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Antigenic Analysis of Punta Toro Virus and Identification of Protective Determinants with Monoclonal Antibodies[1,2]

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Hybridomas producing monoclonal antibodies to the three major structural proteins of Punta Toro virus (PTV) were established by fusion of spleen cells with Sp2/0-Ag-14 mouse plasmacytoma cells. Thirty-six independently derived monoclonal antibodies were evaluated in neutralization, hemagglutination inhibition, and ELISA assays and the isotype, antigen specificities, and cross-reactivities were determined. These antibodies were also assessed for their ability to provide protection in a murine model. Both G1- and G2-specific antibodies were obtained which neutralized virus infectivity *in vitro* and inhibited hemagglutination, whereas nucleocapsid-specific antibodies exhibited neither activity. All of the anti-G1 antibodies were PTV-specific, whereas anti-G2 and anti-nucleocapsid antibodies exhibited varying patterns of cross-reactivity with heterologous phleboviruses. All of the G1-reactive monoclonal antibodies, which bound to epitopes in two distinct topological sites as determined by competitive binding assays, provided efficient protection to both immunocompetent and immunosuppressed mice. In contrast, of the 23 G2-reactive antibodies, only 8 were able to protect immunocompetent mice and only one was able to protect immunosuppressed animals. The degree of protection achieved *in vivo* did not correlate directly with the neutralization titers determined *in vitro*.

INTRODUCTION

Punta Toro virus (PTV) is a member of the *Phlebovirus* genus of the Bunyaviridae family (Bishop and Shope, 1979; Sather, 1970; LeDuc *et al.*, 1982). This genus is currently composed of 38 viruses which have been grouped on the basis of serologic cross-reactions as well as morphologic and biochemical similarities, but which are distinguishable by neutralization tests (Bishop *et al.*, 1980; Casals, 1971; Tesh *et al.*, 1986; Travassos da Rosa, 1983). Phleboviruses have been isolated throughout much of the Old and the New World, and at least eight members of this genus, including PTV, have been associated with febrile disease in humans.

As is the case for other members of the Bunyaviridae family, the genome of PTV is composed of three, unique, single-stranded RNA segments. The 90-nm virus particle is enveloped and contains the genomic RNA complexed with a nucleocapsid protein of 26,000 Da. Associated with the envelope are two glycoproteins, G1 and G2, of 66,000 and 56,000 Da, respectively (Robeson *et al.*, 1979; Ihara *et al.*, 1984). As monitored by negative staining and thin section electron microscopy, these glycoproteins are constructed into closely packed, cylindrical subunits on the virion surface (Smith and Pifat, 1982). In addition to the three major structural proteins, relatively small amounts of a large polypeptide (~200,000 Da) are detected in purified virions, and it is assumed that this protein serves the required polymerase function. Both the S and M genomic RNA segments of PTV have been cloned and sequenced (Ihara *et al.*, 1984, 1985a) and the genetic organization of these two segments has been found to differ markedly. While the M segment, which codes for G1, G2 and a presumptive nonstructural protein (NS_m), was found to be of negative polarity, the S segment, which codes for the nucleocapsid protein and a presumptive nonstructural protein (NS_s), was found to be organized in an ambisense coding strategy (Ihara *et al.*, 1985b). Although the gene coding assignments for these proteins have been defined, the proteins which carry determinants responsible for eliciting neutralizing, hemagglutinin inhibiting, protective, group- and type-specific antibodies remain, for the most part, unknown (Dalrymple *et al.*, 1982). In the studies described here, monoclonal antibodies to the three major structural proteins of PTV were produced and characterized with respect to their antigen specificity, their biological activities, and their reactivity with heterologous pleboviruses. We have recently described an animal model in C57BL/6J mice for PTV infections (Pifat

[1] In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by Committee on Care and use of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

[2] The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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and Smith, 1987), and this model system was used to identify those monoclones capable of protecting otherwise lethally infected mice. Those antibodies found to be protective were further analyzed in competition binding assays to determine the number of distinct topological sites recognized by these antibodies.

MATERIALS AND METHODS

Growth and purification of PTV

The Adames strain of PTV was propagated in Vero cell monolayers, labeled with [^{35}S]-methionine, concentrated by ammonium sulfate precipitation, and purified using sucrose gradients as described previously (Smith and Pifat, 1982; Ihara *et al.*, 1985a).

