JOURNAL OF VIROLOGY, Nov. 1985, p. 482–488
0022-538X/85/110482-07$02.00/0

Vol. 56, No. 2

Isolation and Characterization of Neutralizing Monoclonal Antibodies to Vaccinia Virus

JOSE F. RODRIGUEZ, RICHARD JANECZKO, AND MARIANO ESTEBAN*

Departments of Biochemistry and Anatomy and Cell Biology, Downstate Medical Center, Brooklyn, New York 11203

Received 13 May 1985/Accepted 25 July 1985

Cells producing neutralizing monoclonal antibodies (mAbs) to UV-inactivated vaccinia virus strain WR were derived by fusion of hyperimmunized mouse spleen cells with mouse myeloma cells. Three mAbs that reacted strongly with purified virus envelopes as determined by enzyme-linked immunosorbent assay were studied. The three mAbs recognized a 14,000-molecular-weight (14K) envelope protein of vaccinia virus and were shown to be immunoglobulin G2b (mAbC3 and mAbB11) and immunoglobulin M (mAbF11). By using ascites, one of the antibodies, mAbC3, neutralized (50%) virus infectivity with a titer of about 10^{-4}, whereas the others exhibited lower neutralization titers of 10^{-2} to 10^{-3}. The binding of the mAbs to vaccinia virus did not alter virus attachment to cells. However, virus uncoating was extensively blocked by mAbC3, whereas mAbB11 and mAbF11 had little or no effect. The three mAbs recognized a similar 14K protein in cowpox, rabbitpox, and vaccinia Elstree strains, indicating a high degree of protein conservation among orthopoxviruses. Based on the binding of mAbs to V-8 protease cleavage products of the 14K protein, the extent of protein recognition for other poxviruses, and differences in the degree of virus neutralization and of virus uncoating into cells, we suggest that the three mAbs recognize different domains of vaccinia 14K viral envelope protein. Furthermore, our findings indicate that the 14K protein may play a role in virus penetration.

Smallpox has been the classic example of infectious disease in which recovery is associated with the development of a highly effective and enduring immunity. The immune response is evident in the appearance, during the course of the disease, of complement-fixing and hemagglutinin-inhibiting antibody activity, but these antibodies do not appear to be associated with effective immunity. Rather, protection seems to be correlated with virus-neutralizing antibodies, which, unlike complement-fixing and hemagglutinin-inhibiting antibodies which decline to insignificant levels in about 12 months, are demonstrable for years. On the basis of studies of mousepox, as well as pathologic, virologic, and epidemiologic studies, a reasonable profile of the events in the pathogenesis of smallpox has been constructed (3). Smallpox has been eradicated as a result of a 10-year global vaccination campaign with live vaccinia virus as a vaccine (3). However, recent observations indicate that the development of new strategies may permit even wider use of vaccinia virus as a vaccine. The ability of vaccinia virus to accept and express cloned genes encoding immunologically important proteins of unrelated viruses (15–22, 26–27, 31, 32) and malarial parasites (25) has suggested a novel approach to the development of live vaccines.

Vaccinia virus, a member of the genus *Orthopoxvirus*, is one of the largest and most complex animal viruses. The large 187-kilobase double-stranded genome codes for about 200 genes which are involved in viral infection, viral replication, and virion morphogenesis. About 100 polypeptides are associated with the virion, and approximately 9 polypeptides are incorporated into the outer envelope (6, 18).

Although much is known about the replication cycle of vaccinia virus in tissue culture cells (6, 18), little is known about the viral protein(s) involved in the host immune response and, in particular, the protein(s) which elicits neutralizing antibodies (23). Because the neutralizing response plays a major protective role, characterization of the antibodies, their specificities, and their functional role in the neutralization process is crucial to an understanding of the pathobiology of vaccinia virus. We report here three monoclonal antibodies (mAbs) which recognize an envelope protein of vaccinia virus, have neutralizing activity, and block virus uncoating but not virus attachment to cells. The vaccinia 14,000-molecular-weight (14K) envelope protein is highly conserved among orthopoxviruses and may play a role in virus penetration.

MATERIALS AND METHODS

Virus and cells. Stocks of vaccinia virus strain WR were prepared by infecting HeLa S_3 cells at a multiplicity of 0.05 PFU per cell. Virus was then purified and concentrated by sucrose gradient centrifugation (9, 13). The particle-to-PFU ratio was about 20:1. The Elstree strain of vaccinia virus was grown in the chorioallantoic membrane of eggs. Cowpox and rabbitpox viruses were grown in rabbit kidney cells. Vaccinia (Elstree), cowpox, and rabbitpox viruses were kindly provided by J. A. Holowczak, Rutgers Medical School, New Brunswick, N.J. All viruses were titrated in monkey kidney cells (BSC-40).

Immunization and hybridoma cells. The myeloma cell line P3NI used for fusion is a thyoguanine- and azaguanine-resistant derivative of myeloma cell clones and was kindly provided by T. Easton, Downstate Medical Center, Brooklyn, N.Y. The cells were grown in Dulbecco modified Eagle medium with 20% (vol/vol) heat-inactivated fetal calf serum.

