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THE INTERACTION OF MONOCLONAL ANTIBODIES DIRECTED AGAINST ENVELOPE GLYCOPROTEIN E_1 OF SINDBIS VIRUS WITH VIRUS-INFECTED CELLS

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Two monoclonal antibodies specific for the Sindbis virus envelope glycoprotein E_1 were evaluated for their ability to maintain long-term infection when present in the medium of virus-infected cells. One of them, previously shown to have neutralizing activity and to inhibit haemagglutination, caused suppression of both virus expression at the cell surface and prolonged intracellular virus presence. The other monoclonal antibody which lacked neutralizing activity but inhibited virus-specific haemolysis caused redistribution of viral antigens on the cell surface but only slightly prolonged cell survival. Both epitopes were located on the surface of the virus. By electron microscopy it was demonstrated that the determinant associated with haemolytic activity resided near the virus membrane while the haemagglutination inhibition antibody attached near the apex of the virus spikes.

E_1 monoclonal antibodies Sindbis virus capping

INTRODUCTION

Budding viruses that mature at the cell plasma membrane express antigens on the cell surface. Specific antibodies in the medium of virus-infected cells have been shown to induce polar redistribution of these antigens leading to cap formation, antigen stripping and modulation of virus-coded proteins [7, 9, 11] (for reviews see refs. 10, 12).

To identify and study viral antigens on the surfaces of virus-infected cells, monospecific antisera with a high degree of specific activity for their corresponding antigens are required. The use of monoclonal antibodies in such studies should eventually lead to a precise understanding of virus protein dynamics at the plasma membrane. Moreover, because of their exquisite specificity, monoclonal antibodies may define different functions of antibodies specific for different epitopes on the same antigen. In the present report two monoclonal antibodies to the Sindbis virus E_1 glycoprotein [4] are examined for their effects on virus-infected cells.

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METHODS

Virus and hyperimmune serum

A working stock of Sindbis virus strain AR339 was prepared by inoculation of Vero cell monolayers at an input multiplicity of 0.01 plaque-forming units (p.f.u.)/cell. After incubation for 42 h at 37°C the supernatant medium was clarified by centrifugation at 2000 × *g* for 10 min. Stock virus was stored at -70°C. A suckling mouse brain virus stock was also prepared by intracerebral inoculation of 1–2-day-old mice with 10^3–10^4 LD_{50}/0.02 ml of virus suspension. When approximately 20% of the mice were moribund they were anaesthetized and the brains were removed. A 20% w/v brain suspension was prepared and stored at -70°C. Rabbit hyperimmune antiserum was prepared by inoculating an adult rabbit four times at 2-week intervals with the suckling mouse brain suspension. The first inoculation was administered intramuscularly in complete Freund's adjuvant. This was followed by one intravenous dose and two intradermal inoculations. The rabbit was exsanguinated two weeks after the final inoculation.

Monoclonal antibodies

Two monoclonal antibodies to the E_1 glycoprotein were used; one (30.11) reacted with Sindbis virus haemagglutinin and neutralized virus infectivity. The other (30.12) specifically inhibited haemolysis by Sindbis virus and did not neutralize infectivity. A further monoclonal antibody specific for the capsid protein was also used in some experiments. Their derivation and characterization have already been described [4].

Cell cultures

Vero cells used in virus plaque titrations and immunofluorescence studies were grown in L-15 medium with 5% heat-inactivated fetal calf serum, 10% tryptose phosphate broth and antibiotics. Virus plaque assays were performed as previously described [6], but adapted to multi-test plates (Linbro TC-mulidish Flow Laboratories, Irvine, Scotland). After incubation for 2 days at 37°C, the carboxymethyl-cellulose overlay was removed and the plates were fixed and stained by immersion in a solution of 1% naphthalene black, in an acetic acid-sodium acetate buffer [6].

Indirect immunofluorescence

Infected cells for immunofluorescent staining and capping experiments were seeded on glass coverslips and maintained in L-15 medium. When the effect of antibodies on the virus-infected cells was being studied, the medium was also supplemented with the appropriate antibody and incubated at 37°C. The infected cover-slips were then washed in phosphate-buffered saline (PBS) for 10 min and either fixed in cold acetone for 5 min

or used unfixed for staining. For indirect immunofluorescence, appropriate dilutions of virus-specific antiserum were added for 45 min at 37°C. Coverslips were washed in PBS for 10 min and then covered with fluorescein- or rhodamine-conjugated anti-species globulin (1 : 40 in PBS, Nordic Immunological Reagents, Maidenhead, U.K.). After incubation for a further 45 min at 37°C coverslips were washed in PBS for 10 min, rinsed in distilled water and mounted in glycerol/saline solution (9 : 1) on glass microscope slides. In the case of unfixed cells the water rinse was omitted.

