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In Vitro Studies of Sandfly Fever Viruses and  
Their Potential Significance for Vaccine Development

Annual And Final Report

June 1, 1988

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Jonathan Smith, Ph.D.

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### Abstract/Summary

Punta Toro virus (PTV) infections of inbred strains of mice have been characterized and evaluated as a model in which to study various aspects of the host response to phlebovirus infections and the requirements for protective immunity. The Adames strain of PTV was found to be strongly hepatotropic and lymphotropic and the outcome of infection to be largely a function of age. C57BL/6J mice of less than 5 weeks of age uniformly developed fulminant hepatocellular necrosis with mean survival times of 4.2 days. Resistance to lethal infection increased with age such that >95% of 8 week old animals survived infections. The kinetics of viremia, antibody production, and hematologic changes in 4 and 8 week old mice have also been monitored. The data indicate that the survival of the older animals is related to their ability to delay virus replication and the development of hepatic lesions during the initial 48 hours of infection and their ability to terminate virus replication and clear virus from the circulation 4 to 5 days after infection. The mechanisms responsible for this resistance were studied using anti-interferon serum, immunosuppression and passive immunization.

Thirty-six independently derived monoclonal antibodies with reactivities to PTV G1, G2, or NC were characterized in neutralization, hemagglutination, and elisa assays, and their antigenic specificities and isotypes determined. ( These antibodies were also assessed for their ability to provide protection in the murine model described above.) The results of these studies demonstrated that neutralization epitopes exist on both G1 and G2, and that neutralizing antibodies to both antigens are capable of protecting immunocompetent animals. However, in vivo protection did not correlate directly with in vitro neutralization, and anti-G1 antibodies tended to be much more efficient in protecting immunosuppressed mice.

### General Introduction and Summary of Research Objectives

Among over 400 viruses which are presently classified as arboviruses, approximately 200 belong to the family, *Bunyaviridae*, and at least one-fourth of these are known to cause human disease. Despite their importance as human and animal pathogens, the *Bunyaviridae* remain the least well understood of the major arbovirus groups in terms of their basic virology and biology, the replication patterns they establish in infected cells, and with respect to the approaches which should be considered in immunoprophylaxis. Based on serologic, molecular, and morphological studies, five genera of this family have been defined, *Bunyaviruses*, *Phleboviruses*, *Nairoviruses*, *Uukuviruses*, and *Hantaviruses*. The studies to be reported here have focused on members of the *Phlebovirus* genus, a group previously defined as the phlebotomus or sandfly fever viruses.

There are currently 38 antigenically serologically distinct viruses which have been placed in the *Phlebovirus* genus. As classically described with Naples and Sicilian strains, but also seen with uncomplicated Rift Valley fever, Sandfly fever is acute, incapacitating, but self-limiting febrile disease of 3 to 5 days duration. In most respects the course of disease is similar to dengue fever. There are, in addition, at least four Central or South American phleboviruses which appear to cause a disease similar to sandfly fever: Chagres, Alenquer, Candiru, and Punta Toro viruses. Among the *Phleboviruses*, Rift Valley Fever virus appears to be unique in its capacity to cause severe or fatal disease in humans and epizootics among domestic animals.

The studies covered under this contract have included all of the *Phleboviruses* known to be human pathogens, with the exception of Rift Valley fever virus which requires high level containment. However, for reasons described below, these studies have largely focused on Punta Toro and Karimabad viruses which possess molecular, antigenic, or virulence characteristics which make them particularly desirable for the study of specific aspects of sandfly fever virus replication *in vitro* or *in vivo*.

As has been defined in previous reports, the overall objectives of these studies have been:

1. to describe for this group of viruses, the virus-specific structural and nonstructural proteins, their interrelationships, and post-translational modification and processing.

2. to correlate biochemical data with those obtained from electron microscopy of virions and infected cells to define the interaction of viral components in particle assembly and morphogenesis.

3. to develop an animal model for selected phleboviruses in which to study specific and non-specific effector mechanisms involved in resistance and immunity.

4. to utilize monoclonal antibodies to identify viral antigens and epitopes which are capable of conferring immunologic protection, and to clarify the antigenic relationships among PTV strains and heterologous phleboviruses.

The objectives outlined in 1. and 2. above have been addressed and described at length in previous reports, and are summarized below. The studies to be described in the present report concern 1) the development and characterization of an animal model which utilizes the Adames strain of PTV and inbred C57Bl/6 mice, and 2) the characterization of a panel of monoclonal antibodies to PTV. These studies therefore address the specific aims outlined in 3. and 4. above.

Manuscripts submitted as Appendix 1 and appendix 2 describe these studies in detail, as well as the procedures utilized, and the results obtained. The manuscript submitted as appendix 1 has been published:

Pifat, D.Y. and J.F. Smith (1987). Punta Toro virus infections of C57BL/6J mice: A model for phlebovirus- induced disease. *Microbial Pathogenesis* 3: 409-422.

The manuscript submitted as appendix 2 was submitted 8 April 1988 to Virology for publication:

Pifat, D.Y., Osterling, M.C., and J.F. Smith. Antigenic characterization of Punta Toro Virus and Identification of Protective determinants with monoclonal antibodies.

### Discussion of Results

We have shown that an acute infection can be produced in several inbred strains of mice following a peripheral inoculation of the Adames strain of PTV. However, in most strains examined an age-related resistance develops at 4 weeks of age. Older animals nonetheless undergo an acute infection in which substantial virus replication occurs and in which histopathological changes and viral antigens are readily demonstrable in the major target organ.

The tissue tropism of PTV seems to be largely restricted to the liver, spleen, and other lymphoid organs, despite the presence of high titers of circulating infectious virus for at least 48 hours. The hepatotropism of this strain of PTV is such that even upon intracerebral inoculation, weanling mice nonetheless die of fulminant hepatic necrosis with very little viral antigen demonstrable in the central nervous system.

The leukopenia and lymphopenia observed in PTV - infected mice are similar to those described in humans infected with Sicilian virus and in a variety of animal species infected with Rift Valley fever virus. Such a lymphopenia may be a consistent feature of phlebovirus infections and may be due either to direct killing of these cells by virus infection or to an alteration of normal patterns of lymphocyte migration. In either event, the characteristic hepatic lesions appear to be free of lymphocyte infiltrates as monitored by

histological studies.