BALB/c mice (8 weeks old) were immunized with intact vaccinia virus that was inactivated by UV light (5,760 ergs per mm^2). Mice were injected intraperitoneally with 0.3 ml of virus (30 μg of protein) in Freund complete adjuvant. After 15 days, mice were challenged again with the same amount of viral protein but in Freund incomplete adjuvant. Fifteen days later, mice were boosted with 0.1 ml of virus (30 μg of viral protein) in phosphate-buffered saline (PBS) by the

* Corresponding author.

intravenous route. Three days later, the mice were sacrificed and their spleens were removed and fused with myeloma cells (14). Briefly, 10^8 spleen cells and 2×10^7 myeloma cells were treated with 50% polyethylene glycol 1500. After dilution of polyethylene glycol 1500 PEG in Dulbecco medium, the cells were pelleted, suspended in the same medium supplemented with 20% fetal calf serum, and distributed in 96-well plates. On the next day, an equal volume of medium containing 2 × HAT (100 μM hypoxanthine, 4 μM aminopterin, 16 μM thymidine) was added to all wells. Five to seven days later, the medium was replaced with fresh Dulbecco medium containing 20% fetal calf serum and HAT. After a further incubation of 5 to 7 days, the tissue culture fluid from wells showing cell growth was screened for antiviral antibodies.

Enzyme-linked immunosorbent assay for antienvelope antibodies. Plates (96 wells) were coated with 1 μg of purified UV-inactivated vaccinia virus or purified viral envelopes in 0.1 M carbonate buffer (pH 9) per ml and incubated overnight at 5°C. UV-inactivated vaccinia virus was prepared as previously described (2). In addition, viral envelopes were prepared from 5 mg of purified virus suspended by sonication (500 W for 10 s) in 50 mM Tris hydrochloride (pH 8.5)–10 mM $MgCl_2$–10 mM dithiothreitol–0.5% Triton X-100 (10). After incubation at 37°C for 30 min, the extract was layered on top of a 36% sucrose cushion in the same buffer, and cores were pelleted by centrifugation at 20,000 × *g* for 60 min at 4°C. The upper phase containing the viral envelopes was recovered and dialyzed extensively against PBS. Before the enzyme-linked immunosorbent assay all wells were coated with 5% bovine serum albumin in PBS. Preimmune serum was included as a negative control for each plate. Anti-vaccinia virus serum raised in mice acted as a positive control. Tissue culture medium (1:5 dilution) from hybridomas was added to the plates (50 μl per well) and incubated overnight at 4°C. The plates were washed and goat anti-mouse immunoglobulin cojugated to horseradish peroxidase (Cappel Laboratories, Cochranville, Pa.) was added at a dilution of 1/500. The plates were incubated for 1 h at 37°C and washed. The substrate *o*-phenylenediamine was added, and the plates were incubated for 30 min at 37°C. The reaction was terminated by the addition of 4.5 M H_2SO_4. The wells were scored by using a Multiskan plate reader (Flow Laboratories, Inc., McLean, Va.) Positive hybridomas were propagated, cloned, and used for production of ascites tumors. Briefly, 96 wells containing 100, 500, and 5,000 hybrid cells were prepared. Macrophages were used as feeder cells. Plates that showed 30% of the wells with growing cells were examined by microscopy, and wells containing only one colony were screened and positive cell clones were propagated further. Hybridomas were grown in Pristane-primed syngenic mice as ascites.

Determination of immunoglobulin class. mAbs in culture supernatants were concentrated 10 times by $(NH_4)_2SO_4$ (50%) precipitation. This concentrate then was tested against isotype- and subclass-specific antisera (Bionetics, Kensington, Md) by Ouchterlony double diffusion.

Immunoblotting. Protein samples (5 to 10 μg) were fractionated on 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels. After fractionation, the gel was briefly soaked in 25 mM Tris hydrochloride (pH 8.3)–192 mM glycine–2% (vol/vol) methanol–0.1% SDS, and polypeptides were transferred to nitrocellulose (0.45 μm; Schleicher & Schuell, Inc., Keene, N.H.) by a modification of the procedure originally developed by Towbin et al. (29). The conditions of the transfer were 4 h at room temperature with a current of 40 V and 0.25 A. To visualize the transfered polypeptides, the nitrocellulose paper was either stained with amido black or exposed to X-ray film when proteins were labeled. mAbs were reacted with the nitrocellulose paper as follows. The paper was soaked for 2 h at room temperature with 5% fetal calf serum in PBS and then incubated overnight with the mAbs (1:200 dilution). Thereafter, the paper was washed with PBS four times for 15 min each and then incubated for 2 h at room temperature and for 1 h with goat anti-mouse immunoglobulin conjugated to horseradish peroxidase. After washing out the excess second antibody, the nitrocellulose paper was developed with 0.02% 1-chloro-4-naphthol–0.006% hydrogen peroxide in PBS.

