

Monoclonal antibodies specific for Hantaan virus

(hybridomas/Korean hemorrhagic fever/nephropathia epidemica)

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ABSTRACT Six hybridoma cell lines producing monoclonal antibodies to Hantaan virus were established by fusion of NS-1 mouse myeloma cells with spleen cells of mice immunized with Hantaan virus strain 76-118. The specificity of these monoclonal antibodies was established by immunoblotting analysis and immunofluorescence. Five of the clones reacted with antigens on the cell surface and in the cytoplasm, and one clone reacted with a determinant expressed only in the cytoplasm of the infected cells. Two of the clones produced antibodies that reacted with a M_r 50,000 polypeptide in virus-infected cellular extracts and purified virus preparations. The monoclonal antibodies were used to examine the antigenic relationships among Hantaan virus strains and between Hantaan virus and Prospect Hill virus and the virus of nephropathia epidemica. Three antibodies were capable of distinguishing between the Lee strain and the 76-118 strain of Hantaan virus and three additional antibodies reacted with determinants shared by both virus strains. None of the six reacted with Prospect Hill virus or the virus of nephropathia epidemica.

Hantaan virus, the etiological agent of Korean hemorrhagic fever, is a RNA virus that morphologically resembles the Bunyaviridae (1, 2). It is serologically related to the virus of nephropathia epidemica (NE) (3) and has been shown by indirect immunofluorescence to be related to a recently isolated virus designated Prospect Hill virus (PH virus) in the lungs of North American native meadow voles, *Microtus pennsylvanicus* (4). The availability of serological tests for both Hantaan virus and NE virus has led to the recognition that this recently defined group of rodent viruses is present throughout the world. Antibodies to Hantaan virus have been detected in human sera from Korea, China, the U.S.S.R., Finland, Sweden, Yugoslavia, Greece, India, Iran, Central African Republic, Bolivia, and the United States (5). The antibody patterns of reactivity to Hantaan and NE virus antigens with human sera from America, Sweden, Yugoslavia, and European Russia suggest the possibility of at least a third virus antigenic type as well as the presence of the East Asian type of virus in Europe (6). Recently, work has begun to focus on the molecular biology of the virus to define precisely the biochemical and antigenic nature of Hantaan viral structural components.[†] Using hybridoma technology, we report the derivation and characterization of several monoclonal antibodies with specificity for Hantaan virus and their use in initial studies of the antigenic relatedness of Hantaan virus and other viruses belonging to this group.

MATERIALS AND METHODS

Virus. Hantaan virus strain 76-118 isolated by Lee and Lee (7) and adapted for tissue culture by French *et al.* (8) was used to elicit monoclonal antibodies in these studies. The virus was

passaged 11 times in A549 cells and 16 times in E-6 Vero cells, and it was cloned by the plaque purification technique 3 consecutive times in E-6 cells before preparation of stock virus. Stock virus was propagated in E-6 cells. Virus-infected E-6 cells were maintained in Eagle's minimal essential medium supplemented with 2% fetal calf serum. Virus was harvested on the 12th day after inoculation.

Cell Cultures. The mouse myeloma cell line used in this study was NS-1, a nonimmunoglobulin-secreting cell line of BALB/c origin (9). NS-1 is maintained in Dulbecco's modified Eagle's medium with a high concentration of glucose (4.5 g/liter) (M.A. Bioproducts, Walkersville, MD) supplemented with 15% fetal calf serum/penicillin at 50 units/ml/streptomycin at 50 μ g/ml/1 mM sodium pyruvate/4 mM L-glutamine (Flow Laboratories, McLean, VA).

E-6 cells, a cloned line of Vero cells (CRL-1586), were obtained from the American Type Culture Collection. E-6 cells were cultivated in Eagle's minimal essential medium supplemented with 10% fetal calf serum and 2 mM L-glutamine.

Immunization of Mice. Two-month-old female BALB/c mice were immunized by footpad inoculation with 0.1 ml of a 10% suspension of Hantaan virus-infected lung emulsified in complete Freund adjuvant. The Hantaan virus-infected lung tissue was prepared from *Apodemus agrarius coreae* mice inoculated with the 28th *Apodemus* passage of the 76-118 strain of Hantaan virus. One month later the mice were inoculated intravenously with 0.1 ml of the lung preparation without adjuvant. Spleens were removed 3 days later and cell suspensions were prepared for fusion.