Based on the kinetics of viremia production and accumulation of viral antigen in infected tissues, the ability of the 8 week mice to survive the acute stage of disease appears to be related to at least two distinct mechanisms. One mechanism functions during the first 24 hours after infection, prior to the time when a specific immune response can be detected, and serves to delay the appearance of infectious virus in the circulation. Its effect is short lived, and virus replication, although delayed, progresses to levels comparable with the 4 week animals. The second mechanism, which is functional at 72 to 96 hours after infection, serves to promote virus clearance and restrict further virus spread.

In an effort to identify the early effector mechanism involved in resistance to PTV infection, the role of interferon has been investigated. The experiments involving the treatment of 8 week -old animals with anti-interferon serum have shown that interferon clearly plays a pivotal role in the control of PTV infection. However, the onset of viremia in interferon-treated , 8-week-old mice paralleled the onset of viremia in the animals that did not receive the anti-interferon serum. This result suggests that interferon is not the only mechanism responsible for the increased resistance of the older animals in the initial phases of disease. These studies have also demonstrated that the younger animals do not produce less interferon than the 8 week animals in response to PTV infection, and that treatment with interferon at least in a single dose does not increase the survival rate. The possibility remains that the younger animals are less able to respond to the interferon which is produced, and consequently, are less efficient in establishing the antiviral state.

Other studies involving infection of genetically NK deficient mice or C57Bl/6J mice treated with anti-Sialo GM1 have failed to demonstrate a diminished resistance of either group to PTV infections. These data suggest that NK cells are not responsible for the natural resistance observed in the 8 week animals.

It is clear from the results obtained that a specific immune response plays a

pivotal role in the control of PTV infections. We have shown that antibody alone is capable of providing protection to otherwise lethally infected animals and that immunosuppression of normally resistant animals results in an inability to restrict virus replication. It is remarkable that the passive administration of antibody is effective even when delayed to a time when viremia is at peak titers and liver necrosis is clearly demonstrable.

The manuscript presented in appendix 2 details the results obtained from the study of 36 monoclonal antibodies to the structural proteins of PTV. The characterization of these monoclonal antibodies have allowed us to define certain aspects of the antigenic structure of PTV as well as the identification of at least some of the determinants involved in protection and immunity.

The antigenic analysis of PTV using these monoclonal antibodies has confirmed that both surface proteins possess determinants capable of eliciting neutralizing antibodies as monitored in vitro. As the plaque reduction assays are carried out under physiological conditions, these data indicate that both glycoproteins carry determinants which are assessable at the virion surface. Although antibodies to both glycoproteins also inhibit hemagglutination, such predictions are not readily made from the results of HI assays, which are carried out at reduced pH and may therefore induce conformational changes in the viral antigens. Therefore, both the serological assay which is used to group isolates into the phlebovirus group (HI) and the assay used to define individual viruses (neutralization) monitor determinants on both glycoproteins.

Studies which have monitored the binding of these monoclonal antibodies to heterologous phleboviruses have shown that all of the G1 monoclonal antibodies are PTV-specific and that the anti-nucleocapsid monoclonal antibodies showed very little cross reaction. In contrast 20 of the 27 anti-G2 monoclonal antibodies cross-reacted with at least one and as many as 17 other phleboviruses. The relatively few epitopes held in common between PTV and any other phlebovirus implies that none of the viruses studied here has originated, at least recently, from direct segment reassortment between PTV and a heterologous phlebovirus.

The passive protection studies not only confirm that immunoglobulins alone

can protect mice lethally infected with PTV, but also demonstrate that antibodies to several individual epitopes can provide efficient protection. All five of the G1-specific monoclonal antibodies studied, which define epitopes in two topologic sites, as well as a single G2-specific monoclonal antibody, provide essentially complete protection to otherwise lethally infected mice, irrespective of whether the animals were immunocompetent or immunosuppressed. In contrast, a number of G2-specific monoclonal antibodies provided protection only in the context of an intact endogenous immune response.

Although the capacity of the monoclonal antibodies to provide protection *in vivo* largely correlated with their ability to neutralize virus infectivity *in vitro*, there were some significant exceptions noted. The mechanism by which non-neutralizing antibodies may protect, in this system or other, has not been determined with certainty, but most theories have focused on the presumed ability of these antibodies to bind to viral antigens on the surface of infected cells, thereby rendering these cells to cellular or complement-mediated lysis.

In any event, 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 group of viruses. 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.

Publications emanating from this Contract

Smith, J.F. and D.Y. Pifat (1982). Morphogenesis of Sandfly Fever Viruses (Bunyaviridae Family). *Virology* 121:61-81.

Pifat, D.Y. and J.F. Smith (1987). Punta Toro virus infections of C57BL/6J mice: A model for phlebovirus- induced disease. *Microbial Pathogenesis* 3: 409-422.

Pifat, D.Y., Osterling, M.C., and J.F. Smith. Antigenic characterization of Punta Toro Virus and Identification of Protective determinants with monoclonal antibodies. Submitted to Virology for publication.

Dalrymple, J.M. C.J. Peters, J.F. Smith, and M.K. Gentry (1981). Antigenic Components of Punta Toro Virus. in *The Replication of Negative Strand Viruses*, D.H.L. Bishop and R.W. Compans, eds. Elsevier North Holland Press.

## Punta Toro virus infection of C57BL/6J mice: a model for phlebovirus-induced disease

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Punta Toro virus infections of inbred strains of mice have been characterized and evaluated as a model in which to study various aspects of the host response to phlebovirus infections and the requirements for protective immunity. The Adames strain of Punta Toro virus was found to be strongly hepatotropic and lymphotropic and the outcome of infection was largely a function of age. C57BL/6J mice of less than 5 weeks of age uniformly developed fulminant hepatocellular necrosis with mean survival times of 4.2 days. Resistance to lethal infection increased with age such that >95% of 8-week-old mice survived challenge. The kinetics of viremia, antibody production, and hematological changes in 4- and 8-week animals indicated that the survival of the older animals is related to their ability to delay virus replication and the development of hepatic lesions during the initial 48 h of infection and their ability to terminate virus replication and clear virus from the circulation 4 to 5 days after infection. The mechanisms responsible for this resistance were studied using anti-interferon serum, immunosuppression, and passive immunization.

**Key words:** Punta Toro virus; phlebovirus; Bunyaviridae; pathogenesis; interferon; immune response.

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### Introduction

Punta Toro virus belongs to the *Phlebovirus* genus of the Bunyaviridae family which currently encompasses 38 antigenically distinguishable viruses. These viruses have been shown to be related by reciprocal serological reactions and to possess similar biochemical and morphological characteristics.<sup>1-4</sup> As classically described with Naples and Sicilian strains, but also seen with Rift Valley fever virus, Sandfly fever is an acute, incapacitating, but self-limiting febrile disease of 3 to 5 days duration.<sup>5,6,7</sup> There are at least four Central or South American phleboviruses which appear to cause a disease

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.