Plaque neutralization assay. About 200 PFU of vaccinia virus were incubated for 60 min at 37°C with serial dilutions of mAbs in 0.5 ml of Dulbecco medium containing 2% heat-inactivated calf serum. The virus-antiserum sample was allow to adsorb to monkey cells (BSC-40) plated in Linbro plates (6 by 6 wells) for 60 min at 37°C, and assays were carried out in triplicate. The inoculum was removed, and the wells were overlaid with fresh medium containing 0.9% agar. After 48 h of incubation at 37°C, the agar was removed, and plaques were visualized by staining with 1% crystal violet in 2% ethanol.

Analysis of vaccinia virus uncoating by sucrose gradients. BSC-40 cells were grown in 100-mm dishes and infected with [^{3}H]thymidine-labeled vaccinia virus (specific activity, 2×10^5 cpm/μg of DNA) that previously was incubated for 1 h at 37°C with normal mouse serum or with a 1:50 dilution of mAbs. Virus adsorption was carried out in the presence of 100 μg of cycloheximide per ml, virus inoculum was removed after 1 h at 37°C, and 10 ml of Dulbecco medium containing 2% newborn calf serum and 100 μg of cycloheximide per ml was added. At 4 h postinfection, cytoplasmic extracts were prepared by washing three times with cold PBS (4°C) and once with cold hypotonic RSB medium (10 mM KCl, 10 mM Tris hydrochloride [pH 7.4], 1.5 mM $MgCl_2$). The monolayers were scraped into RSB medium, adjusted to 0.5% Nonidet P-40, vortexed, and allowed to stand for 10 min on ice. Subviral particle formation was determined by established protocols (2, 11, 24). After spinning at 800 × *g* for 10 min, the supernatants were sonicated three times for 5 s each at 50 W (Biosonick II; Bronwill Scientific Inc., Rochester, N.Y.), layered onto a 25 to 40% (wt/wt) sucrose gradient (0.01 M Tris hydrochloride [pH 9]), and centrifuged at 5°C for 22 min at 20,000 rpm (SW41 rotor). The fractionated gradients were precipitated with 10% trichloroacetic acid (4°C) and harvested by filtration through GF/C filters (Whatman, Inc., Clifton, N.J.) which were dried and counted in a liquid scintillation counter.

RESULTS

Isolation of mAbs against envelope proteins of vaccinia virus. Initial screening of hybridoma supernatants was carried out against UV-inactivated vaccinia virus. Clones which exhibited strong activity were subcloned twice by serial dilution. The positive clones were grown, and the supernatants were tested by Western blotting against purified vaccinia envelopes, virus cores, and total viral proteins. Three mABs apparently recognize the same viral protein of 14K (Fig. 1). The protein is localized in the envelope of vaccinia virus since a strong reaction was observed with purified envelopes but not with cores. Two other proteins of about 28K and 11K were found to react with mAbs but to a lesser extent than was the 14K protein. The specific reactivities of

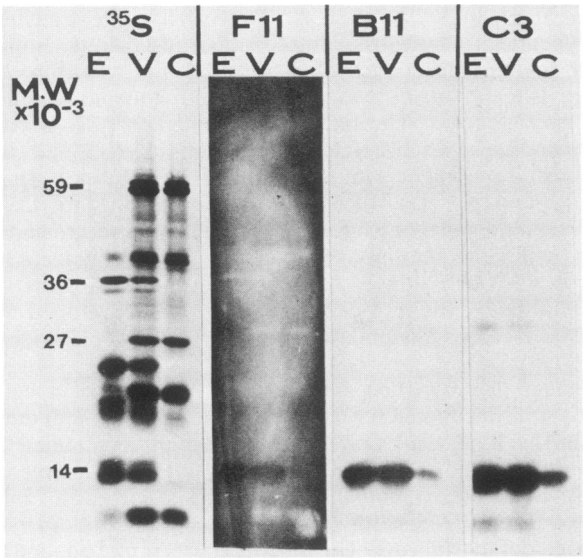

FIG. 1. Antigen specificities of three mAbs against vaccinia virus. Purified [^{35}S]-labeled vaccinia virus, viral cores, and viral envelopes were separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose. The nitrocellulose strips were incubated with a 1:50 dilution of hybridoma antibodies as ascites, and binding was detected by the immunoperoxidase staining method. ^{35}S refers to an autoradiogram of labeled polypeptides. The different mAbs are given on top. E, Viral envelopes; V, whole virus; C, viral cores. The molecular weight (MW) of some vaccinia polypeptides are noted. The dark background consistently observed with F11 is attributed to a characteristic property of immunoglobulin M.

28K and 11K proteins with B11 and C3 is under investigation. To obtain high yields of antibodies, ascites fluid was prepared from the clones. After protein purification and immunodiffusion testing, two of the clones (B11 and C3) were found to express immunoglobulin G2b whereas the other clone (F11) expressed immunoglobulin M. To determine whether the three mAbs recognize the same protein, the 14K band was excised from the 12% gel, run on a 15% gel after partial digestion with *Staphylococcus aureus* V-8 protease (4), transferred to nitrocellulase paper, and finally reacted with the three different mABs. Two fragments of about 11.9K and 10.7K were recognized by the mAbs (Fig. 2A). One of the fragments (11.9K) reacted intensely with B11 and C3, whereas the other fragment (10.7K) reacted only with C3. Neither of these fragments reacted with F11 (not shown). Failure to detect the 10.7K band in lane B11 relative to a weak band in lane C3 is not the result of differences in amount of cleavage products but rather of a lack of reactivity, as shown by the autoradiograph of the cleavage products (Fig. 2B). These findings provided evidence that we isolated three distinct mAbs reactive against at least two distinct epitopes of the 14K envelope protein of vaccinia virus.