Production of Hybridomas. The hybridization technique used was a modification of that described by Kennett *et al.* (10). The modifications have been described in detail (11).

The heavy chain isotype of the monoclonal immunoglobulin was determined by the Ouchterlony double-immunodiffusion technique. Rabbit antisera for mouse IgM, IgG1, IgG2a, IgG2b, and IgG3 were purchased from Litton Bionetics.

Purification of Viruses. Fluid from cultures was harvested on the 12th day after infection and clarified by centrifugation at 12,000 \times g for 30 min at 4°C. The supernatant from the above step was then centrifuged at 49,000 \times g for 90 min in a Beckman type 42 rotor at 4°C. The pellet was resuspended in 0.01 M Tris-HCl/0.1 M NaCl/1 mM EDTA at pH 7.4 (TNE buffer) and was layered on a 30-50% (wt/wt) sucrose density gradient (prepared in TNE buffer) over a cushion of 1 ml of 60% (wt/wt) sucrose in TNE buffer. The sucrose gradient was centrifuged at 110,000 \times g for 18 hr in a Beckman SW 41 rotor at 4°C.

Abbreviations: NE, nephropathia epidemica; PH virus, Prospect Hill virus; IF, immunofluorescence.

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The virion buoyant density was 1.18 g/cm³. The resulting virus band was harvested and pelleted at 49,000 × g for 90 min in a Beckman type 42 rotor. Pelleted virus was resuspended in TNE buffer.

Immunofluorescence (IF). Immunofluorescent staining of Hantaan virus-infected E-6 cells was used to screen the hybridoma-derived antibodies and to assess antibody to membrane and cytoplasmic viral antigens. For membrane staining, E-6 cells infected 11 days earlier were monodispersed by trypsinization, washed with phosphate-buffered saline, and then reacted in suspension with hybridoma culture supernatants or control antiserum for 30 min at 37°C. After being washed three times, the cells were reacted with fluorescein-labeled rabbit anti-mouse IgG (heavy and light chains), rewashed three times, and examined. Staining for cytoplasmic antigens was performed with the same antibodies on Hantaan virus-infected E-6 cells that were fixed with acetone for 7 min and stained as above. Uninfected E-6 cells and infected cells incubated with fluorescein-labeled rabbit anti-mouse IgG alone or the parental NS-1 culture fluid or mouse IgG and IgM were used as negative controls. Lung tissues from sero-positive *Clethrionomys glareolus* and *M. pennsylvanicus* were employed in the indirect IF technique to demonstrate NE and PH virus antigens, respectively.

Electrophoretic Blotting Procedure. Hantaan virus-infected and uninfected E-6 cells were disrupted with 1% NaDodSO₄/1% 2-mercaptoethanol/62.5 mM Tris·HCl, pH 6.8, then electrophoresed on 10% polyacrylamide gel to resolve the polypeptides (12), and transferred electrophoretically to nitrocellulose according to the method of Towbin *et al.* (13). The electrophoretic blots were incubated with 3% bovine serum albumin in 0.15 M NaCl/0.01 M Tris·HCl, pH 7.4 (Tris/NaCl/albumin), and 0.1 mM phenylmethylsulfonyl fluoride for 1 hr at 40°C to saturate the remaining protein binding sites. The blots were incubated for 6 hr at room temperature with the monoclonal antibodies or control antiserum appropriately diluted in Tris/NaCl/albumin containing 10% fetal calf serum. After washing with Tris/NaCl/albumin the blots were overlaid with ¹²⁵I-labeled rabbit anti-mouse IgG (10⁶ cpm/ml) for 3 hr at room temperature. The blots were washed, dried, and exposed to Kodak X-Omat film.

RESULTS

The supernatants of 1,000 hybridoma cultures were tested for antibody to Hantaan virus by indirect IF. Twenty hybridomas were positive in the initial IF test and, after cloning in soft agarose, 6 of the 20 clones maintained specific antibody secretion and remained stable cell lines after several months in culture.