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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**Table 1** Influence of age on the outcome of infection with Punta Toro virus<sup>a</sup>

Age	D/T <sup>b</sup>	Day of Death	
		Mean	Range
3 weeks	5/5	3.4	3-4
4 weeks	5/5	4	4
5 weeks	3/5	4.3	4-5
6 weeks	1/5	4	4
7 weeks	0/5	—	—
8 weeks	0/5	—	—

<sup>a</sup> Groups of C57BL/6J male mice were inoculated subcutaneously with 4000 PFU of PTV.

<sup>b</sup> Dead/Total.

similar to sandfly fever: Chagres, Alenquer, Candiru, and Punta Toro viruses.<sup>8-11</sup> The capacity to cause fatal disease in humans and widespread epizootics appears to be unique to Rift Valley fever virus.

Phleboviruses are usually transmitted either by phlebotomine flies or mosquitoes,<sup>12</sup> although aerosol infection is well documented with Rift Valley fever virus.<sup>13</sup> Although the ecology and epidemiology of infections induced by phleboviruses have been examined by several investigators,<sup>8,12,14-18</sup> little is known of the specific and non-specific effector mechanisms which influence resistance and immunity, or the antigens and epitopes which confer immunological protection. In order to study these aspects of phlebovirus infections, we have established a model requiring only P2 containment levels, which utilizes Punta Toro virus infections of inbred strains of mice. Reported here are the results of studies which have identified tissue tropisms and characterized the progression of histopathological lesions and hematological changes, as well as the development of the host immune and interferon responses. We also examined the influence of age and genetic background on the outcome of infection. Initial studies designed to identify non-specific effector mechanisms involved in the natural resistance to phlebovirus infections and to evaluate the role of the immune response in the survival of resistant animals are also presented.

## Results

### *The susceptibility of C57BL/6J mice to lethal infection is age dependent*

C57BL/6J mice, 3- to 8-weeks old, were infected subcutaneously with 4000 plaque forming units (PFU) of Punta Toro virus (PTV), and monitored for illness and death over a 4-week period. Typical results of such experiments are shown in Table 1, in which animals of 4-weeks of age or less showed uniform susceptibility to a lethal infection following a peripheral challenge with PTV. These animals exhibited massive hepatocellular necrosis at necropsy. Animals older than 4 weeks showed increasing resistance to lethal infection which was complete by 7-weeks of age. Surviving animals successfully cleared all circulating virus by day 6 post-infection. The resistance of the older animals to a peripheral challenge with PTV could not be overcome either by increasing or decreasing the virus inoculum.

### *Susceptibility of inbred strains of mice*

Sixteen inbred strains of mice were examined for their susceptibility to peripheral challenge with PTV to determine whether the age-dependent susceptibility observed

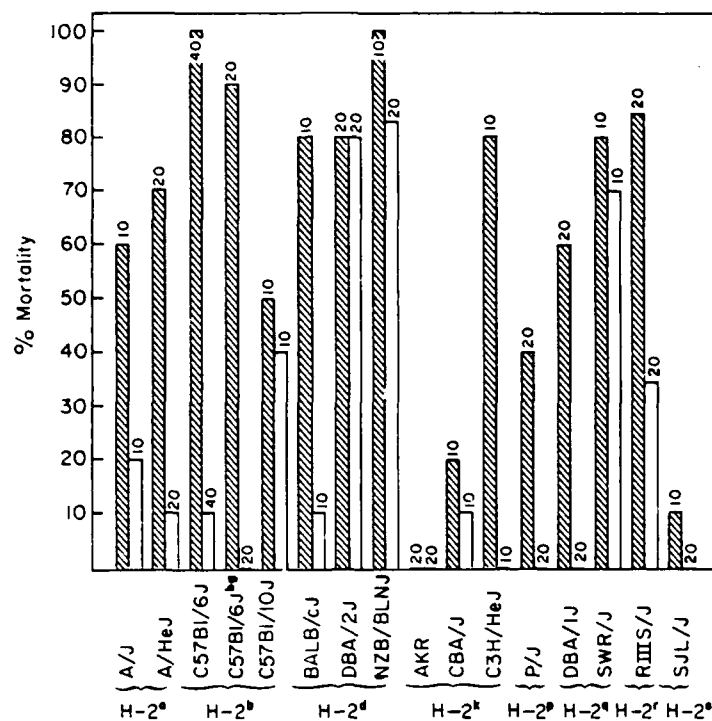


Fig. 1. Influence of genetic background on the outcome of infection of 4- and 8-week-old mice with PTV. Four- and eight-week-old male mice of the various strains indicated were inoculated subcutaneously with 4000 PFU of PTV. The mice were monitored for illness and/or death for a period of 30 days. The number of animals in each group is given at the top of the bar graphs. Open bars represent 8-week animals, hatched bars represent 4-week animals.

was unique to C57BL/6J mice and whether the susceptibility to lethal infection could be linked to either genetic background or H-2 haplotype. Among the strains examined, ten exhibited a marked age-dependent susceptibility to lethal infection but none was found to be uniformly susceptible when challenged at 8-weeks of age (Fig. 1). The degree of susceptibility exhibited by the various inbred strains of mice did not appear to correlate with H-2 haplotype. Several strains of mice, including C57BL/6J and NZB/BLNJ, were highly susceptible to a peripheral challenge with PTV at 4-weeks of age (Fig. 1). C57BL/6J mice were utilized for further studies as infection of 4-week animals of this strain had a uniformly lethal outcome, and 4- and 8-week animals showed the greatest differential response.

#### *Histopathological lesions induced by PTV infection*

Complete necropsies were performed on groups of 4- and 8-week-old animals at 24-h intervals after inoculation. Gross lesions were observed only at 4 days post-infection when livers from animals of both ages were discolored and mottled. The younger animals consistently exhibited hemorrhage in the proximal duodenum, when moribund.