Neutralization of vaccinia virus infectivity by mAbs. Since the 14K protein is located in the envelope of vaccinia virus, it was of interest to examine the mAbs for neutralizing activity. This was evaluated afer incubation (at 37°C) of appropriate dilutions of the mABs with 200 PFU of vaccinia virus, followed by virus titration on BSC-40 cells. C3 exhibited strong neutralizing activity (50% at a $1{:}10^{-4}$ dilution), whereas B11 and F11 had lower neutralizing activity at 10^{-2} to 10^{-3} dilutions (Fig. 3). With a 10^{-1} dilution we obtained a neutralization of about 90% with C3. The resistance of about 10% of the infectious virus could be attributed to other sites on the 14K viral protein which are required for complete neutralization. Indeed, we observed a synergistic effect with C3 and B11 (90% at a $1{:}10^{-2}$ dilution). Resistance of the infectious virus to neutralization by mABs also could be due to generation of mutant viruses. This possibility is under investigation. These findings establish that vaccinia virus infectivity in tissue culture cells can be blocked by mAbs reactive against the 14K envelope protein.

mAbs blocked vaccinia virus uncoating in cells. Inhibition of vaccinia virus infectivity by the mAbs could occur at the level of virus attachment or uncoating. These various steps during virus-cell interaction were measured with mAbs in comparison with polyclonal antibodies by using [^{3}H]thymidine-labeled vaccinia virus. The kinetics of virus attachment to cells is presented in Fig. 4. It is clear that while polyclonal antibodies raised against whole vaccinia virus strongly prevented virus attachment, in constrast, the mAbs had no effect on either the rate or extent of virus adsorption to cells. Virus uncoating was estimated from the extent of conversion of vaccinia virus particles to subviral cores, as determined by velocity sedimentation in sucrose gradients (Fig. 5). In cells infected with nonneutralized vaccinia virus, most of the virus particles were converted into cores. In cells infected with vaccinia virus neutralized with mAbs, a drastic effect was found with clone C3. This mAb greatly impaired the extent (62%) of conversion of virus into cores. This inhibition of virus uncoating is similar to that observed in cells inoculated with vaccinia virus

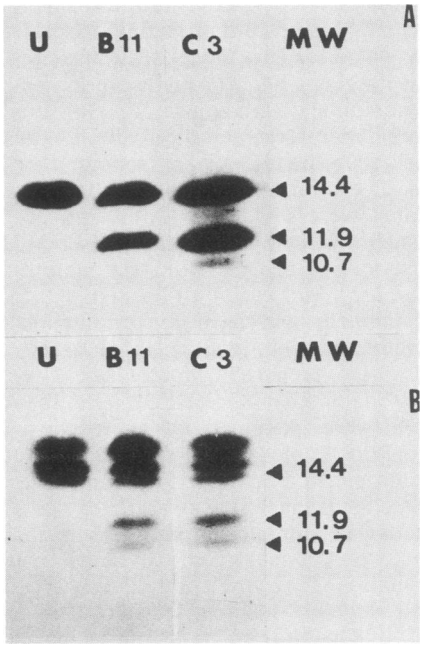

FIG. 2. Peptide recognition by mAbs on the 14K vaccinia envelope protein. The 14K protein labeled with [^{35}S]methionine was separated on 12% SDS–polyacrylamide gels from purified envelopes. The band was scissored and then digested with V-8 protease, and the cleavage fragments were separated on 12% SDS–polyacrylamide gels (4) and transferred to nitrocellulose. (A) Reacted with the mAbs and visualized by the immunoperoxidase method: unreacted (U) and reacted with the mAbs (B11 and C3). (B) Autoradiograph of the V-8 cleavage products and of the undigested (U) band. The molecular weights (MW) are given.

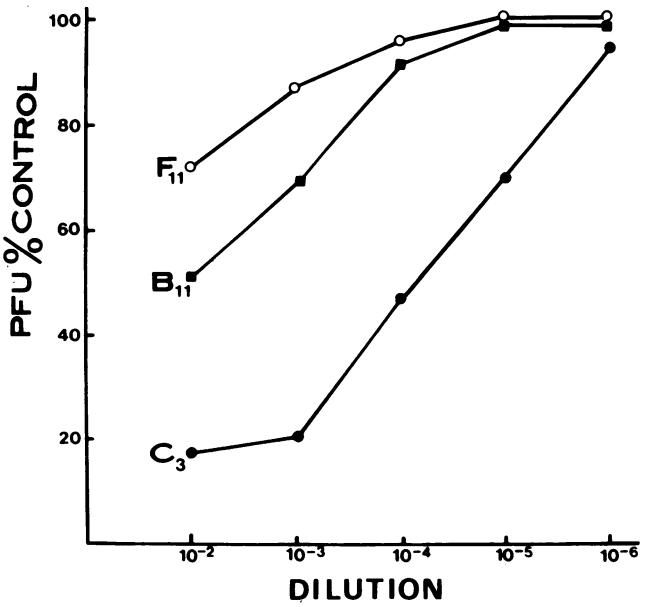

FIG. 3. Neutralization titers of vaccinia virus infectivity by mAbs. The titer of the neutralizing antibody present in ascites was measured by a plaque reduction assay against 200 PFU of vaccinia virus. Titers refer to the dilution of ascites which causes 50% plaque reduction.