Preparation of viral glycoproteins and immunization of Balb/cJ mice

Eight-week-old male Balb/cJ mice were infected subcutaneously with 4000 plaque-forming units (PFU) of PTV. After 4 weeks those animals which exhibited the highest plaque reduction neutralization titers (PRNT) were given a secondary intravenous immunization consisting of either gradient-purified, γ-irradiated PTV or purified viral glycoproteins. Purified glycoprotein and nucleocapsid preparations were obtained by the disruption of 2.8 mg of purified virus with 2% octylglucoside (Calbiochem, La Jolla, CA) in TNE (0.1 *M* NaCl, 0.01 *M* Tris, 0.001 *M* EDTA, pH 7.2). This lysate was layered over a discontinuous CsCl gradient consisiting of 2.5 ml of 35% (w/w) CsCl and 4.5 ml of 20% (w/w) CsCl, and centrifuged at 15° for 4 hr at 35,000 rpm in a Beckman SW41 rotor. Nucleocapsid complexes banded at the 35–20% interface and were not detectably contaminated with viral glycoproteins as monitored by SDS–polyacrylamide gel electrophoresis. Supernatant fractions above the 20% CsCl interface, which contained both G1 and G2, were dialyzed against TNE and concentrated by ultrafiltration.

Production of anti-PTV hybridomas

Three days after secondary immunization, mice were anesthetized and spleens were processed as described previously (Earley and Osterling, 1986). Spleen cells from three mice were processed independently, and the procedure for cell fusion with Sp2/0-Ag14 myeloma cells (Shulman *et al.*, 1978) was essentially that described by Kennett *et al.* (1980) and Earley and Osterling (1986). The hybridoma cultures were incubated at 37° with several changes of HAT medium, and the supernatant fluids were screened by ELISA for the presence of anti-PTV antibodies. Selected hybridoma cultures were cloned by limiting dilution using Balb/cJ mouse peritoneal macrophages as feeder layers. All monoclonal antibodies in which the designator is preceded by I or III were produced from animals boosted with purified virions, whereas those preceded by II were obtained from an animal boosted with precipitated viral glycoproteins. In addition, five monoclonal antibodies reactive with PTV glycoproteins were prepared against the prototype Balliet strain of PTV (ATCC VR-559) using the P3X63Ag-8 fusion partner (Dalrymple *et al.*, 1982); their designators are preceded by "A." Ascitic fluids were produced in adult Balb/cJ mice using standard procedures.

Enzyme-linked immunoassay

Hybridoma cultures were screened for anti-PTV antibody using an enzyme-linked immunosorbant assay (ELISA) with gradient-purified PTV (400 ng per well) or purified nucleocapsid antigen, as described previously (Pifat and Smith, 1987).

Plaque reduction neutralization test (PRNT)

This test was performed in Vero cell monolayers, essentially as described by Earley *et al.* (1967) with a constant virus inoculum treated with varying dilutions of monoclonal ascitic fluids. The titers are expressed as the reciprocal of the highest antibody dilution resulting in either 80 or 50% plaque reduction.

Hemagglutination inhibition (HI) test

This procedure utilized a modification of the method described by Clark and Casals (1955). Purified virus was used as the source of viral hemagglutinin and ascitic fluids were extracted twice with acetone. The test was performed at the predetermined optimum pH for the Adames strain of PTV (pH 6.2). Antigen–antibody mixtures were incubated overnight at 4°, mixed with gander erythrocytes, and the plates were incubated at 37° for 60 min.

Determination of antibody class and isotype

Antibody class and isotype were determined by the ELISA procedure described above except that rabbit antisera specific for mouse heavy and light chains were used (Boehringer-Mannheim, Indianapolis, IL) and detected with a peroxidase-labeled goat anti-rabbit IgG.

Specificity determination by immunoprecipitation

Radiolabeling of viral antigens, immunoprecipitation, and SDS–polyacrylamide gel electrophoresis were carried out as previously described (Smith and Pifat, 1982). Immunoprecipitates with monoclonal antibodies which were not bound by protein A were collected on agarose beads containing goat anti-mouse immunoglobulins (Cappel Laboratories, Malvern, PA).

Indirect immunofluorescence assay (IFA)

Confluent Vero cell monoloayers were infected with heterologous phleboviruses, incubated for 48 hr at 37°, harvested, and mixed with an equal number of uninfected cells which served as an internal control. Cell suspensions were spotted on Teflon-masked glass slides, air dried, and fixed with acetone at −20°. Individual monoclonal ascitic fluids diluted 1:100 were reacted with the fixed cells and incubated for 30 min at room temperature in a humidified chamber. The slides were then washed for 30 min in phosphate-buffered saline, pH 7.4 (PBS), and overlaid with fluorescein-conjugated, goat anti-mouse immunoglobulin. After 30 min the slides were again washed in PBS and mounted in carbonate-buffered glycerol.