Electron microscopy

Infected Vero cells for electron microscopy were processed using standard procedures [5].

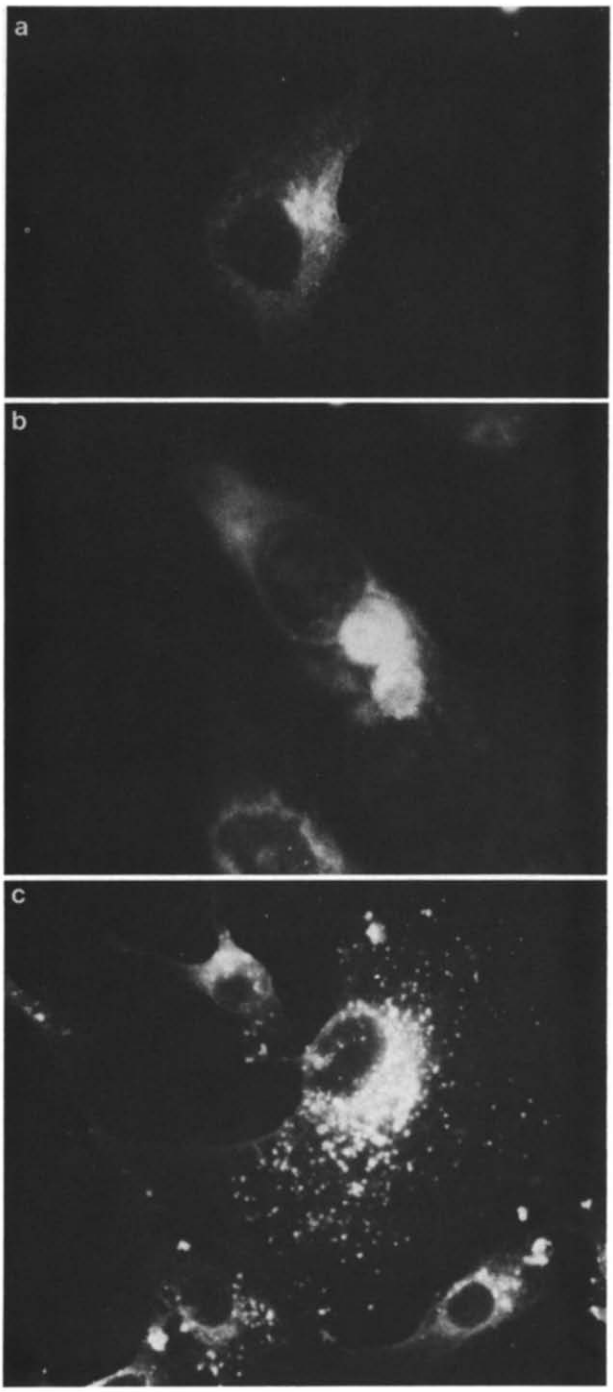
RESULTS

Survival of infected Vero cells in the presence of monoclonal antibodies

The incubation period before the appearance of cytopathic effect (CPE) after Sindbis virus infection of Vero cell cultures was directly related to virus input multiplicity. At high multiplicity (5–10 p.f.u./cell) CPE, characterized by contracted cytoplasm and increased cellular refractoriness appeared 12–18 h post-infection. At lower multiplicities (0.1–0.01 p.f.u./cell) the time was extended to 36–48 h. Total destruction of the cell monolayer occurred after a further 12 h incubation.

Incorporating either of the two E_1 monoclonal antibodies in the medium of Sindbis virus-infected cultures (imput multiplicity 5–10 p.f.u./cell) 4 h post-infection had different effects on the outcome of the infection. Monoclonal antibody 30.12 at a final concentration in the medium of 2% merely delayed the CPE by 12–18 h. In contrast, the presence of monoclonal antibody 30.11 at similar concentrations resulted in the complete inhibition of cell destruction. Seven days post-infection these antibody-protected cultures were subcultured in either antibody-free or antibody containing (30.11) media resulting respectively in either the total destruction of the cell monolayer within 24 h, or continued protection.