irradiated with UV light (2). A small inhibitory effect (9%) on virus uncoating was observed with vaccinia virus neutralized with clone B11. However, no alterations in the degree of virus uncoating were observed with clone F11. These findings suggest that virus uncoating is the level at which vaccinia virus infectivity is inhibited by mAbs raised against the 14K envelope protein.

The 14K vaccinia envelope protein is conserved in orthopoxviruses. To assess the specificity of the mAbs and to test whether the 14K envelope protein of vaccinia virus was conserved among poxviruses, Western blots were carried out with other members of the *Orthopoxvirus* genus. The results are shown (Fig. 6) for cowpox, rabbitpox, and vaccinia virus (Elstree strain). The most significant observation is that the three mAbs recognized the same 14K protein in the different virus isolates, indicating that this protein is highly conserved. Other differences in the extent of reactivity of mABs for the 14K and 11K protein were found within the viruses, again suggesting that the three mAbs are directed against different protein domains. When the total viral proteins were stained with amido black it became clear that there are qualitative and quantitative differences in the polypeptide composition of the different virus strains. However, these differences do not correlate with changes in the extent of reactivity of mAbs for the 14K envelope protein.

DISCUSSION

In this paper we describe the isolation of three mAbs directed against vaccinia virus. All three antibodies appear to recognize different domains of the same protein. The protein has a molecular weight of about 14,000, and based on analysis of intact virus and detergent-extracted envelope components, we localized the antigen within the virus envelope.

Despite the fact that all three mAbs appear to recognize the same protein, large differences in neutralizing activity were observed among the three mAbs. C3 was found to possess the strongest neutralizing activity, producing about 50% inhibition at titers as low as 10^{-4}. In contrast, 100-fold higher titers of B11 were required to obtain similar levels of inhibition, whereas F11 at the highest titer tested produced less than 30% inhibition. The differences in neutralizing activity of the mAbs cannot be explained in terms of different affinities of the mAbs, since in radioimmunoassays against purified envelope proteins, no evidence for such differences was obtained (not shown). More likely, differences in neutralizing activity of the mAbs are related to the fact that the mAbs clearly recognized different epitopes on the 14K envelope protein. This suggestion is supported by the observation that the three antibodies exhibit different binding patterns with respect to V-8 digestion products of the 14K protein.

The mechanism by which C3 and to a lesser extent B11 inhibit vaccinia virus infection is not due to an inhibition of virus attachment to cells, ruling out the possibility that mAbs act by a simple steric hindrance mechanism. Incomplete blocking of virus infection by low dilutions of mAbs may be explained in terms either of formation of virus aggregates, which, in turn, prevent binding of mAbs to the cognate protein or of the existence of more than one route for the virus penetration into cells. Two mechanisms, phagocytosis and cell fusion, have been proposed to explain vaccinia virus

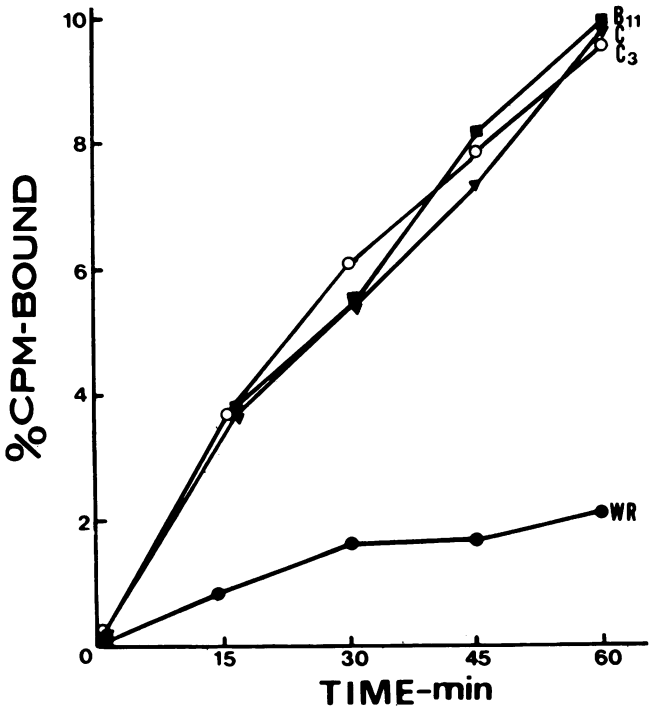

FIG. 4. Kinetics of vaccinia virus attachment to cells in the presence of mAbs. [^{3}H]thymidine-labeled vaccinia virus was incubated at 37°C for 1 h with a 1:50 dilution of mAbs. BSC-40 cells in 24 Linbro plates were infected with 2,000 particles of the virus-antiserum complex per cell. At various times postinfection, inoculum was removed; cells were washed three times with medium; 0.5 ml of 5% trichloroacetic acid was added; the monolayer was washed in ethanol, dried, and dissolved in 0.3 N NaOH–0.5% SDS; and the radioactivity was counted. The amount of radioactivity bound was determined from the input radioactivity. Vaccinia virus was incubated with mouse serum (▼), mAbB11 (■), mAbC3 (○) and rabbit anti-vaccinia virus strain WR antiserum (●).