By using the indirect IF test and employing both acetone-fixed and unfixed Hantaan virus-infected E-6 Vero cells, the six antibody-producing clones could be divided into three groups. As shown in Table 1, groups I and II monoclonal antibodies reacted with antigens on the cell surface and in the cytoplasm of virus-infected cells; group III monoclonal antibody reacted with

a determinant expressed only in the cytoplasm of virus-infected cells. The three antibodies making up group I (24E2, 28D7, and 9G4) demonstrated pronounced discrete patches of cytoplasmic staining and a granular-to-clumped staining of the membrane of virus-infected cells (Fig. 1 *a* and *b*). The two antibodies in group II (10C5 and 25F4) produced a fine speckled fluorescent staining in the cytoplasm completely surrounding the nucleus and discrete granular membrane staining (Fig. 1 *c* and *d*). Group III antibody (6E2) gave a well-defined dot-like cytoplasmic staining pattern; the staining was often pronounced in the perinuclear area (Fig. 1*e*). Antibody 6E2 did not stain the surface of unfixed cells (Fig. 1*f*). None of the six monoclonal antibodies studied gave intranuclear staining. In comparison, the positive control rat anti-Hantaan virus antiserum gave a pattern of speckled cytoplasmic and plasma membrane staining. No fluorescence was seen when using uninfected cells.

To study Hantaan virus production, the expression of virus-specific antigens in infected cells was followed by using monoclonal antibody 24E2 (group I). Monolayers of E-6 cells were infected with 76-118 strain of Hantaan virus at a multiplicity of 0.1. Infected E-6 cell cultures were processed for examination at various intervals up to 10 days after infection. At each interval, 100 cells were scored for cytoplasmic and plasma membrane fluorescence; culture fluids were collected and virus titers measured by indirect IF. Cytoplasmic antigen was first detected 4 days after infection when 5% of the cells showed staining with antibody 24E2. Surface antigen was first detected 5 days after infection when 1% of the cells showed membrane fluorescence and 10% cytoplasmic fluorescence. The percentage of cells demonstrating cytoplasmic antigen increased to 95% by the 10th day after infection. In contrast, maximal accumulation of surface antigen was observed at 10 days when 30% of the cells gave positive IF. As shown in Fig. 2, the time of the appearance of antigen in the infected cells approximated virus production, with the highest concentration of detectable extracellular virus reached on the 9th day after infection. The intensity of staining increased with time, suggesting increased accumulation of antigen first in the cytoplasm and only later at the surface of the infected cells (Fig. 2).

Each of the six antibody-producing clones was analyzed for its immunoglobulin isotype by Ouchterlony double-diffusion analysis. Antibodies 24E2, 6E2, and 10C5 were of the IgG1 subclass, whereas 9G4, 28D7, and 25F4 were of the IgM class.

To test the fine specificity of the monoclonal antibodies in this study and to examine the antigenic differences in Hantaan virus strains, we analyzed two strains of Hantaan virus. The Lee and the 76-118 strains of Hantaan virus are indistinguishable serologically by using rat anti-Hantaan virus antiserum. The ability of the monoclonal antibodies to select out strain differences was demonstrated by IF against target cells infected with the Lee strain or the 76-118 strain of Hantaan virus. Three antibodies—10C5, 9G4, and 24F4—reacted in cytoplasmic IF with the 76-118 strain but not with the Lee strain. Antibodies 24E2, 28D7, and 6E2 were unable to define determinants unique to one strain or the other and reacted with cells infected with either strain of virus (Table 2).

Because Hantaan virus has been shown by the indirect IF test to be serologically related to NE virus and PH virus, the specificity of the Hantaan virus-derived monoclonal antibodies would be strengthened by the failure of each to react with NE virus and PH virus. Thus, when the monoclonal antibodies were reacted with NE virus in lung sections of *C. glareolus* and PH virus in lung sections of *M. pennsylvanicus* in the IF test, none of the monoclonal antibodies crossreacted with the NE and PH viruses, suggesting that these antibodies recognize antigenic determinants unique to Hantaan virus.