After further incubation for 4 days the antibody-treated cells were again subcultured in antibody-free and antibody (30.11)-containing media. In the presence of the monoclonal antibody, no CPE was observed. However, cells incubated in the absence of antibody were destroyed within 48 h. Examination of these cultures by immunofluorescence with hyperimmune antiserum 15 days post-infection demonstrated that 100% of the cells contained viral antigens. The intracellular distribution of viral antigens stained by hyperimmune antiserum differed from that seen in cytolytic infection (Fig. 1a). One feature was the presence of one or two large inclusions near the nuclear membrane often distorting the nucleus (Fig. 1b). Similar inclusions were observed when using anti-NP and anti-E_1 monoclonal antibodies for staining. A large number of smaller inclusions distributed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in the cytoplasm were also observed but these were not stained by anti-NP monoclonal antibody (Fig. 1c).

The long-term maintenance of infected cultures was dependent upon the continued presence of monoclonal antibody 30.11 in the culture medium. Some infected cultures were thus maintained for up to 3 months, though most cultures became free of detectable virus antigen after a period of 6–8 weeks. This type of immune pressure, however, did not result in the selection of virus lacking the corresponding epitope as judged by immunofluorescence using monoclonal antibody 30.11.

Antibody-mediated redistribution of viral antigens

Both monoclonal antibodies were evaluated for their ability to cap Sindbis virus antigens on the cell surface. Vero cells inoculated with 5 p.f.u./cell, 4 h previously were then incubated in the presence of one or other of the two monoclonal antibodies for 4–18 h at 37°C. They were then examined unfixed by immunofluorescence after addition of rhodamine- or fluorescein-labelled rabbit anti-mouse globulin. Detached cells showed distinct areas of cap formation on the surface of virus-infected cells (Fig. 2a, b). After 18 h up to 15% of the cells incubated in the presence of monoclonal antibody 30.12 were capped. In the presence of monoclonal antibody 30.11 approximately 5% of the cells were capped and there was a marked reduction in the number of cells expressing surface antigens. In cells still attached to the coverslips most virus-specific antigens were restricted to the polarized microvilli on one side of the cells giving them a braided appearance. In some instances the antigens could be seen after they were expelled from the cells (Fig. 2c, d, arrowed).

The ability of the two monoclonal antibodies to maintain cap formation for up to 36 h post-infection was also examined by immunofluorescence. In the presence of monoclonal antibody 30.12 up to 1% of surviving cells showed capping. In contrast cells incubated in the presence of monoclonal antibody 30.11 had undetectable levels of the corresponding antigen on their surface.

Ultrastructural studies

It has already been shown that Sindbis virus-infected Vero cells show abundant microvilli distributed around the cell surface. Virus particles budding from the plasma membranes utilize small preferred sites randomly arranged on the cell surface, though viral

Fig. 1. Indirect immunofluorescence using a rabbit hyperimmune antiserum of Sindbis virus infected cells cultured on glass coverslips and fixed with acetone. a) 24 h post-infection. b) 15 days post-inoculation and maintained in medium containing 2% monoclonal antibody 30.11 (similar inclusions were seen using an anti-capsid and anti-E_1 monoclonal antibodies). c) As (b) stained by both hyperimmune serum or either anti-E_1 monoclonal antibodies.