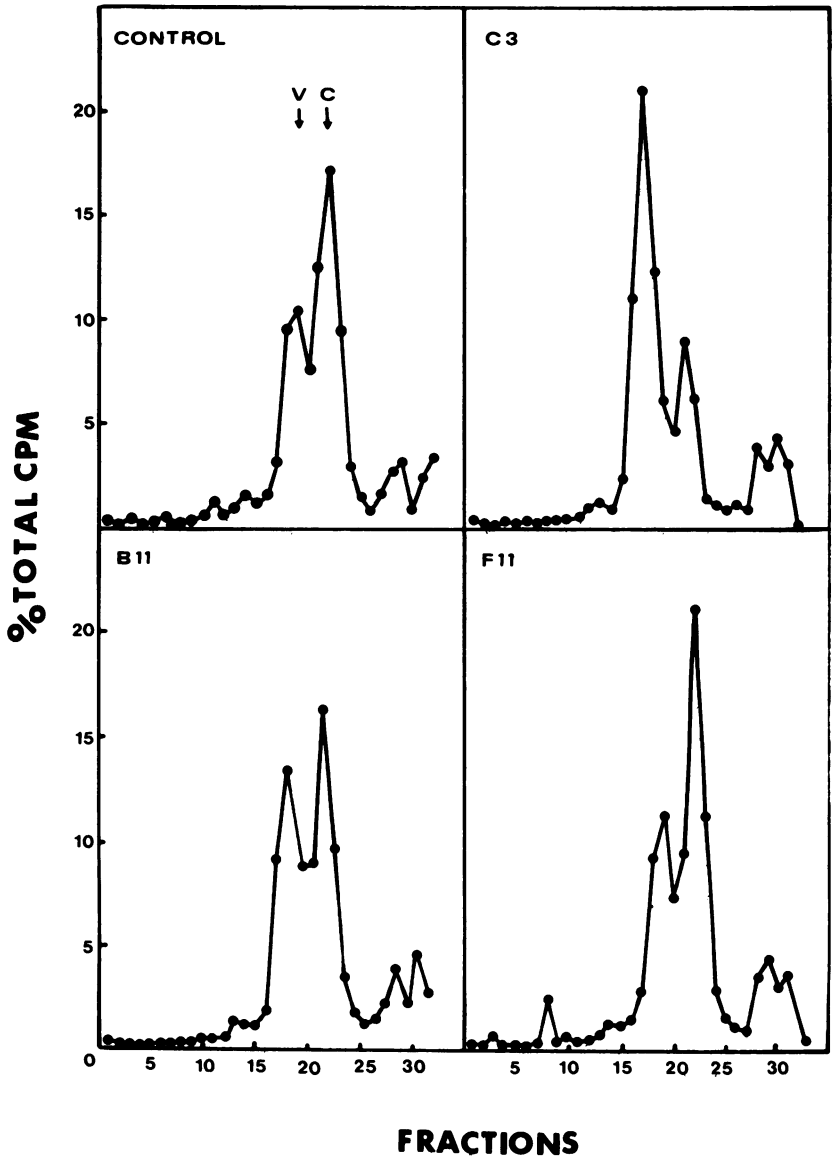

FIG. 5. Alteration of vaccinia virus uncoating into cells by mAbs. [^{3}H]thymidine-labeled vaccinia virus was incubated with normal mouse serum (control) or with the various mAbs. Conditions for virus infection and analysis of virus particles in cytoplasmic extracts by velocity sedimentation in sucrose gradients were described in Materials and Methods. The sedimentation of intact virus particles (V) and of viral cores (C) are indicated with arrows.

attachment and penetration into cells (1, 5, 6). The failure of lysosomotropic agents to inhibit vaccinia virus infectivity and the fact that in subcellular fractionation in Percoll gradients vaccinia virus does not appear in the endosome fraction strongly suggest that vaccinia penetration does not occur via an endocytic mechanism as found for other animal viruses. Instead, we found that most of vaccinia virus enters the cell by fusion with the plasma membrane (manuscript submitted for publication).