Microscopic lesions were not detectable in 4- or 8-week animals sacrificed 24 to 48 h after infection. However, by day 3, moderate midzonal hepatocellular necrosis, which appeared to involve both hepatocytes and sinusoidal cells, was observed in 4-week animals. These lesions also contained eosinophilic "Councilman-like" bodies similar to those classically observed in yellow fever and seen in Rift Valley fever infections of newborn lambs and susceptible rats.<sup>19,20</sup> Mononuclear cell infiltrates were absent (Fig. 2). The red pulp of the spleen showed small multifocal areas of necrosis, but identification of the cell types involved was not possible by standard hematoxylin and eosin staining techniques. In 4-week animals sacrificed on day 4, histological

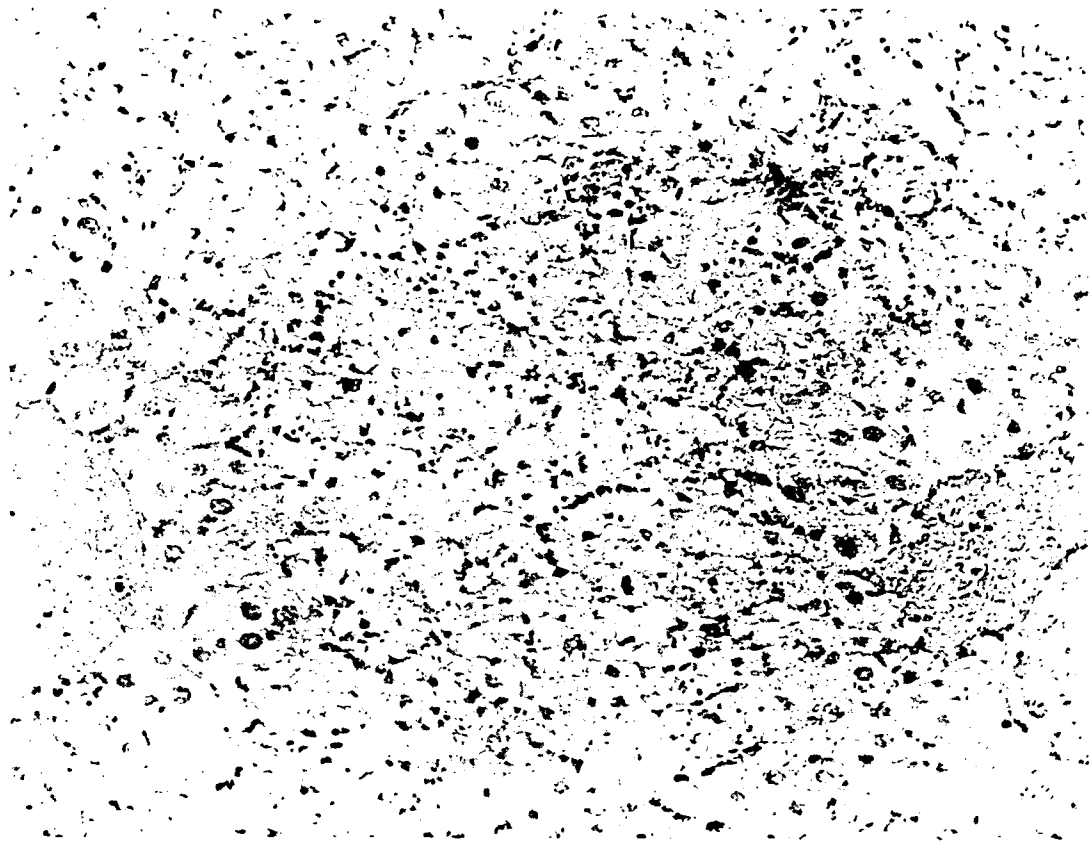


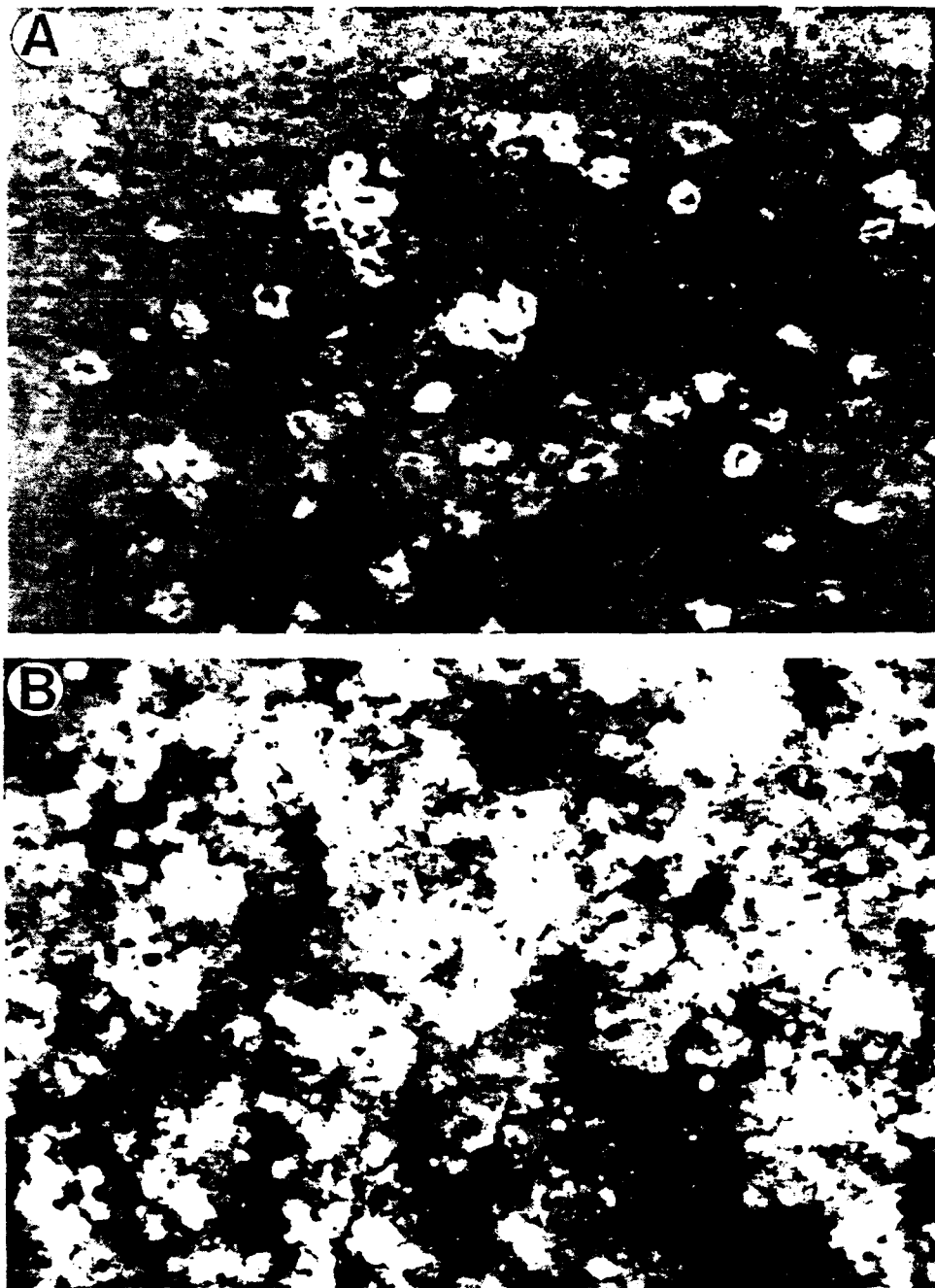
Fig. 2. Hepatocellular necrosis in PTV-infected, 4-week-old male C57BL/6J mice. Liver tissue was removed at 72 h post-infection and processed for hematoxylin and eosin staining. Lesions were initiated midzonally with relative sparing of hepatocytes immediately adjacent to central veins.