Table 1. Immunofluorescent staining patterns produced by anti-Hantaan virus monoclonal antibodies in infected E-6 cells

Hybridoma	Cellular location of virus antigens	
	Cytoplasm	Plasma membrane
Group I: 24E2, 28D7, and 9G4	Pronounced discrete patches	Granular to clumped
Group II: 10C5 and 25F4	Fine speckled granular	Granular
Group III: 6E2	Well-defined dots	Negative

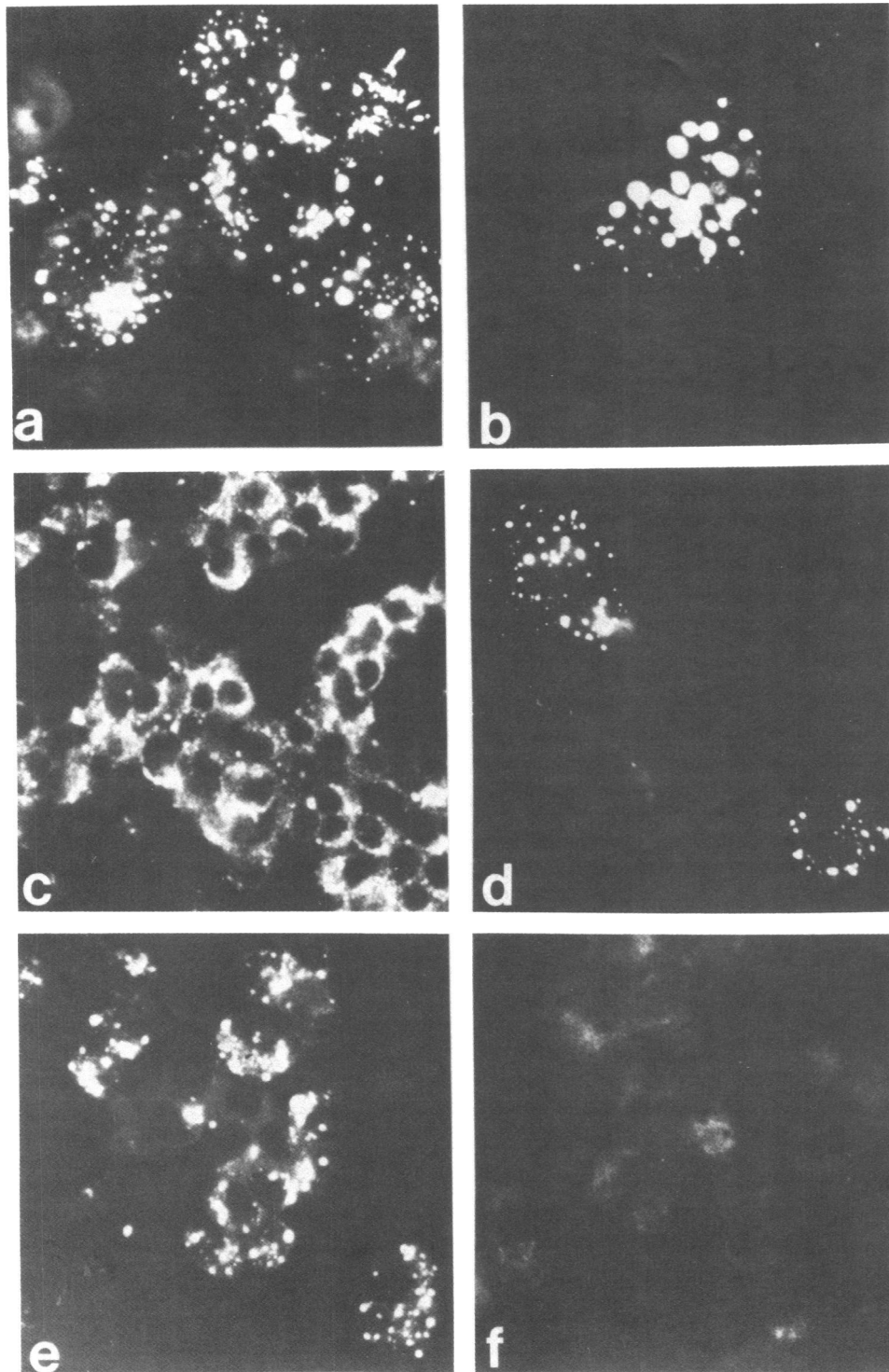


FIG. 1. Immunofluorescent staining patterns produced by anti-Hantaan virus monoclonal antibodies in infected E-6 cells. Group I antibody 24E2 reacted with antigens found in the cytoplasm of E-6 cells infected with Hantaan virus and fixed in acetone (a) and with cells stained in suspension (b). Group II antibody 25F4 stained both antigens found in the cytoplasm of acetone-fixed cells (c) and with antigens found on the cytoplasmic membrane of infected cells (d). Group III antibody 6E2 reacted with antigens found in the cytoplasm of acetone-fixed cells (e) but not with antigens expressed at the surface of infected cells (f). ($\times 390$.)