Passive immunization with monoclonal antibodies

As an initial identification of protecting antibodies, a single large dose of 0.2 ml of undiluted ascitic fluid was administered intraperitoneally to normal or immunosuppressed 4-week-old C57BL/6J mice, 24 hr after virus challenge. Immunosuppression was achieved by irradiation with a Gamma Cell 40 (Atomic Energy of Canada, Ottawa, Canada), which was used to deliver 750 rads, 4 hr prior to virus challenge. Irradiated mice were housed in sterilized cages and were given sterile water. In all cases the virus challenge consisted of 4000 PFU of the Adames strain of PTV (approximately 200 LD_{50}) given subcutaneously.

Those monoclonal antibodies which were found to have a protective effect in both immunocompetent and immunosuppressed animals were purified by affinity chromatography using protein A-Sepharose (Sigma, St. Louis, MO). Four-week-old C57BL/6J mice were passively immunized with 1 to 100 μg of purified monoclonal antibody given 24 hr after challenge. Animals were challenged subcutaneously as described above and monitored for illness or death for 30 days.

Labeling of monoclonal antibodies with [^{35}S]methionine

Selected monoclonal antibodies which were found to be protective were metabolically labeled with [^{35}S]-methionine as described by Yelton *et al.* (1981).

Competitive binding assays

^{35}S-labeled monoclonal antibodies were utilized in competitive binding assays after the appropriate dilution required to saturate the binding sites of a standard antigen preparation (400 ng of gradient-purified PTV per well) was determined. Unlabeled monoclonal antibodies (as ascitic fluids) were diluted 10^{-1} to 10^{-7} and allowed to react for 1 hr at 37°. Negative controls consisted of equivalent dilutions of normal ascitic fluid prepared with SP2/0 cells. Plates were drained and subsequently incubated with 50 μl of the appropriate dilution of ^{35}S-labeled monoclonal antibody. Following 1 hr at 37°, the plates were washed with BBS containing 0.5% Tween 80, and individual wells were excised from the plate and counted in a scintillation counter. The competing titer of each monoclone was calculated as the last dilution of unlabeled ascitic fluid to cause a 50% reduction in bound radioactivity. The radioactivity bound with ^{35}S-labeled PTV-specific monoclones varied between 35,000 and 99,000 cpm per well compared with 400 cpm obtained with a similarly labeled heterologous (Sindbis-specific) monoclonal antibody.

RESULTS

Production, screening, and selection of hybridomas

From twenty 96-well plates initially seeded, 254 wells were found to contain antibodies reacting with virion antigens in ELISA assays. Media fractions from these hybridomas were subsequently assayed for the presence of neutralizing antibodies and for antibodies reactive with purified PTV nucleocapsid complexes. All hybridomas secreting neutralizing antibodies, or antibodies reacting with purified virions but not with nucleocapsid antigens, were selected for subcloning, expansion, and ascitic fluid production. Several hybridomas secreting anti-nucleocapsid antibodies were similarly processed. A total of 36 independently derived hybridomas were selected, and multiple clones of each parent hybridoma were expanded for ascites production.

Antigen specificity, isotype, and binding characteristics of anti-PTV monoclonal antibodies

Immunoprecipitation of [^{35}S]-methionine-labeled, PTV-infected cell lysates revealed that 23 of the monoclonal antibodies reacted with the G2 (56K) surface glycoprotein, while four reacted with the G1 (66K) glycoprotein. Nine hybridomas with demonstrable anti-nucleocapsid activity in screening assays were subcloned and their expected specificity was confirmed by immunoprecipitation as above. Monoclonal antibodies were not obtained which reacted with the large, 200,000-Da protein, nor were any monoclones obtained which reacted with presumptive nonstructural proteins. Similarly, none of these monoclones precipitated polypeptides of higher molecular weight than the

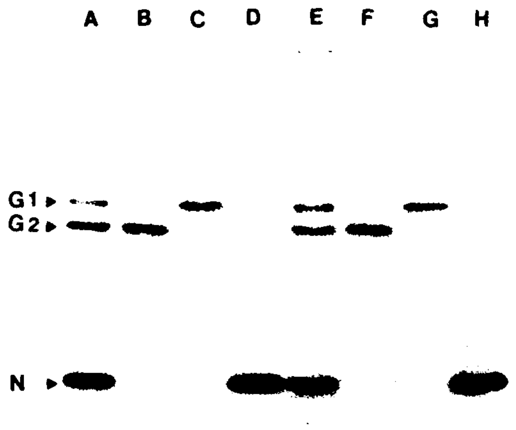

FIG. 1. Immunoprecipitation of PTV polypeptides with polyclonal and monoclonal antibodies. The antigen specificity of PTV-reactive monoclonal antibodies was determined by immunoprecipitation of PTV-polypeptides from ^{35}S-labeled, infected Vero cell lysates. Lanes A and E show the polypeptides immunoprecipitated with polyclonal hyperimmune mouse ascitic fluid. Lanes C and G, B and F, and D and H are aliquots of the same cell lysate immunoprecipitated with G1, G2, and nucleocapsid-reactive monoclones, respectively.

mature structural proteins and identifiable as potential precursor molecules, although the labeling conditions used in these experiments would likely preclude the detection of rapidly processed species. The specificity of the immunoprecipitation procedures, as analyzed on 13% polyacrylamide gels, is shown in Fig. 1, and the antigen specificity of each monoclonal antibody is listed in Tables 1–4. These tables also list the isotype of the monoclones as determined in ELISA assays with isotype and class-specific rabbit antisera.