lesions were observed in the liver, spleen, small intestine, lymph nodes, and thymus. The liver showed massive hepatocellular and sinusoidal cell necrosis with hemorrhage. Lesions in the spleen consisted of diffuse hypocellularity and mild necrosis in the red pulp as well as moderate lymphoid necrosis in the white pulp. Mild multifocal necrosis was also seen in the villous crypt intestinal epithelial cells. None of the other tissues sampled showed any evidence of virus-induced histological alterations.

In 8-week animals, histological lesions were first apparent 4 days postinfection. At this time, mild hepatocellular necrosis and necrosis of the lymphoid organs were observed which were similar to lesions seen 3 days postinfection in the 4-week animals. Again, histopathological lesions were not evident in any other organ or tissue.

#### *Tissue tropism of PTV as monitored by immunofluorescence*

In 4-week animals infected with PTV, antigen-positive cells were demonstrable in the liver beginning at 48 h after inoculation. At 72 h post-infection, liver sections of 4-week animals showed numerous fluorescent cells and larger fluorescent foci [Fig. 3(a)]. By day 4 after infection, when these mice were moribund, greater than 90% of the cells in the liver contained viral antigen [Fig. 3(b)]. Significant amounts of specific fluorescence could not be consistently demonstrated in other tissues. Infected cells were first demonstrable in liver sections from 8-week animals 3 days after infection. By day 4 after infection, the number of antigen-positive cells increased but rarely exceeded 15–20%. As with the younger animals, specific fluorescence was not observed in other tissues.



**Fig. 3.** Accumulation of viral antigen in the liver of PTV-infected, 4-week-old C57BL/6J mice. Liver tissues were excised, frozen and processed for indirect immunofluorescence. Tissues were removed at 72 h after infection (panel A) or 96 h after infection (panel B).

#### *PTV-induced hematological changes*

The infection of both 4- and 8-week-old animals with PTV was accompanied by a significant decrease in the total number of circulating leukocytes, beginning 24 h after infection in the 4-week animals ( $P < 0.007$ ) and 72 h after infection in the 8-week animals ( $P < 0.0001$ ) (Fig. 4). This decrease was primarily due to a significant drop in the number of circulating lymphocytes (Fig. 4). By day 3 after infection in both the 4- and the 8-week animals, the number of circulating lymphocytes was less than 10% of that of normal, age-matched controls. However, the lymphopenia was transient in the older animals as demonstrated by the rising number of circulating lymphocytes present on days 4 and 5. Animals of both ages also showed a significant decrease in

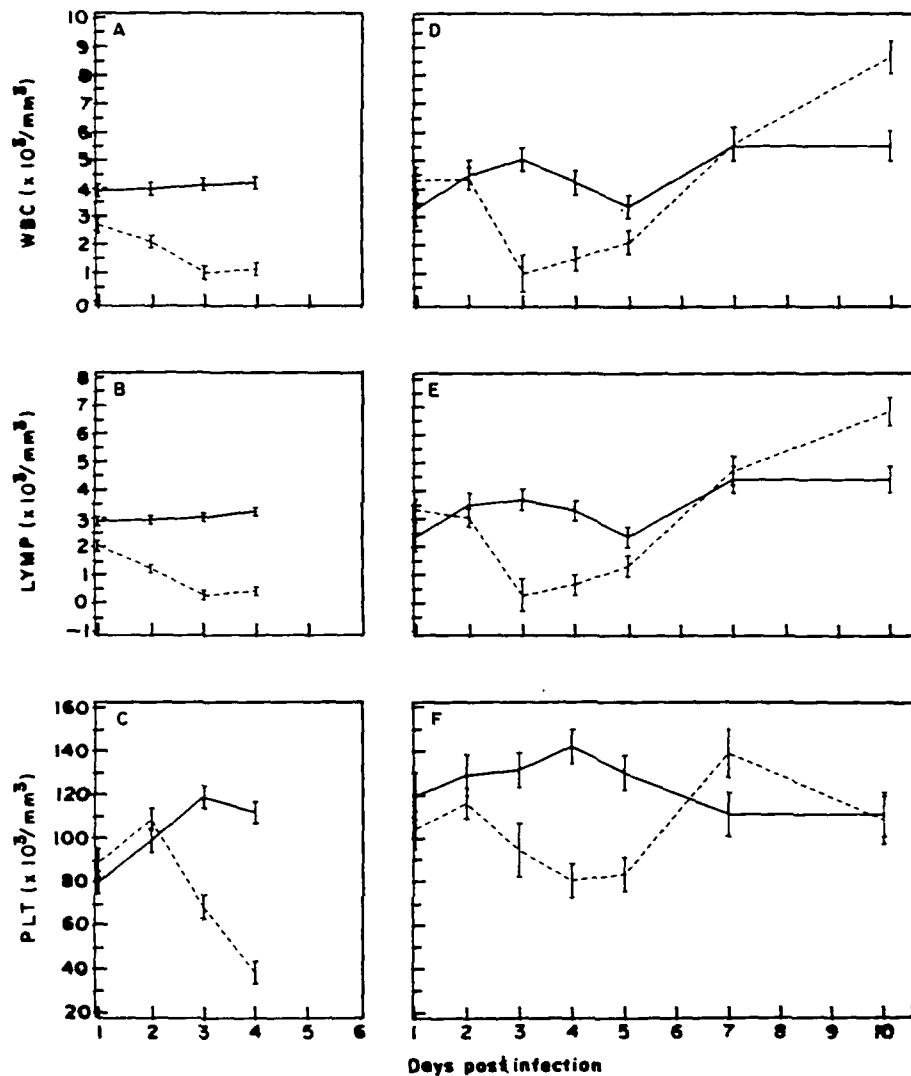


Fig. 4. PTV-induced hematological changes in 4- and 8-week-old mice. Four- and eight-week-old male C57BL/6J mice were inoculated subcutaneously with 4000 PFU of PTV. Groups of mice were killed at 24-h intervals and blood samples were taken for hematological analysis. Panels A, B and C represent white blood cell (WBC), lymphocyte, and platelet counts, respectively, for the 4-week animals. Panels D, E and F represent WBC, lymphocyte, and platelet counts, respectively, for the 8-week animals. Solid lines represent results from uninfected control animals; discontinuous lines represent results from PTV-infected animals.