Polyacrylamide gel electrophoresis and immunoblotting analysis techniques were employed to determine the polypeptide of Hantaan virus recognized by the individual antibodies. Whole cell lysates of Hantaan virus-infected and uninfected E-6 Vero cells were separated on a 10% NaDodSO₄/polyacrylamide gel and transferred to nitrocellulose membranes to give electrophoretic blots. As shown in Fig. 3, antibodies 6E2 and 24E2 and the rat anti-Hantaan virus antiserum bound to a sin-

gle polypeptide band in infected cell lysates. The M_r of the polypeptide relative to the gel markers was 50,000. This band was not apparent in the uninfected cell lysate or in the control done with normal rat serum. However, the M_r 50,000 protein was shown to be virus specific in that antibodies 6E2 and 24E2 applied to nitrocellulose transfers of NaDodSO₄/polyacrylamide gels containing purified preparations of the virus bound to a viral protein of M_r 50,000 (Fig. 3). In these experiments

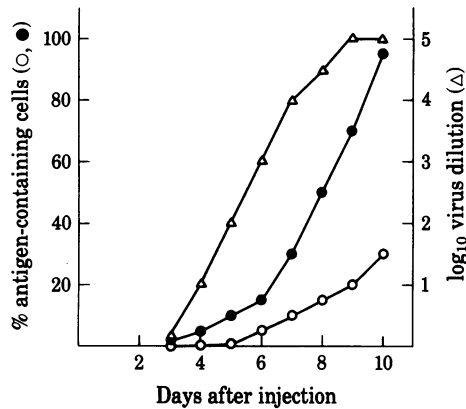


FIG. 2. Kinetics of Hantaan virus replication and expression of virus-specific antigens in infected E-6 cells. E-6 cells were infected with Hantaan virus, strain 76-118, at a multiplicity of 0.1. Virus-specific antigens were detected by IF by using monoclonal antibody 24E2. Illustrated are the percentages of infected E-6 cells showing cytoplasmic (●) and membrane (○) IF at intervals after infection, in relation to the appearance of extracellular virus (Δ).

we were unable to identify the viral polypeptides recognized by the remaining four antibodies. It is possible that these antibodies cannot recognize the antigen in its dissociated form.

DISCUSSION

The present study describes the derivation and initial characterization of six hybridoma cell lines that react with Hantaan virus, the etiological agent of Korean hemorrhagic fever. To date there have been very limited data to define the distribution and antigenic nature of the structural components of this virus. The monoclonal antibodies described herein were employed to elucidate antigenic determinants of Hantaan virus proteins. In addition, the present study provides information with respect to (i) the synthesis of the viral antigen, (ii) the kinetics of virus maturation and release, and (iii) the distribution and probable orientation of viral antigen in cellular membranes.

Monoclonal antibodies to Hantaan virus can be grouped according to the pattern of their reactivity on acetone-fixed and unfixed virus-infected E-6 Vero cells. Five of the antibodies generated reacted against antigens expressed both in the cytoplasm and at the cell surface. One hybridoma, 6E2, recognized an antigen expressed only in the cytoplasm of infected cells. The demonstration of specific cytoplasmic IF is in agreement with previous reports of intracellular localization of Hantaan virus antigen (8) and is consistent with the site of virus replication for Bunyaviruses (14). Furthermore, the observation of viral antigen first in the cytoplasm at 4 days after infection, later followed by detection of viral antigen in the plasma membrane and accumulation of extracellular virus, is consistent with the sequence of virus maturation described for Bunyaviruses, which consists essentially of virus budding into the Golgi cisternae

Table 2. Reactivity of six hybridoma antibodies to the 76-118 and the Lee strains of Hantaan virus

Hybridoma	Hantaan virus strain*	
	76-118	Lee
24E2, 28D7, and 6E2	+	+
10C5, 9G4, and 25F4	+	-

* E-6 cells were infected with 76-118 strain or Lee strain of Hantaan virus. Virus-specific antigens were detected by the IF test.