The ELISA titers obtained under conditions of antigen excess varied between 1×10^3 and 1.6×10^5, and were independent of the antigen specificity or the isotype of the monoclones (Tables 1–4). Approximate antibody concentrations present in IgG2a or IgG2b monoclonal ascites were determined by preparative protein A-affinity chromatography and were found to vary between 0.48 and 4.6 mg of immunoglobulin per milliliter of ascitic fluid. The much wider variation in end point ELISA titers of these same ascites suggests that a substantial range of affinities is represented by this panel of antibodies.

All monoclones were tested for cross-reactivity with 35 heterologous phleboviruses. Twenty-five of the antibodies were found to be reactive with at least one phlebovirus other than PTV (Fig. 2), while 16 were found to be PTV-specific (not shown). All five of the G1-reactive monoclones were PTV-specific. Among cross-reactive antibodies, five reacted with the nucleocapsid protein, while all others monitored determinants on the G2 glycoprotein. However, the cross-reacting, nucleocapsid-specific monoclones each reacted with a single heterologous phlebovirus, whereas many of the G2-reactive monoclonal antibodies were broadly cross-reactive. Among the 20 G2-specific antibodies, none exhibited the same pattern of cross-reactivity. One monoclone (5F3) reacted with all but one of the viruses belonging to the subgroup of viruses of which Punta Toro is a member (Bishop *et al.*, 1980), and in general, cross-reactions were much more frequent among viruses in this subgroup. Of the 36 phleboviruses tested, 31 were recognized by at least one of the antibodies used in this study. Cross-reactivity patterns did not discriminate Old and New World phlebovirus isolates (Tesh *et al.*, 1982).

Biological activities of PTV-reactive monoclonal antibodies

Neutralization. All five monoclonal antibodies reactive with G1 were found to exhibit 80% plaque-reduction titers (Table 1). Ten of the 23 G2-reactive monoclones also exhibited 80% endpoint titers (Table 2), whereas 17 additional G2-specific monoclones demonstrated 50% endpoint titers only (Table 3). The 80% neutralization titers varied between 40 and 2560 for G1-reactive antibodies and between 0 and 640 for the G2-reactive antibodies. Antibodies reacting with determinants on the nucleocapsid protein showed no demonstratable neutralization activity (Table 4).

Hemagglutination inhibition. With one exception (I 4C6), all glycoprotein-reactive monoclonal antibodies had demonstrable HI activity. Therefore, the serological assay which has been used to classify viruses as members of the *Phlebovirus* genus monitors multiple determinants on both glycoproteins (Tables 1–3). The HI titers of the G1-reactive monoclones varied between 640 and 2560 (Table 1), while the titers for the G2-reactive monoclones varied between 10 and 10,240 (Ta-

TABLE 1

CHARACTERISTICS OF G1-REACTIVE MONOCLONAL ANTIBODIES

Designator	ELISA titer	Neutralization titer 80%	Neutralization titer 50%	HI titer	Isotype
II 5F9-2-3	80,000	2560	>10,240	1280	IgG2a, k
II 2F9-2	160,000	640	>10,240	1280	IgG2a, k
A. 7F5	10,000	640	2,560	1280	IgG2a, k
III 1D10-1	40,000	40	2,560	640	IgG2b, k
I 3E8	160,000	2560	>10,240	2560	IgG1, k

