific anti-capsid protein serum, nucleocapsids were prepared from Triton X-100-treated purified virions and used to inhibit ADCMC. The titration of the inhibition (Fig. 5) shows that 0.6 μg per assay inhibited ADCMC by 90%, and 0.2 μg inhibited ADCMC by 50%. The inhibition was not observed when the concentration of nucleocapsid was less than 0.03 μg per assay. Intact virions (between 10^7 and 10^8 PFU/ml at 11 h) released during infection had no apparent inhibitory effect on ADCMC caused by anti-capsid protein antibodies.

Comparison of capsid protein inhibition of heterologous and homologous ADCMC induced by hyperimmune anti-SIN serum. The heterologous ADCMC reactions with hyperimmune anti-SIN serum on SF-infected cells were also inhibited by purified capsid protein, although the homologous (anti-SIN) ADCMC reactions were not inhibited at all (Fig. 5). These data suggest that the ADCMC caused by hyperimmune anti-SIN serum was probably directed primarily at glycoproteins (E1, E2) in the homologous reaction and to capsid protein in the cross-reaction. The inhibition by capsid protein was not attributed to anti-complement activity since the preparations had no effect on a sheep erythrocyte-hemolysin-complement system (data not shown).

RIA on glutaraldehyde- and methanol-fixed infected cells. The reactivity of anti-capsid protein serum in ADCMC suggested that a part of the capsid protein may have been exposed on the plasma membrane and represented a fraction of the total amount of capsid protein in the infected cell. To estimate this fraction on the cell surface, glutaraldehyde was used to fix the cells as a cross-linking agent, whereas methanol fixation was used to allow the antibody to react which capsid protein both on the surface and intracellularly. The solid-phase RIA described above also provided a second method, independent of ADCMC, for detecting the presence of capsid protein on the surface of infected cells.

The effect of various concentrations of glutaraldehyde on RIA detected by monospecific anti-capsid protein and by hyperimmune anti-SIN sera on SIN-infected cells at 11 h after infection was determined (Fig. 6). At a 0.025% concentration, anti-capsid protein serum at a 1:20 dilution detected about 30% of the capsid protein found with methanol treatment. Note that the amount of capsid protein detectable decreased rapidly as the concentration of glutaraldehyde was increased so that only 5% was found at 0.125% glutaraldehyde. In contrast, anti-SIN serum at a 1:50 dilution on SIN-infected cells detected 88% of the viral antigens detected with methanol treatment at 0.025% glutaraldehyde, 69% of viral antigens at 0.1% glutaraldehyde, and 60% of viral antigens at 0.8% glutaraldehyde. Similarly, anti-SF serum on SF-infected cells detected 94% of viral antigens at 0.01% glutaraldehyde and 60% of viral antigens at 0.8% glutaraldehyde (data not shown). The extent of reactivity of anti-virion sera is presumably due to the detection of glycoproteins expressed during the budding process.

As a control, the temperature-sensitive mutant of SIN, *ts*23, was examined. This maturation defective mutant makes capsid protein and glycoproteins at nonpermissive temperatures, but does not have glycoproteins in the plasma membrane or orientation of nucleocapsids at the plasma mem-

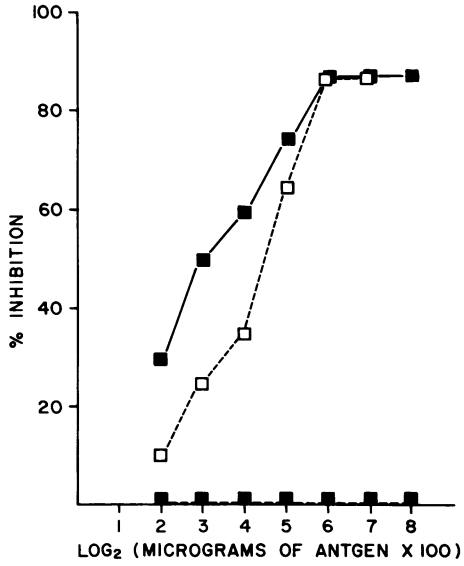

FIG. 5. Ability of various concentrations of purified capsid protein to inhibit ADCMC by hyperimmune anti-SIN serum on SIN (■—■)- or SF (■—■)-infected cells and by hyperimmune anti-capsid protein serum on SIN-infected cells (□—□). Cytolysis in the absence of added capsid protein was 74 and 42% on SIN- and SF-infected cells, respectively, with anti-SIN serum and 30% with anti-capsid serum.