Uncoating of vaccinia virus was clearly inhibited by C3 and B11, but to different degrees, suggesting that the mAbs act by interfering with the uncoating mechanism or at some point in virus penetration prior to this step. Furthermore, these observations suggest that the 14K protein may have an important function in virus penetration. A 37K glycoprotein and an unidentified penetration protein have been suggested to be involved in virus attachment and penetration (12). A possible role of a 58K surface tubule protein also has been proposed in vaccinia virus-cell fusion during the initial phases of virus penetration (28). Although polyclonal antibodies raised against the 37K and 58K vaccinia proteins have neutralizing activity, no information was obtained on the mechanism of virus neutralization (12, 18). It is likely that virus attachment to cells is prevented by polyclonal antibodies via steric hindrance by coating the virus particle with multiple bivalent antibody molecules. After exposure of vaccinia virus to polyclonal antibodies, a large fraction of the inoculum fails to become cell associated (5, 6) as shown here (Fig. 4), whereas the particles of inoculum which are internalized fail to fuse themselves out of the endosome and become degraded in lysosomes (5, 6).

The mechanism of neutralization of virus infectivity for most animal viruses remains obscure (7). Our data imply that in a complex virus, such as vaccinia, a 14K envelope protein

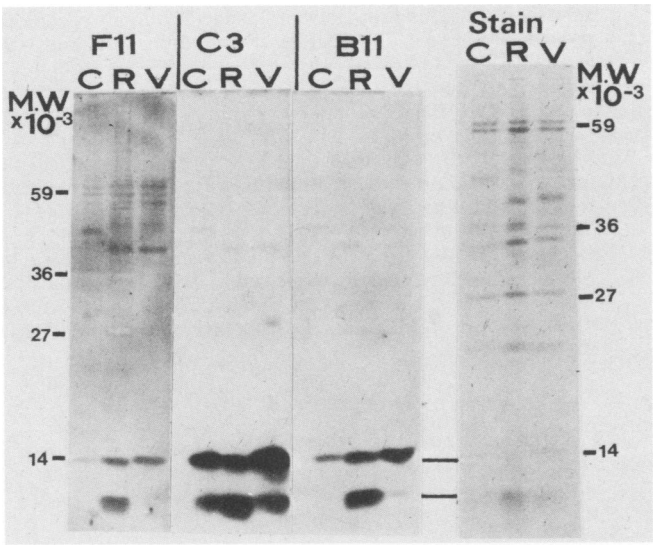

FIG. 6. Antigen specificities of mAbs for orthopoxviruses. The polypeptides of purified cowpox (C), rabbitpox (R), and vaccinia (V) Elstree strain viruses were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The nitrocellulose strips were incubated with a 1:50 dilution of mAbs, and the reacted proteins were visualized by the immunoperoxidase method. On the right are the polypeptides of the different viruses stained with amido black. The molecular weights (MW) are given for some vaccinia polypeptides.

specifically induces antibodies that can neutralize virus infectivity, and virus attachment and penetration can be dissociated as separate events in what is apparently a multistep process.

Different members of the poxvirus family can be distinguished on the basis of protein composition, genomic analysis, and immunogenicity (8, 30, 31). The observation that mAbs to the 14K protein cross-react strongly with a similar-molecular-weight protein species from cowpox, rabbitpox, and vaccinia Elstree strain suggests that this protein has been conserved in the evolution of these viruses. Furthermore, the retention of this protein on the envelope of the virus implies an important biological function for this protein.

The use of mAbs against the 14K protein will be important to unravel the mechanism of virus penetration and to identify the receptor site on the cell. Identification of a biologically functional domain of the 14K protein which confers neutralizing activity will be important to determine whether only one antibody per site is required to neutralize virus infectivity and to determine the nature of the epitope and how conserved this region is in the poxvirus group; identification also will aid in the development of live recombinant vaccinia virus as potential vaccines.

ACKNOWLEDGMENTS

We thank Tom Easton and Josef Michl for much help and stimulating discussions and Victoria Jimenez for excellent technical assistance and for graphic art.

J.F.R. was supported by a fellowship from the Consejo Superior de Investigaciones Científicas, Spain. This investigation was supported by Public Health Service grant RO216780 from the National Institutes of Health.