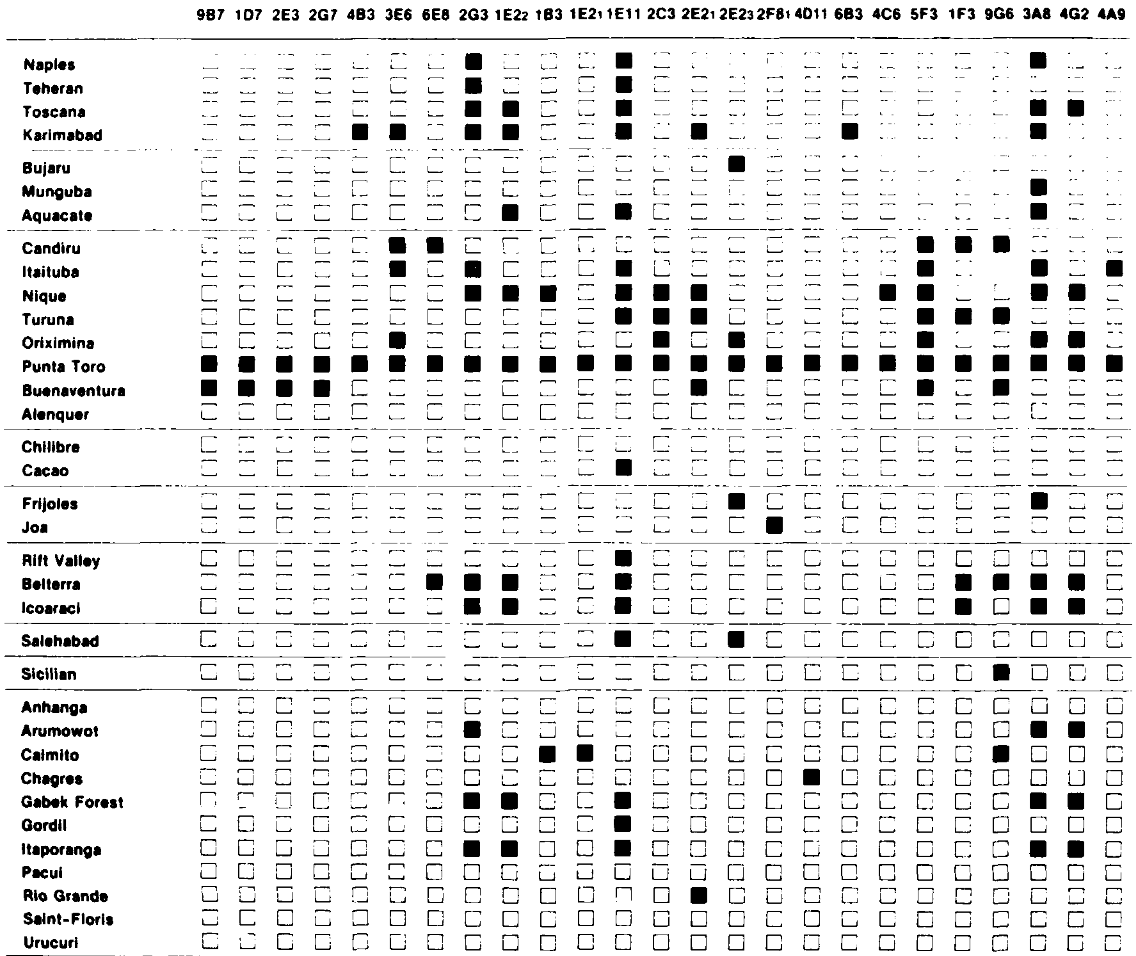

FIG. 2. Cross-reactivity of PTV-reactive monoclonal antibodies with heterologous phleboviruses. Confluent Vero cell monolayers were infected with individual phleboviruses, and antigen slides were prepared by mixing (1:1) infected and uninfected cells. Individual monoclonal ascitic fluids were diluted 1:100 and tested for their reactivity with each phlebovirus in indirect immunofluorescence assays. Strongly positive (■) or negative (□) fluorescence was observed, with essentially no intermediate reactions. Horizontal bars reflect subgroups as described by Bishop *et al.* (1980).

bles 2 and 3). None of the nucleocapsid-reactive monoclones showed evidence of HI activity (Table 4).

Passive transfer of monoclonal antibodies to immunocompetent PTV-infected mice. Passive immunization with polyclonal PTV-specific antisera has been shown previously to protect mice from lethal infection (Pifat and Smith, 1987). To identify the specific viral antigens carrying protective determinants, monoclonal antibodies were administered to immunocompetent, 4-week-old C57BL/6J mice 24 hr after an otherwise lethal virus challenge. Postinfection administration ensured that target organs were infected. All of the G1-reactive monoclones provided efficient protection (Table 5). In addition, most of the neutralizing G2-specific monoclones also provided significant protection (Table 6). However, the IgM monoclonal antibody, 4G11, which possessed a high *in vitro* neutralization titer (1:640), protected only 20% of the animals, and provided only a slight delay in the mean time to death when compared to mice receiving virus alone. In contrast, the monoclone 6E8, which had very low neutralization activity (1:10), protected all mice from an otherwise lethal virus challenge.