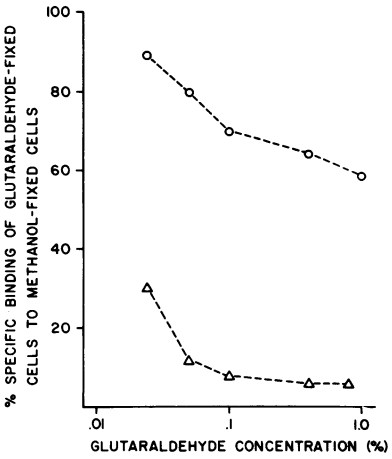

FIG. 6. RIA of SIN-infected cells with anti-SIN (○—○, 1:50) or anti-capsid protein (○- -○, 1:20) sera at 11 h postinfection as a function of glutaraldehyde concentration in fixation.

branes (41). Only 5 to 7.5% of the total capsid protein could be detected with the lowest concentration of glutaraldehyde with monospecific anti-capsid protein serum (Fig. 7).

Similar results were obtained with hyperimmune anti-SIN sera on SIN *ts*23-infected cells. These findings suggest that the relatively low, but significant, levels observed (i.e., about 2,000 cpm above a background of 1,000 cpm) were due to small amounts of capsid protein on the surface of SIN *ts*23-infected cells, probably owing to leakiness of the mutant.

Cytolysis by anti-capsid protein serum is not due to absorbed nucleocapsid. The results thus far suggest that some determinants of capsid protein are exposed on the external surface of the plasma membrane of infected cells. One possibility to explain this observation is that nucleocapsids without envelopes are released from disrupted virus-infected cells and absorb to the outer surface of intact cells.

We infected L929 cell monolayers with either SIN or SF at an MOI of 20, and the supernatant fluids were harvested 13 h postinfection, when the cells had undergone an extensive cytopathic effect. Uninfected cells of the same age were

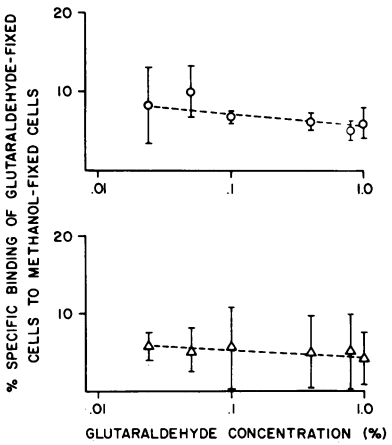

FIG 7. RIA of SIN *ts*23-infected cells using anti-SIN (○, 1:50) or anti-capsid protein (△, 1:20) sera at 13 h postinfection as a function of glutaraldehyde concentration in fixation. The data represent the results of three experiments with the standard deviations indicated.

incubated with the supernatant fluids for 1.5 h at 37°C, the cells were washed, and ADCMC assays were done with hyperimmune anti-SIN or anti-SF sera. No specific cytolysis of these cells was observed with either hyperimmune antiserum. Thus, it is unlikely that passive absorption of nucleocapsids to the outer surface of cells is the explanation for the reactivity with anti-capsid protein serum.

DISCUSSION

The present findings suggest that determinants of the capsid protein of alphaviruses are accessible to specific antibody produced after prolonged hyperimmunization (as opposed to standard immunization) and the outer plasma membrane surface of infected cells. Two independent techniques were used for detection of capsid protein, ADCMC and RIA with monospecific rabbit anti-capsid protein serum shown to be free of anti-E1 and anti-E2 activity. Further, the addition of purified capsid protein inhibited the ADCMC. Although the anti-capsid protein serum was prepared to purified SIN capsid protein, prolonged hyperimmunization was required to produce the antibodies reactive in ADCMC. These data are the first to suggest that cross-reactive capsid antigenic determinants may be on the surface of alphavirus-infected cells and may have in vivo significance as discussed later.

It should be noted that there are several recent reports on analogous observations with other viruses. Internal matrix and nucleocapsid antigens appear to be expressed on cells infected with influenza virus (1, 5, 6, 21, 44, 45). The matrix antigen was demonstrated on the surface of infected P815 cells by ADCMC with anti-matrix serum and by binding of radiolabeled monoclonal anti-matrix antibody. Further, Ada and Yap (1) precipitated the matrix protein from the outer surface of influenza-infected L929 cells with purified anti-matrix antibody. Nucleoprotein was detected by immunofluorescence on the surface of infected cells 2 h after infection (44), but it could not be demonstrated by ADCMC (6). (Perhaps extended hyperimmunization, as in our system, would have allowed detection by ADCMC.) Immunization with matrix protein did not confer protection against clinical disease in ferrets or against lethal infection in mice, nor did it reduce the severity of lung lesions in mice. However, mice immunized with the purified protein were able to clear challenge virus more rapidly from their lungs than were unimmunized animals (45). These findings were interpreted to indicate that cell-mediated immunity, rather than humoral immunity, was more important in cross-protection with influenza virus (13, 45). Other data that support this interpretation (27) showed that extensive cross-reactivity by cytotoxic T lymphocytes (CTL) was observed with target cells infected with influenza virus having internal, but not external, virion determinants homologous with priming virus. Likewise, influenza virus polymerase (internal), P3, was found to be a target antigen for CTL (4).