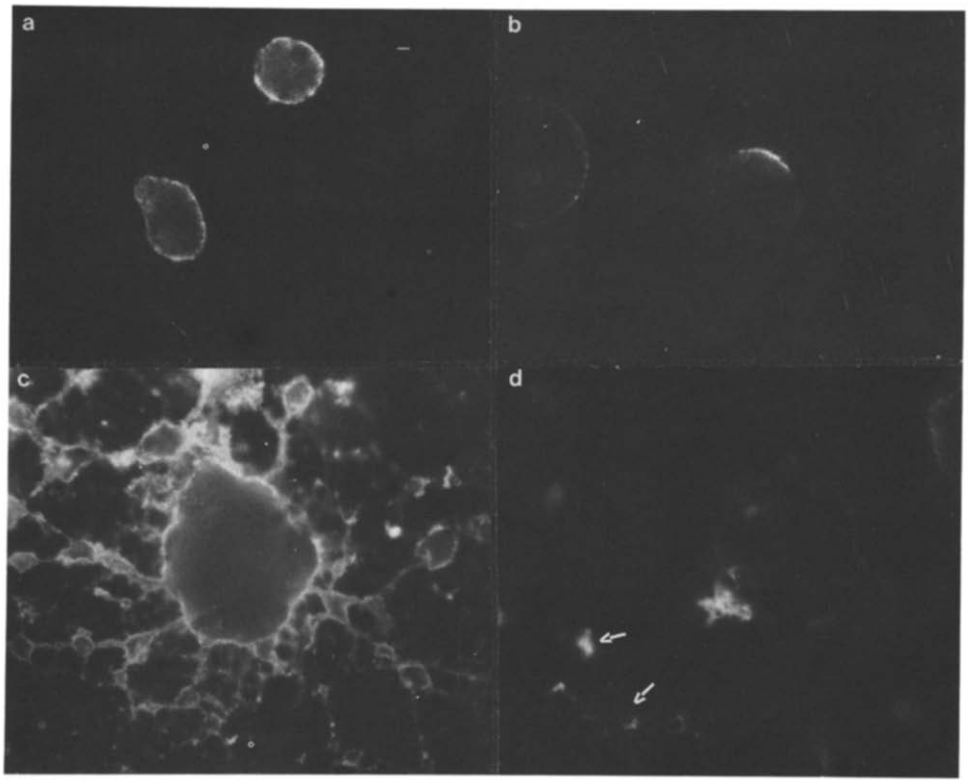


Fig. 2. a) Detached unfixed Sindbis virus infected Vero cell stained by indirect immunofluorescence with monoclonal antibody 30.12. b) As a), except that cells were incubated for 4 h with 2% anti-E_1 monoclonal antibody (either 30.11 or 30.12) prior to addition of rhodamine conjugate. c) Anchored cell stained as (a). d) Anchored cell maintained in medium containing anti-E_1 monoclonal antibody 30.11 overnight, prior to adding anti-mouse conjugate. Arrows indicate expelled microvilli.

antigen is usually more evenly distributed in the plasma membrane [3]. In our study such viral patching was distinguishable from antibody-induced redistribution by the lack of involvement of the microvilli and the presence of a number of 'patches' on each cell (Fig. 3a). Following antibody-induced cap formation, the site of viral patching was usually restricted to one area. This area was characterized by compacted microvilli which were aligned along the cell surface and could be seen in cross-section. On the cytoplasmic side of the plasma membrane large accumulations of viral nucleocapsids, immediately below the cap, were observed (Fig. 3b, arrowed). Negatively stained preparations of pellets obtained after centrifugation (15,000 × *g* for 2 h) of cell supernatants 24 h post-infection showed a large number of aggregated virus particles. When the cells were maintained in the presence of monoclonal antibody 30.12, these consisted entirely of complete mature particles characterized by a protein halo not wider than the existing spikes. Pellets from culture supernatants incubated for 24 h at 37°C in the presence of