the number of platelets beginning on day 3 after infection for the 4-week animals ( $P < 0.001$ ) and day 4 after infection for the 8-week animals ( $P < 0.0001$ ). All other hematological values measured remained within normal limits throughout the course of the infection. By day 7 post-infection, all hematological values for the 8-week animals had returned to normal ranges, although on day 10, leukocyte counts were slightly elevated.

#### *Development of viremia and kinetics of specific antibody synthesis*

Serum samples from individual 4- and 8-week animals sacrificed at 24-h intervals after infection were assayed for infectious virus and PTV-specific antibody. An ELISA assay was utilized to measure both IgG and IgM antibody levels. The viremia titers in the 4-week animals rose over four orders of magnitude between 24 and 48 h after infection, were maintained or increased slightly on day 3, and began to decline on

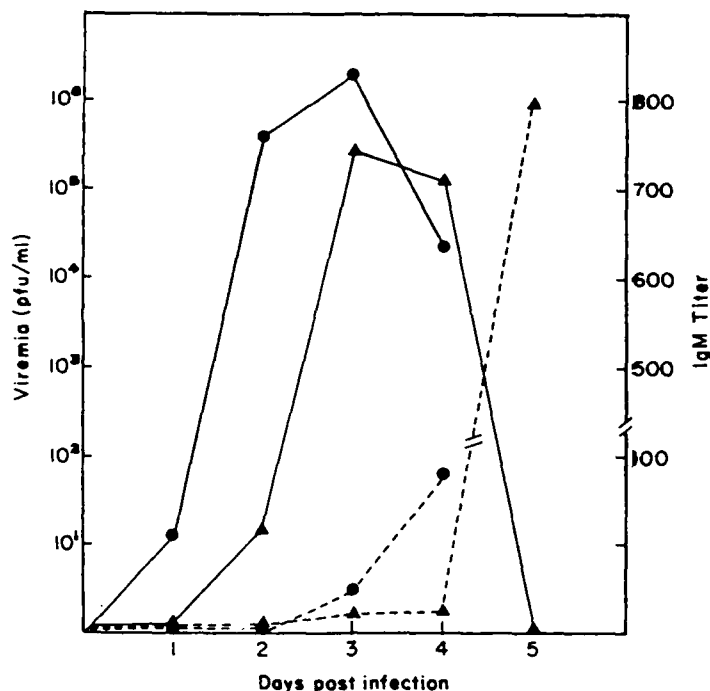


Fig. 5. Kinetics of viremia and virus-specific IgM synthesis in C57BL/6J mice infected with PTV. Groups of 4- and 8-week-old mice were inoculated subcutaneously with 4000 PFU of PTV in 0.2 ml of EMEM and were killed at 24 h intervals. The viremia levels reported here represent geometric mean values calculated for each time point and age group. The IgM titers correspond to arithmetic means for each time point and age group. (Viremia: ●—● 4-week; ▲—▲ 8-week; IgM Synthesis: ●---● 4-week; ▲---▲ 8-week).

day 4 when the animals were moribund (Fig. 5). Similar kinetics were observed in several independent experiments with peak geometric mean titers of  $3 \times 10^6$  to  $4 \times 10^7$  PFU/ml of serum. Viremia titers also reached high levels in 8-week-old animals although consistently somewhat lower (5- to 10-fold) than those seen in the younger animals. However, in the older animals, the appearance of infectious virus was consistently delayed and viremia was abruptly cleared by 5 days postinoculation (Fig. 5).

Virus-specific IgG antibody was not detectable in serum samples during the 5-day period after infection but was detectable on day 6 in the 8-week animals (data not shown). Specific IgM antibody, on the other hand, was detected between 48 and 72 h after infection in the 4-week animals, and rose to maximum titers on day 4. The kinetics of IgM synthesis were similar in the older animals in which maximum titers were reached 5 days after infection, which was coincident with the clearance of infectious virus from the circulation. The first appearance of neutralizing antibody in the 8-week animals occurred on day 8.

#### *Kinetics of the interferon response in 4- and 8-week-old mice following PTV infection*

Serum interferon levels were monitored in both 4- and 8-week animals at 24 h intervals after PTV infection. As shown in Table 2, significant interferon titers were not demonstrable in animals of either age group 24 h after infection. However, 48 h after infection, interferon titers of 960 (arithmetic mean) were detected in the 4-week animals, whereas the 8-week animals showed interferon titers of  $< 40$ . By 3 days after infection, all animals showed high titers of circulating interferon which generally remained high on day 4. The 4-week animals succumbed to infection despite high

**Table 2** Kinetics of interferon response in 4- and 8-week-old C57BL/6J mice infected with Punta Toro virus<sup>a</sup>

Age	Day 1	Day 2	Day 3	Day 4	Day 5
4-weeks	< 40	960	> 2560	2560	n.d. <sup>b</sup>
8-weeks	< 40	< 40	> 2560	1920	160

<sup>a</sup> Mice were inoculated subcutaneously with 4000 PFU of PTV.

<sup>b</sup> Not done; 4-week-old animals died between 4 and 5 days post infection.

**Table 3** Effect of anti-interferon serum on the outcome of infection of 8-week-old C57BL/6J mice with Punta Toro virus<sup>a</sup>

Treatment	D/T <sup>c</sup>	Day of Death	
		Mean	Range
PTV <sup>a</sup> alone	0/10	—	—
Anti-Inf alone <sup>b</sup> 4.8 × 10 <sup>3</sup> U	0/10	—	—
PTV+Anti-Inf 4.8 × 10 <sup>3</sup> U	10/10	5	5
2.4 × 10 <sup>3</sup> U	10/10	4.5	4-5
1.2 × 10 <sup>3</sup> U	6/10	5	5
0.6 × 10 <sup>3</sup> U	6/10	5	5

<sup>a</sup> Infected mice were given 4000 PFU of PTV subcutaneously 4 h after treatment with anti-interferon serum.