In another example, internal virion core antigen, p30, has been demonstrated with antibody on the surface of cells transformed with retroviruses (20, 23, 31). The p30 was also specifically recognized by CTL (38).

Although explanations for the presence of internal viral antigenic determinants on the outer cell surface have been minimal, how do we account for the detection of capsid protein on the surface of alphavirus-infected cells? We have considered three possibilities. The first possibility is that during prolonged hyperimmunization with presumably purified capsid protein, low levels of antibody are made to a minor contaminant, such as E1 glycoprotein. However, if

cross-reacting antibody to a viral glycoprotein was present in the anti-capsid protein serum, it was not detectable in the immunoblot technique. In addition, cross-cytolysis of SF-infected cell was abrogated by native purified capsid protein free of detectable glycoprotein. A plausible explanation is that very low, nondetectable concentrations of anti-glycoprotein antibody could be reacting with the glycoprotein on the cell surface and cause a perturbation, which allows determinants of the capsid protein to be exposed and reactive with anti-capsid protein antibody. In support of this explanation, Clegg et al. (10) found that two different monoclonal antibodies to E1 stimulated each other's binding. Their data suggested that binding of either of the monoclonal antibodies altered the conformation of E1 in such a way as to increase its affinity for the other and at the same time to release PE2.

A second possibility to explain the detection of capsid protein on the cell surface is that, during prolonged immunization, antibodies are made to antigenic determinants of the capsid protein, which ordinarily are cryptic (for example, a sequence of amino acid residues that is not processed and presented in normal immunization because it lacks three-dimensional conformation [29]). Once the antibody is produced, it could react with homologs of similar sequence, which are known to be present in the primary amino acid sequences of both SIN and SF capsid proteins (34).

The third possible explanation for our results is that the detection of capsid protein per se on the surface of infected cells may be due to a more or less natural event in which the sealing of the lipid bilayer of the plasma membrane after budding could become progressively inefficient, especially as the cytopathic effect develops when the cell is metabolically as well as morphologically impaired. This impairment may allow capsid to enter the site by direct insertion, membrane inversion, or some other mechanism.

Previously, we proposed that E1 glycoprotein on the surface of infected cells is one cross-reactive entity among alphaviruses detected by ADCMC. Several studies suggest that E1 exposed on the outer cell surface has unique immunological characteristics, because it is conformationally different from the E1 in virions: (i) the binding of E1-specific monoclonal antibody appears to dissociate PE2-E1 antigenic sites (10); (ii) the amount of glycosylation of E1 in infected cells was found to be reduced from that found on virions, and it was suggested that the differential glycosylation contributed to the availability or expression of E1 antigenic sites on infected cells compared with mature virions (25, 26); (iii) a majority of immunologically relevant E1 epitopes present on SIN-infected cells become cryptic during SIN maturation (12); and (iv) we have reported that a native conformation of cell-associated E1 is necessary for cross-reactive immune precipitation, whereas virion, but not cell-associated, E1 retains immunologic cross-reactivity after denaturation as detected in the immunoblot technique (50).

E1 glycoprotein on the cell surface during SIN infection is detected early with homologous hyperimmune anti-SIN sera (18, 50). Similarly, E1 glycoprotein is the probable target antigen detected early in the cross-reactivity of hyperimmune anti-SIN sera with SF-infected cells and the reactivity of anti-SF sera with SIN- or SF-infected cells. However, cytolysis caused by hyperimmune anti-capsid protein serum is delayed about 2 h beyond that with hyperimmune anti-virion sera. These results suggest that there are two cross-reactions at the infected cell surface: the early one detects cross-reaction to unique native E1 (50), and the later one detects cross-reaction to exposed capsid protein, native or denatured, a major point of this paper.

Although the presence of capsid protein on the cell surface may be the consequence of a cytopathic effect that is not related to virus maturation per se, the cross-reactivity of the exposed capsid with immune effector systems in vivo could still play a significant role in cross-protection among alphaviruses regardless of the mechanism that accounts for its presence on the infected cell surface.

ACKNOWLEDGMENTS

We thank John Bulrice and Rene Crombie for technical assistance.

This work was supported in part by U.S. Department of Agriculture grant CRSR-2-2456.

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