Although these studies were conducted with unfractionated ascitic fluids, there was no indication that nonimmunoglobulin components contributed to the protection observed. Twenty-three other ascitic fluids containing nonneutralizing monoclonal antibodies reactive either with G2 or the nucleocapsid proteins, or normal ascitic fluids produced with Sp2/0 myeloma cells, were tested as above and were found to provide no protection (not shown). In addition, the monoclonal antibodies 7F5 (G1-specific) and 9G2 (G2-specific) were purified by protein A-Sepharose affinity chromatography,

TABLE 2

CHARACTERISTICS OF NEUTRALIZING[a] G2-REACTIVE MONOCLONAL ANTIBODIES

Designators	ELISA titer	Neutralization titer 80%	Neutralization titer 50%	HI titer	Isotype
II 4G11	5,000	640	>10,240	5120	IgM, k
A. 9G6	10,000	640	2,560	1280	IgG2a, k
II 3E6	15,000	160	>10,240	2560	IgG2a, k
I 1E11	30,000	160	2,560	10240	IgG2a, k
II 1F3	80,000	160	2,560	320	IgG2a, k
II 1B5	40,000	160	2,560	40	IgG2a, k
I 2F8-1-5	160,000	40	>10,240	2560	IgG2a, k
I 2E2-3	40,000	40	640	1280	IgG2a, k
I 2F8-1-1	40,000	10	640	2560	IgG2a, k
II 6E8	40,000	10	160	1280	IgG2a, k

[a] Defined as 80% plaque-reduction neutralization.

TABLE 3

CHARACTERISTICS OF NONNEUTRALIZING[a] G2-REACTIVE MONOCLONAL ANTIBODIES

Designators	ELISA titer	Neutralization titer 80%	Neutralization titer 50%	HI titer	Isotype
II 5F3	10,000	<10	2,560	640	IgM, k
II 2F9-3	80,000	<10	>10,240	2560	IgG2a, k
I 2G3	20,000	<10	2,560	2560	IgG2a, k
II 1E2	2,500	<10	160	320	IgG2a, k
I 1E2	30,000	<10	160	320	IgG2a, k
I 2E2-1	20,000	<10	160	320	IgG2a, k
A. 4G2	1,000	<10	160	80	IgG2a, k
I 1B3	30,000	<10	40	320	IgG2a, k
II 5F7	20,000	<10	10	10	IgG2a, k
I 2C3	40,000	<10	10	320	IgG2a, k
I 6B3	10,000	<10	<10	10	IgG2a, k
I 3D5	1,600	<10	<10	<10	IgG2b, k
A. 3A8	2,000	<10	160	640	IgG1, k
A. 4A9	4,000	<10	40	320	IgG1, k
I 4D11	10,000	<10	40	320	IgG1, k
II 4C6	80,000	<10	40	<10	IgG1, k
I 2G5	20,000	<10	<10	1280	IgG1, k

[a] Defined as 80% plaque-reduction neutralization.

were found to elute in a single peak by low-pressure cation exchange chromatography and were found to protect all mice in doses of less than 1 μg per animal (data not shown).

Passive transfer of monoclonal antibodies to immunocompromised PTV-infected mice. To determine whether these monoclonal antibodies in and of themselves were capable of providing protection, or whether protection required an intact endogenous immune response, 4-week-old animals were immunosuppressed with whole-body irradiation prior to infection and passive immunization. The mean survival time of animals receiving radiation alone was 15 days, whereas the mean survival time of irradiated and infected animals was 4 days (Tables 5 and 6).

As was seen in immunocompetent animals, the G1-reactive monoclonal antibodies provided substantial or complete protection in immunosuppressed recipients with mean time to death equivalent to that seen in animals receiving radiation only. However, most of the G2-specific monoclones which protected immunologically intact animals failed to provide protection to immunosuppressed animals. Some protection and delay in death was observed with 9G6, 2F8-1-5, and 2E2-1. However, the most efficient protection with G2-specific monoclones was observed in mice receiving the monoclonal antibody 6E8, which exhibited one of the lowest *in vitro* neutralization titers. All other nonneutralizing, G2-reactive monoclones described previously, and several selected nucleocapsid-reactive monoclones, were tested in these studies and were found to provide no protection (data not shown).

Competitive binding assays. Ten monoclones which either provided protection or longer survival times when passively transferred to immunosuppressed, PTV-infected mice, were analyzed in competitive binding assays (Table 7). By this definition, protective epitopes were found to reside in at least two distinct topological sites on the G1 glycoprotein and at least three topological sites on the G2 glycoprotein. All monoclonal antibodies defining a given topological site were reciprocally competitive. No competition was observed among antibodies binding to different glycoproteins.