LITERATURE CITED

1. **Armstrong, J. A., D. H. Metz, and M. R. Young.** 1973. The mode of entry of vaccinia virus into L cells. J. Gen. Virol. **21**:533–537.
2. **Bablanian, R., G. Coppola, S. Scribani, and M. Esteban.** 1981. Inhibition of protein synthesis by vaccinia virus. III. The effect of ultraviolet-irradiated virus on the inhibition of protein synthesis. Virology **112**:1–12.
3. **Behbehani, A.** 1983. The smallpox story: life and death of an old disease. Microbiol. Rev. **47**:455–509.
4. **Cleveland, D., S. Fisher, M. Kirschner, and U. K. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252**:1102–1106.
5. **Dales, S.** 1965. Penetration of animal viruses into cells. Prog. Med. Virol. **7**:1–43.
6. **Dales, S., and B. G. T. Pogo.** 1981. Biology of poxvirus. *In* D. W. Kingsbury and J. Zur Hansen (ed.), Virology monographs, vol. 18. Springer-Verlag, Vienna. York.
7. **Dimmock, N. J.** 1984. Mechanisms of neutralization of animal viruses. J. Gen. Virol. **65**:1015–1022.
8. **Esposito, J. J., J. F. Obijeski, and J. H. Nakano.** 1978. Orthopoxvirus DNA: strain differentiation by electrophoresis of restriction endonuclease fragmented virion DNA. Virology **89**:53–66.
9. **Esteban, M.** 1984. Defective vaccinia virus particles in interferon-treated infected cells. Virology **133**:220–227.
10. **Golini, F., and J. R. Kates.** 1984. Transcriptional and translational analysis of a strongly expressed early region of the vaccinia virus genome. J. Virol **49**:459–470.
11. **Holowczak, J. A.** 1972. Uncoating of poxviruses. I. Detection and characterization of subviral particles in the uncoating process. Virology **50**:216–232.
12. **Ichihashi, Y., and M. Oie.** 1980. Adsorption and penetration of the trypsinized vaccinia virion. Virology **101**:50–60.
13. **Joklik, W. K.** 1962. The purification of four strains of poxvirus. Virology **18**:9–18.
14. **Kohler, G., and C. Milstein.** 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature (London) **256**:495–497.
15. **Mackett, M., G. L. Smith, and B. Moss.** 1982. Vaccinia virus: a selectable eukaryotic cloning vector. Proc. Natl. Acad. Sci. USA **79**:7415–7419.
16. **Mackett, M., G. L. Smith, and B. Moss.** 1984. A general method for the production and selection of infectious vaccinia virus recombinants expressed foreign genes. J. Virol. **49**:857–864.
17. **Mackett, M., F. Yilma, J. K. Rose, and B. Moss.** 1985. Vaccinia virus recombinants: expression of VSV genes and protective immunization of mice and cattle. Science **227**:433–435.
18. **Moss, B.** 1984. Principles of virus replication: poxviruses. *In* B. M. Fields, R. Chanock, R. Shope, and B. Roizman (ed.), Human viral diseases. Raven Press, New.
19. **Moss, B., G. L. Smith, J. L. Gerin, and R. H. Purcell.** 1984. Live vaccinia virus recombinant protects chimpanzees against hepatitis B. Nature (London) **311**:67–69.
20. **Panicali, D., S. W. Davis, R. L. Weinberg, and E. Paoletti.** 1983. Construction of live vaccines by using genetically engineered poxviruses: biological activity of recombinant vaccinia virus expressing influenza virus hemaglutinin. Proc. Natl. Acad. Sci. USA **80**:5364–5368.
21. **Panicali, D., and E. Paoletti.** 1982. Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene of herpes simplex virus into the DNA of infectious vaccinia virus. Proc. Natl. Acad. Sci. USA **79**:4927–4931.
22. **Paoleti, E., B. R. Lipinskas, C. Samsonoff, S. Mercer, and D. Panicali.** Construction of live vaccines using genetically engineered poxviruses: biological activity of vaccinia virus recombinants expressing the hepatitis B virus surface antigen and the herpes simplex virus glycoprotein D. Proc. Natl. Acad. Sci. USA **81**:193–197.
23. **Rondle, C. J. M., and K. R. Dumbell.** 1982. A poxvirus antigen associated with pathogenicity for rabbits. J. Hyg. **89**:383–388.
24. **Sarov, I., and W. K. Joklik.** 1972. Characterization of intermediates in the uncoating of vaccinia virus. Virology **50**:593–602.
25. **Smith, G. L., G. N. Godson, V. Nussenzweig, R. Nussenzweig, J. Barnwell, and B. Moss.** 1984. *Plasmodium knowlesi* sporozoite

antigen: expression by an infection recombinant vaccinia virus. Science **224**:397–399.
26. **Smith, G. L., M. Mackett, and B. Moss.** 1983. Infection vaccinia virus recombinants that express hepatitis B virus surface antigen. Nature (London) **302**:490–495.
27. **Smith, G. L., B. R. Murphy, and B. Moss.** 1983. Construction and characterization of an infectious vaccinia virus recombinant that expresses the influenza hemagglutinin gene and induces resistance to influenza infection in hamsters. Proc. Natl. Acad. Sci. USA **80**:7155–7159.
28. **Stern, W., and S. Dales.** 1976. Biogenesis of vaccinia: isolation and characterization of a surface component that elicits antibody suppressing infectivity and cell-cell fusion. Virology **75**:232–241.
29. **Towbin, H., T. Staelelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
30. **Turner, A., and D. Baxby.** 1979. Structural polypeptides of orthopoxvirus: their distribution in various members and location within the virion. J. Gen. Virol. **45**:537–545.
31. **Walls, H. H., D. W. Ziegler, and J. M. Nakano.** 1981. Characterization of antibodies to orthopoxviruses in human sera by radioimmunoassay. Bull. W.H.O. **59**:253–262.
32. **Wiktor, T., R. I. MacFarlan, K. J. Reagan, B. Dietzschold, P. J. Curtin, W. H. Wunner, M. P. Kieny, R. Lathe, J. P. Lecoca, M. Mackett, B. Moss, and H. Koprowski.** 1984. Protection from rabies by a vaccinia virus recombinant containing the rabies virus glycoprotein gene. Proc. Natl. Acad. Sci. USA **81**:7194–7198.