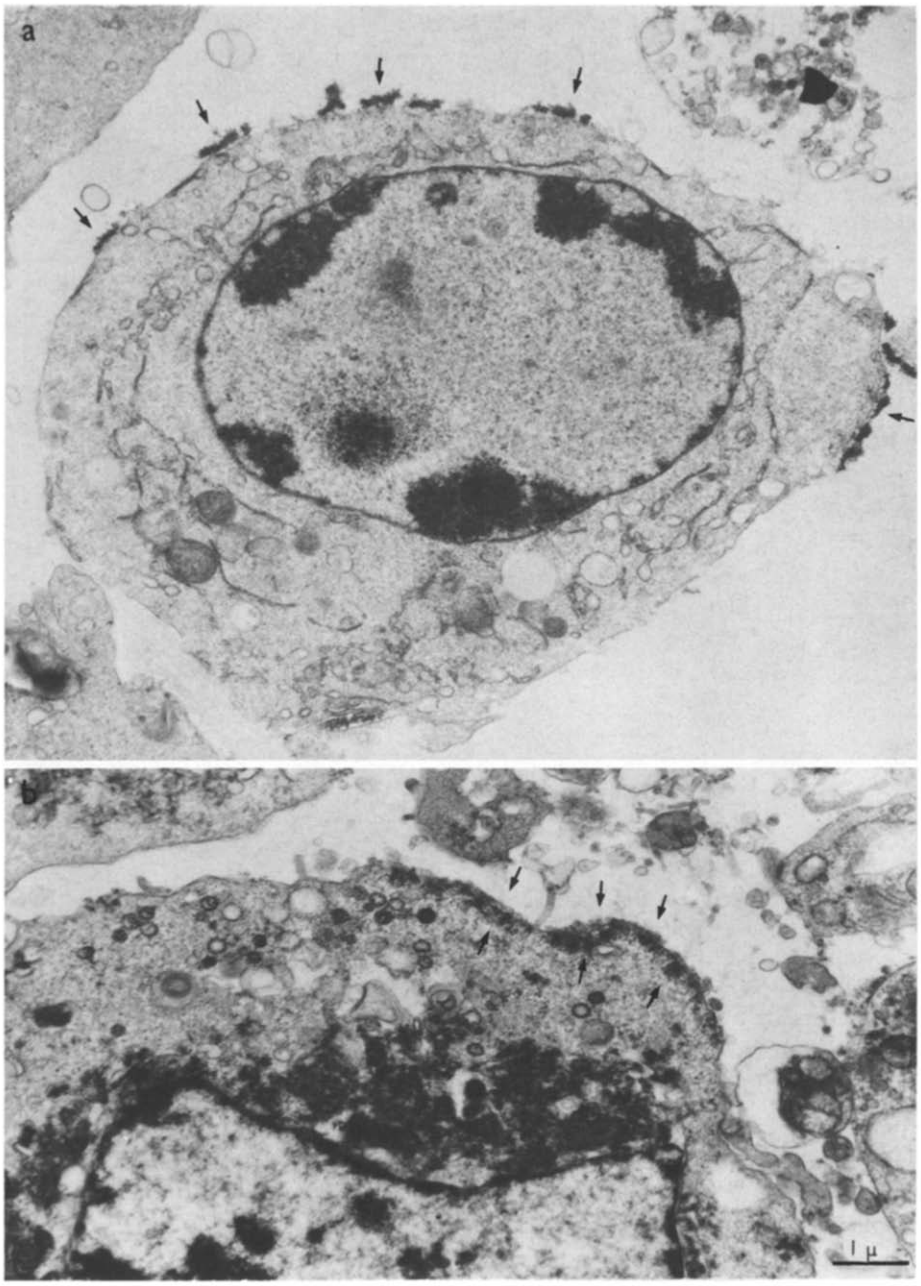


Fig. 3. a) Thin section electron micrograph of Sindbis virus-infected cell showing normal virus patching. b) Thin section electron micrograph of Sindbis virus-infected cell incubated for 4 h at 37°C in the presence of 2% anti-E_1 monoclonal antibody (either 30.11 or 30.12) prior to fixation. Note accumulation of viral capsids immediately below the compacted microvilli seen in cross-section.