TABLE 4

CHARACTERISTICS OF NUCLEOCAPSID-REACTIVE MONOCLONAL ANTIBODIES

Designators	ELISA titer	Neutralization titer 80%	Neutralization titer 50%	HI titer	Isotype
II 1D7	160,000	<10	<10	<10	IgG2a, k
II 5F9-2-1	160,000	<10	<10	<10	IgG2a, k
II 2E3	80,000	<10	<10	<10	IgG2a, k
II 2G7	80,000	<10	<10	<10	IgG2a, k
II 4B3	40,000	<10	<10	<10	IgG2a, k
III 1D10-2-2	40,000	<10	<10	<10	IgG2a, k
II 3C4	20,000	<10	<10	<10	IgG2a, k
III 3D4	40,000	<10	<10	<10	IgG2b, k
I 9B7	80,000	<10	<10	<10	IgG1, k

TABLE 5

PASSIVE IMMUNIZATION WITH G1-REACTIVE MONOCLONAL ANTIBODIES

Monoclone[a]	Normal mice[b] protected/total	Mean day to death	Range	Irradiated mice[c] protected/total	Mean day to death	Range
I. 7F5	9/9	—	—	10/10	—[d]	—
II 5F9-2-3	10/10	—	—	9/10	6	6
II 2F9-2	10/10	—	—	9/10	6	6
I 3E8	10/10	—	—	7/10	6.3	6–7
III 1D10-1	10/10	—	—	10/10	—	—
PTV alone	0/10	3.9	3.5	0/10	4.3	4–5

[a] Undiluted ascitic fluid (0.2 ml) was transferred 24 hr after viral challenge.
[b] Four-week-old C57BL/6J mice were inoculated subcutaneously with 4000 PFU of PTV.
[c] Four-week-old C57BL/6J mice were irradiated (750 rads) 4 hr prior to PTV inoculation. Mean survival time of animals receiving radiation alone was 15 days.
[d] Survival equivalent to animals receiving radiation alone.

DISCUSSION

In this study we produced and characterized 36 monoclonal antibodies to the major structural proteins of PTV, a virus which has previously served as a prototype phlebovirus to study morphological (Smith and Pifat, 1982), biochemical (Ihara *et al.*, 1985a) and pathological (Pifat and Smith, 1987) properties of the *Phlebovirus* genus. The characterization of this group of monoclones allowed us to define certain aspects of the antigenic structure of PTV-coded proteins as well as to identify at least some of the determinants involved in protection and immunity.

A previous effort to generate monoclonal antibodies to PTV antigens yielded 99 PTV-reactive monoclonal antibodies of which 94 reacted with the nucleocapsid polypeptide, 4 with the G2 glycoprotein, and a single antibody was found to be G1-specific (Dalrymple *et al.*, 1981). In the present study, immunization and screening procedures were designed to increase the yield of glycoprotein-reactive monoclones. Although this was successful in that 27 glycoprotein-specific monoclones were obtained, most of the positive hybridomas nonetheless secreted antibodies directed at the nucleocapsid polypeptide. This has also been found to be the case with the related phlebovirus, Rift Valley Fever

TABLE 6

PASSIVE IMMUNIZATION WITH G2-REACTIVE MONOCLONAL ANTIBODIES

Monoclone[a]	Normal mice[b] protected/total	Mean day to death	Range	Irradiated mice[c] protected/total	Mean day to death	Range
II 4G11	2/10	5.5	5–6	0/8	4.4	4–5
A. 9G6	10/10	—	—	3/10	6.1	4–7
II 3E6	10/10	—	—	0/8	5.1	4–6
I 1E11	10/10	—	—	0/10	6.2	4–8
II 1F3	10/10	—	—	0/10	4.4	4–5
II 1B5	10/10	—	—	0/8	5.2	4–6
I 2F8-1-5	4/10	6	6	6/10	6.4	6–7
I 2E2-3	10/10	—	—	0/10	5.6	4–8
I 2F8-1-1	10/10	—	—	0/10	6.5	4–8
II 6E8	10/10	—	—	9/10	7	7
I 2E2-1	n.d.[d]	n.d.	n.d.	3/10	8.6	7–9
A. 4G2	1/10	5.5	3–7	0/10	8.4	5–11
I 1B3	n.d.	n.d.	n.d.	0/10	8.0	5–11
PTV alone	0/10	3.9	3–5	0/10	4.3	4–5

[a] Undiluted ascitic fluid (0.2 ml) was transferred 24 hr after inoculation.
[b] Four-week-old C57BL/6J mice were inoculated subcutaneously with 4000 PFU of PTV.
[c] Four-week-old C57BL/6J mice were irradiated (750 rads) 4 hr prior to inoculation. Mean survival time of animals receiving radiation alone was 15 days.
[d] n.d., not done.