<sup>b</sup> Anti-interferon serum ( $\alpha$  and  $\beta$ ), at the doses indicated, were administered by intraperitoneal inoculation.

<sup>c</sup> Dead/Total.

interferon titers. In the 8-week animals, interferon titers declined on day 5 after infection, at a time when these animals had successfully cleared circulating virus.

#### *Treatment with anti-interferon serum ablates the resistance of 8-week animals*

Injection of rabbit anti-mouse interferon ( $\alpha$  and  $\beta$ ) markedly affected the outcome of PTV infection in otherwise resistant 8-week mice (Table 3). All animals receiving  $2.4 \times 10^3$  neutralizing units of anti-interferon or more, died between 4 and 5 days after infection. The effect of lower doses of anti-interferon was less dramatic, but nonetheless resulted in the death of 60% of the animals. The animals which succumbed to PTV infection after anti-interferon treatment had viremia titers as high as  $10^9$  PFU/ml of serum 3 days after infection which is two to three orders of magnitude higher than that seen in untreated animals, irrespective of age. However, the onset of viremia in the treated 8-week-old animals was essentially the same as that seen in untreated animals and remained delayed by 24 h relative to untreated 4-week animals. In parallel experiments, treatment of 4-week animals with single interferon ( $\alpha$  and  $\beta$ ) doses ranging from 1000 to 10000 units, did not affect their survival rate or time of death when compared to animals receiving virus alone (data not shown).

#### *Immunocompromised 8-week-old C57BL/6J mice undergo a lethal infection with PTV*

The results shown in Table 4 indicate that a uniformly lethal infection is established in 8-week-old animals which have been immunosuppressed by whole-body irradiation and subsequently challenged with 4000 PFU of PTV. These animals died within 4 to

**Table 4** Effect of immunosuppression on the outcome of infection of 8-week-old C57BL/6J mice with Punta Toro virus.

Treatment	D/T <sup>b</sup>	Day of Death	
		Mean	Range
PTV <sup>a</sup>	0/10	—	—
PTV+			
950 rads	10/10	5.4	4-8
950 rads	10/10	9.7	8-11

<sup>a</sup> Mice were inoculated subcutaneously with 4000 PFU of PTV 4 h after receiving 950 rads (one LD<sub>100</sub>).

<sup>b</sup> Dead/Total.

**Table 5** Protective effect of passive immunization with PTV-specific anti-serum.<sup>a</sup>

Treatment	D/T <sup>b</sup>	Day of Death	
		Mean	Range
PTV alone	10/10	4	4
PTV+HMAF			
-4 h	0/10	—	—
+24 h	0/10	—	—
+48 h	0/10	—	—

<sup>a</sup> 4-week-old C57BL/6J mice were inoculated intraperitoneally with hyperimmune mouse ascitic fluid (HMAF). The times indicated are relative to infection with 4000 PFU subcutaneously.

<sup>b</sup> Dead/Total.

5 days post-infection, whereas the mean survival time of uninfected animals receiving radiation alone was 10 days. Liver sections from the immunosuppressed and infected animals, when tested for the presence of viral antigen by immunofluorescence, exhibited strong fluorescence in > 90% of the cells, resembling the response of untreated 4-week animals. The immunosuppressed mice developed higher peak viremia titers ( $10^8$  PFU/ml of serum), and these titers were maintained in moribund animals, demonstrating their total failure to clear circulating virus. However, the onset of viremia in the 8-week immunosuppressed animals remained delayed by 24 h when compared to that seen in 4-week infected animals. In addition, the mean survival time for the 8-week animals was approximately 24 h longer than that seen in the younger animals, suggesting that an early effector mechanism responsible for the initial delay in virus replication seen in untreated 8-week animals is radioresistant.

*Passive immunization with PTV-specific antibody protects otherwise lethally infected animals*

The results shown in Table 5 demonstrate that the administration of specific antibody alone, even delayed to a time when hepatic lesions are rapidly progressing and virus replication is in logarithmic phase, is capable of protecting all infected mice. Similar experiments involving the transfer of glycoprotein-specific monoclonal antibodies to mice which have been both lethally infected and lethally irradiated have shown that

some of these antibodies are capable of providing protection even when the endogenous immune response of the recipient is abrogated (manuscript in preparation).

## Discussion

Many phleboviruses are capable of inducing a lethal encephalitis in mice upon direct intracerebral inoculation.<sup>21,22</sup> However, with the exception of Rift Valley fever virus,<sup>23,24</sup> previously tested phleboviruses were not lethal for weanling or adult mice when inoculated peripherally. With rare exceptions in the case of Rift Valley fever virus<sup>25</sup> and possibly Toscana virus,<sup>26</sup> human infections with phleboviruses do not result in encephalitis and therefore an animal model was sought in which a non-encephalitic disease would result following a peripheral challenge.

We have shown here that an acute infection can be produced in several inbred strains of mice following a peripheral inoculation of the Adames strain of PTV. However, in most mouse strains examined, an age-related resistance to lethal infection develops at approximately 4 weeks of age. As demonstrated here with C57BL/6J mice, the older resistant animals nonetheless undergo an acute course of infection in which substantial virus replication occurs and in which histopathological changes and viral antigens are readily demonstrable in the major target organ. It has been shown that inbred strains of rats show dramatic differences in susceptibility to RVFV and that susceptibility is governed by a single mendelian gene.<sup>19</sup> Although some variation in susceptibility to PTV was apparent among the mouse strains examined, no strain was found to be uniformly susceptible to PTV at 8-weeks of age.

The tissue tropism of PTV appears to be largely restricted to the liver, spleen, and other lymphoid organs, despite the presence of high levels of circulating infectious virus for at least 48 h. The hepatotropism of PTV is such that, upon an intracerebral inoculation of the Adames strain of PTV, weanling mice nonetheless die of fulminant hepatocellular necrosis with very little viral antigen found in the central nervous system at the time of death (data not shown). The histopathological lesions seen in the lymphoid organs in the absence of detectable viral antigen (by immunofluorescence) could reflect a non-productive infection in which virus-induced destruction of cells occurs without the accumulation of large amounts of viral proteins.