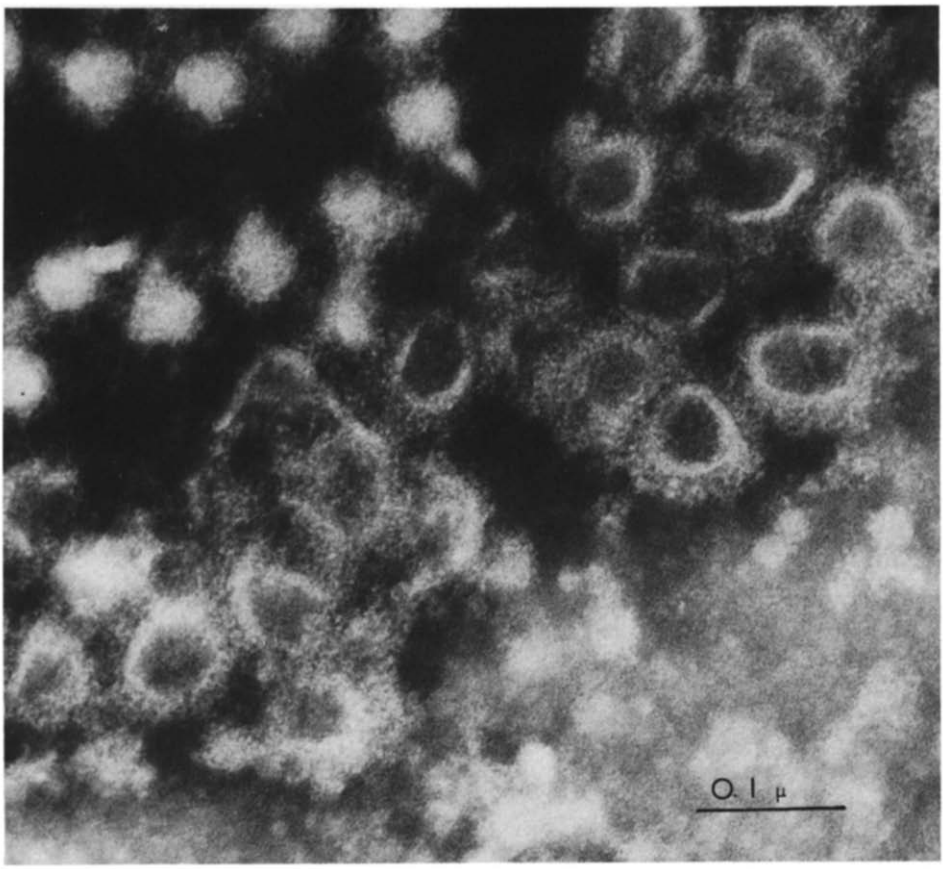
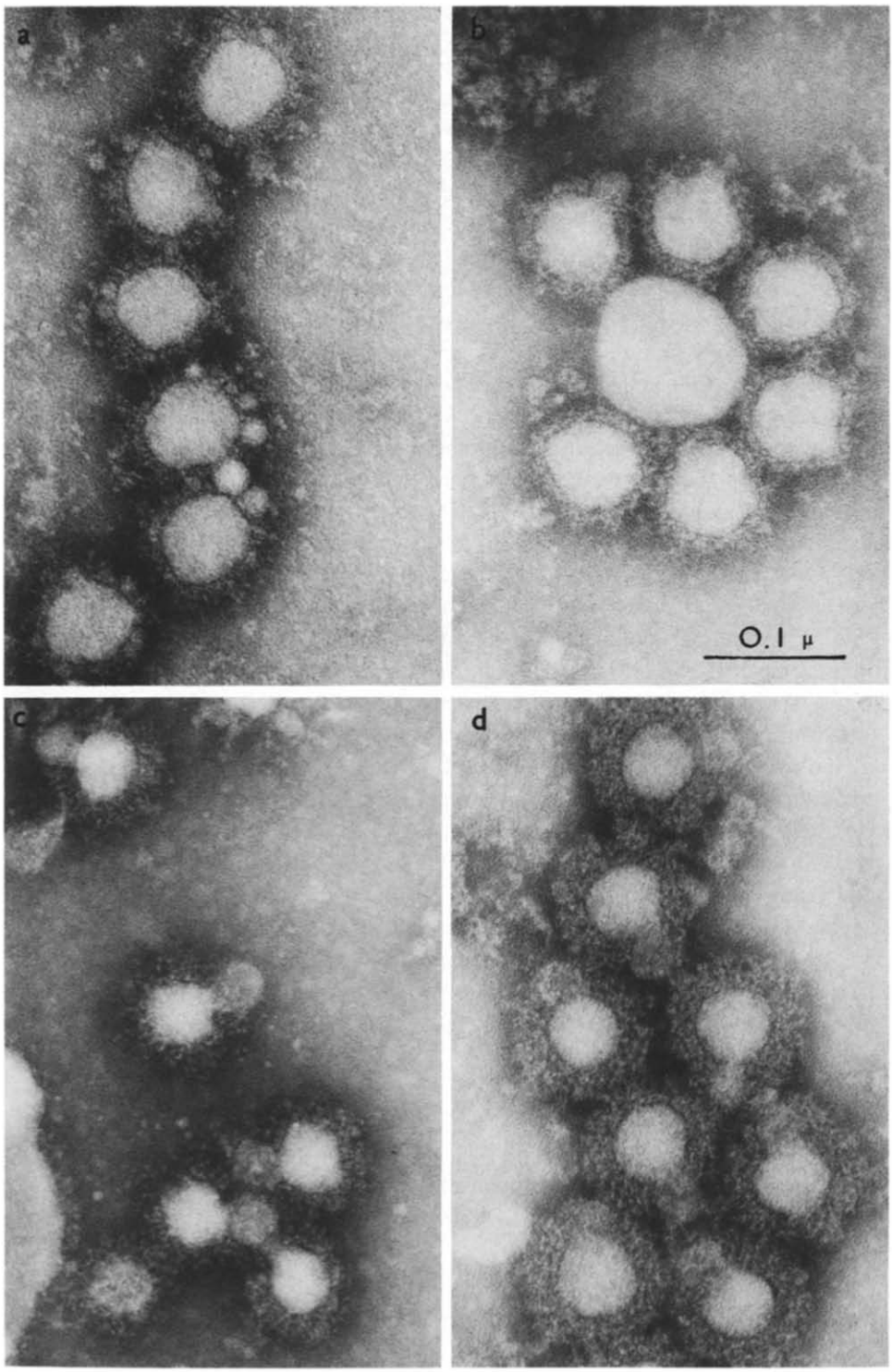