TABLE 7

COMPETITIVE BINDING ANALYSIS[a] WITH SELECTED PTV-REACTIVE MONOCLONAL ANTIBODIES

	^{35}S-Labeled monoclonal antibodies									
	I 2E2-1	I 1B3	I 2F8-1-5	II 6E8	A. 9G6	III 1D10-1	I 3E8	II 5F9-2-3	II 2F9-2	A. 7F5
I 2E2-1 (G2)	10^2	10^2	—	—	—	—	—	—	—	—
I 1B3 (G2)	10^3	10^2	—	—	—	—	—	—	—	—
I 2F8-1-5 (G2)	—	—	10^3	—	—	—	—	—	—	—
II 6E8 (G2)	—	—		10^2	10^3	—	—	—	—	—
A. 9G6 (G2)	—	—	—	10^2	10^2	—	—	—	—	—
III 1D10-1 (G1)	—	—	—	—	—	10^3	10^3	10^3	10^3	—
I 3E8 (G1)	—	—	—	—	—	10^2	10^2	10^2	10^2	—
II 5F9-2-3 (G1)	—	—	—	—	—	10^3	10^2	10^2	10^2	—
II 2F9-2 (G1)	—	—	—	—	—	10^3	10^3	10^3	10^2	—
A. 7F5 (G1)	—	—	—	—	—	—	—	—	—	10^2

[a] The competing titer of each monoclone was calculated as the highest dilution of unlabeled ascitic fluid to cause a 50% reduction in bound radioactivity when compared to controls.

virus, and with other members of the Bunyaviridae family such as Crimean Congo hemorrhagic fever virus (unpublished observations), and presumably reflects the fact that the nucleocapsid protein is highly immunogenic relative to the viral glycoproteins.

As the plaque-reduction assays are carried out under physiological conditions, data from these assys indicate that both glycoproteins carry determinants which are accessible at the virion surface. The results presented also indicate that both the serological assay which is used to group isolates into the *Phlebovirus* genus (HI) and the assay used to define individual viruses (neutralization) monitor determinants on both glycoproteins.

Cross-reaction studies have shown that all of the G1 monoclones were PTV-specific and that the anti-nucleocapsid monoclones showed very limited cross-reaction. In contrast, 20 of the 27 anti-G2 monoclones cross-reacted with at least one and as many as 17 other phleboviruses. Among the 20 G2-specific antibodies, none exhibited the same pattern of cross-reactivity. The observation that the PTV G2 glycoprotein appears to be antigenically more conserved than the G1 glycoprotein is consistent with existing sequence information which has shown that the predicted amino acid sequences of PTV G1 and G2 are, respectively, 35 and 49% homologous with their RVF counterparts (Ihara *et al.*, 1985a). Determinant conservation does not necessarily imply functional equivalence, as the monoclone 1E11, which neutralizes PTV and cross-reacts with RVFV in ELISA or IFA, fails to neutralize RVFV *in vitro* or protect mice challenged with RVFV (data not shown).

The passive immunization studies not only confirm that immunoglobulins alone can protect mice lethally infected with PTV, but also demonstrate that antibodies to several individual epitopes are sufficient to provide efficient protection. All five of the G1-specific monoclones studied here, which bind to at least two topological sites, as well as a single G2-specific monoclone, provided essentially complete protection to otherwise lethally infected mice, irrespective of whether the animals were immunocompetent or immunosuppressed. In contrast, a number of G2-specific monoclones provided protection only when the endogenous immune response of the host was left intact. A similar reduction in protective efficacy in immunosuppressed mice has been reported for a number of monoclonal antibodies reactive with herpes simplex virus (Balachandra *et al.*, 1982). Monoclonal antibodies able to protect only immunocompetent animals are assumed to exert their protective effect by delaying virus replication and restricting tissue injury to sublethal levels until an appropriate and protective immune response is initiated.

Although the capacity of the monoclonal antibodies to provide protection *in vivo* largely correlated with their ability to neutralize virus *in vitro*, there were significant exceptions. One monoclone (6E8) which exibited very low *in vitro* neutralization capacity, nonetheless protected all immunocompetent mice and 90% of the immunosuppressed animals. In contrast, an IgM monoclone (II 4G11), with a relatively high neutralization titer, was able to protect only 20% immunocompetent and none of the immunosuppressed mice. Nonneutralizing antibodies which protect *in vivo* and neutralizing antibodies which fail to provide protection have both been described in other virus systems (Buchmeier *et al.*, 1984; Boere *et al.*, 1983; Schmaljohn *et al.*, 1982). The ability of antibody alone to arrest an ongoing infection indicates that immunogens inducing primarily or solely humoral antibody would likely be suitable vaccines for this virus group. Furthermore, the observation that antibodies to single determinants, even in minute amounts, are capable of providing complete protection, suggests that immunogens composed of synthetic or expressed products may well be feasible vaccine candidates.

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