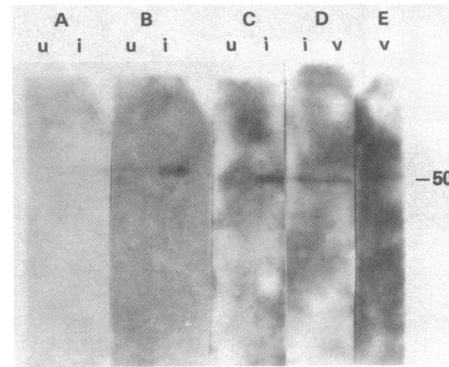


FIG. 3. Autoradiographs of immunoblots showing a Hantaan virus-specific polypeptide. Autoradiographs of nitrocellulose blots of electrophoretically separated polypeptides from uninfected E-6 cells (u), Hantaan virus-infected E-6 cells (i), and purified Hantaan virus (v). Immunoblots were processed with normal rat serum (A), rat anti-Hantaan serum (B), monoclonal antibody 24E2 (C and E), and monoclonal antibody 6E2 (D). $M_r \times 10^{-3}$ is indicated on the right.

and migrating to the plasma membrane (14, 15).

It has been reported that several RNA viruses, such as measles (16), lymphocytic choriomeningitis virus (17), and Venezuelan encephalitis (18), have intracellular localization of their envelope and nucleocapsid proteins. During virus maturation, there is a subsequent migration of these proteins to the cell membrane. In contrast to envelope proteins, nucleocapsid proteins have not been observed on the external surface of the infected cell membrane. In this study, five of the six clones produced antibodies that detected viral antigens on the cell membranes of infected cells. Because we are using monoclonal antibodies, the presence of antibodies to other viral or cellular antigens can be excluded. These results suggest the antibodies produced by the five clones are detecting an envelope protein of Hantaan virus, and antibody 6E2, which reacted with an antigen expressed only in the cytoplasm, is recognizing the viral nucleocapsid protein.

Viral-specific proteins in Hantaan virus-infected cells and purified virus particles were identified by the immunoblotting assay. The viral antigens recognized by antibodies 6E2 and 24E2 and rat anti-Hantaan virus antibody correlate to proteins of $M_r \approx 50,000$. Although these data are not complete, the IF staining results indicate the antigenic determinants characteristic of both 6E2 and 24E2 antibodies are located on two polypeptides of similar mobilities, which are not resolved by standard discontinuous polyacrylamide gel electrophoresis and by the immunoblotting technique.

The monoclonal antibodies proved capable of distinguishing between two strains of Hantaan virus: the Lee strain of Hantaan virus isolated from a human patient and 76-118 strain of Hantaan virus isolated from the rodent *Apodemus agrarius coreae*. Lee and 76-118 strains of Hantaan virus are indistinguishable serologically by using conventional rat anti-Hantaan virus antisera. Among the six antibodies, we found three that recognized antigens expressed on both virus strains. This finding implies the conservation of certain viral antigenic determinants during host adaptation.

NE virus and PH virus are antigenically related viruses that are distinguishable serologically from the Hantaan virus (3, 4). Although it was hoped that the monoclonal antibodies to Hantaan virus would clarify to an extent the relationship among these viruses, the antigenic determinants recognized by the monoclonal antibodies were not found on NE virus and PH virus. However, it is difficult to draw inferences on antigenic disparities when examining only a few monoclonal antibodies.

Antibodies 24E2 and 28D7 were reactive with both strains of Hantaan virus; antibody 24E2 was found by immunoblotting analysis to be directed against a determinant on a polypeptide of $M_r \approx 50,000$. If the determinants recognized by antibodies 24E2 and 28D7 are in fact located on the same viral protein, the results suggest that antibodies 28D7 and 24E2 bind to a group-specific portion of the polypeptide. Additional characterization of the remaining three antibodies is needed to determine if there is an invariant sequence of the M_r 50,000, which carries group-specific determinants as well as variable sequences responsible for type-specific determinants.

At present, we do not know the exact epitopes of the monoclonal antibodies. Our results do show that these antibodies react specifically with Hantaan virus and can identify structural characteristics of the virus as well as serve as immunological reagents in Hantaan virus serology.

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