Fig. 4. Electron micrograph of negatively stained preparation from a Sindbis virus-infected culture maintained for 24 h at 37°C in the presence of 2% monoclonal antibody 30.11.

monoclonal antibody 30.11, however, consisted of aggregated viral membranes and particles devoid of nucleocapsids (Fig. 4). By contrast, the appearance of immune aggregates obtained after incubation for only 4 h at 37°C is shown in Fig. 5. With monoclonal antibody 30.12 again the appearance of the virions was not significantly different from control preparations (Fig. 5a, b). However, a significant increase in the halo-surrounding virus particles in the presence of monoclonal antibody 30.11 was observed (Fig. 5c). The presence of monoclonal antibody 30.12 was evident in mixtures of the two monoclonal antibodies by a substantial augmentation of the density but not the width of the immune halo (Fig. 5d).

Fig. 5. Attachment of two E_1 monoclonal antibodies to the surface projections of Sindbis virus. Negatively stained preparations from infected culture supernatants. a) Sindbis virus only. b) Culture maintained in the presence of 2% monoclonal antibody 30.12 for 4 h before harvest. c) As (b) except that monoclonal antibody 30.11 was used. d) As (b) except that a mixture of the two monoclonal antibodies was used. Note augmentation of immune halo.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DISCUSSION

The interaction of the two E_1 monoclonal antibodies with virus-infected cells can be considered with the results obtained in neutralization experiments previously described [4]. Thus the neutralizing antibody (30.11), when acting on infected cells diminished virus production, disrupted virus maturation, caused the redistribution of intracellular virus antigens (Figs. 1b, 2b, 2d and 3b) and stripped viral antigens from the cell surface (Figs. 2d and 4). The non-neutralizing monoclonal antibody (30.12) was more efficient at maintaining capping. Virus cytopathology was not markedly inhibited, and virus yields were not reduced when compared with control cultures (data not shown). By electron microscopy both epitopes were demonstrated on the surface of the virus. However, the extent of the immune halo observed in negatively stained preparations when the antibodies were used individually or in combination with each other suggests that the antibody to Sindbis haemagglutinin attached on the apex of the virus spike, while that inhibiting haemolytic activity attached nearer the virus membrane (Fig. 5).

Though the biological function of capping is not fully understood, the evidence available implies that it is a prerequisite in the activation of the alternate pathway of complement and possibly other effector systems (reviewed by Sissons and Oldstone [12]). In an in vivo situation therefore non-neutralizing antibodies may play an essential role in the elimination of virus-infected cells by the immune system before full virus production is completed and without concomitant suppression of viral antigens on the cell surface. The importance of this becomes apparent when the long-term effects of the neutralizing monoclonal antibody on Sindbis virus-infected cells are considered. By stripping the corresponding virus antigens from the cell and modulating their intracellular expression, a 'dormant' viral stage was induced that resulted in prolonged intracellular virus survival. It has been proposed by Barrett and Atkins [2] that persistent infection of mammalian cells by alphaviruses is an equilibrium state between virus multiplication and factors such as DI particles and interferon production. If the essential prerequisite for persistent infection is the survival of infected cells, then some antibodies (such as 30.11) may also be involved. Prolonged maintenance of the persistent state might then depend on subsequent selection pressures on the virus to evolve to a less cytopathic agent involving a combination of other factors such as DI particles, small plaque variants, *ts*-mutants and interferon [1, 2, 8].

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