The leukopenia and lymphopenia described here in PTV-infected mice are similar to those described in humans infected with Sicilian virus<sup>5,6</sup> and in a variety of animal species infected with Rift Valley fever virus.<sup>20,27</sup> Such a lymphopenia may be a consistent feature of phlebovirus infections and may be due to the direct killing of these cells by virus infection, or to an alteration of the normal pattern of lymphocyte migration. In either event, hepatic lesions appear to be free of lymphocyte infiltrates as monitored by histological studies.

Based on the kinetics of viremia production and accumulation of viral antigen in infected tissues, the ability of the 8-week-old mice to recover from the acute infection induced by PTV appears to be related to at least two distinct mechanisms. One mechanism functions during the first 24 h after infection, prior to the time when a specific immune response can be detected, and serves to delay the appearance of infectious virus in the circulation. Its effect is short lived, and virus replication, although delayed, progresses to levels comparable to those seen in 4-week animals. The second mechanism, which is functional at 72 to 96 h after infection, serves to promote virus clearance and restrict further virus spread.

In an effort to identify the early effector mechanism(s) involved in natural resistance to PTV infection, we have investigated the role of interferon. Experiments involving the treatment of 8-week-old animals with anti-interferon serum have shown that

interferon clearly plays a pivotal role in the control of PTV infection. However, the onset of viremia in anti-interferon-treated, 8-week-old mice paralleled the onset of viremia in the animals that did not receive the anti-interferon serum. This result suggests that interferon is not the only early effector mechanism responsible for the increased resistance of the older animals in the initial stages of the disease. The studies presented here also show that 4-week-old animals do not appear to produce less endogenous interferon than the 8-week animals in response to PTV infection, and that the treatment of 4-week-old animals with a single dose of 10 000 units of exogenous interferon does not result in an increased survival rate. The possibility exists that the 4-week animals are less able to respond to the interferon which is produced and consequently less efficient at establishing an antiviral state.

Other experiments involving infection of either NK cell-deficient C57BL/6<sup>bg/bg</sup> mice<sup>28</sup> or C57BL/6J mice treated with anti-asialo GM1 (data not shown) have failed to demonstrate a diminished resistance of either group and therefore suggest that NK cells are probably not responsible for the natural resistance observed in the 8-week animals.

It is clear from the experiments described here that a specific immune response plays a vital role in the eventual control of PTV infections. We have shown that antibody alone is capable of providing protection to otherwise lethally infected animals and that immunosuppression of normally resistant animals results in the inability to restrict viral replication. It is remarkable that the passive administration of antibody is effective even when delayed to a time when viremia is at peak titers and liver necrosis is clearly demonstrable. In addition, monoclonal antibodies are capable of protecting PTV-infected animals whose endogenous immune response has been abrogated (data to be presented elsewhere).

It is anticipated that the model described here will allow the characterization of mechanisms involved in the natural resistance to phleboviruses and the identification of epitopes on PTV proteins responsible for eliciting protective immunity. In addition, PTV infections of 4-week-old animals may also serve as a model in which to test the efficacy of potential immunomodulators and antiviral compounds against viruses of the phlebovirus genus.

## Materials and methods

*Virus strains and assays.* The Adames strain of PTV was provided by Dr C. J. Peters (USAMRIID, Frederick, MD). This strain of Punta Toro virus was isolated from the serum of a patient infected in eastern Panama. The initial isolation was performed on Vero cell monolayers and the virus used in these experiments was in the fourth Vero passage.

Infectivity titers were determined by plaque assay in confluent Vero cell monolayers overlaid with 0.5% agarose (Marine Colloids, Rockland, ME) in Eagles' minimal essential medium (EMEM) containing Earle's salts, penicillin (200 U/ml) and streptomycin (200 ug/ml). Cultures were incubated at 37°C for days then stained with neutral red (0.01%) in a 0.5% agarose overlay, and counted after 15 to 24 hours. The titer of virus stocks used in these studies was  $5 \times 10^7$  PFU/ml.

*Inoculation of mice and collection of tissue samples.* Inbred male mice (A/J, A/HeJ, C57BL/6J, C57BL/6J<sup>bg</sup>, C57BL/J10, Balb/cJ, DBA/2J, NZB/BLNJ, AKR, CBA/J, C3H/HeJ, P/J, DBA/1J, SWR/J, RIIS/J, SJL/J) of 4- or 8-weeks of age were obtained from Jackson Laboratories (Bar Harbor, ME.). Unless otherwise indicated, all mice were inoculated subcutaneously with 0.2 ml of EMEM containing 4000 PFU of PTV; control mice received EMEM alone. To determine the kinetics of virus infection or antibody synthesis, groups of 10 mice, either 4- or 8-weeks old, were killed at 24-h intervals after infection, for 5 days. Blood samples were obtained from methoxyflurane (Abbot Laboratories, North Chicago, IL.)—anesthetized mice by axillary section or by retro-orbital bleeding. Serum samples were frozen at

-70°C for subsequent infectivity, antibody, and interferon titrations. Hematological values were obtained from EDTA-anticoagulated blood with a Technicon H-6000 hematology analyzer which had been standardized with blood samples from untreated 4- and 8-week C57BL/6J mice. Values were obtained for WBC, RBC, MCV, MCH, MCHC, RDW, PLT, MPV, PDW, HCT, differential, and total neutrophil, lymphocyte, eosinophil, basophil, and monocyte counts. The accuracy of the values obtained for leukocyte parameters was periodically confirmed by direct visual examination of blood smears.

For histological examination, sections of lung, liver, heart, spleen, central nervous system, salivary glands, lymph nodes, skin, thymus, intestine, kidney, pancreas, skeletal muscle, peripheral nerve, sternal bone marrow, testes, bladder and adrenal gland, were removed and fixed with a buffered formaldehyde/glutaraldehyde (4%/1%) solution. Paraffin sections were prepared and stained with hematoxylin and eosin by standard procedures.

*Immunofluorescence assays.* Indirect immunofluorescence assays were used for the detection of PTV antigen in frozen sections obtained from the tissues listed above. Hyperimmune mouse ascitic fluid (HMAF), prepared as described by Brandt *et al.*,<sup>29</sup> was used as the primary antibody. Cryostat tissue sections (6  $\mu\text{m}$ ) were fixed in acetone at -4°C for 10 mins, washed in phosphate buffer, pH 7.4, containing 1M NaCl (PBHS), overlaid with the primary antibody, and maintained in a humidified chamber for 1 h at 37°C. Sections were then washed in PBHS for 1 h and overlaid with fluorescein-conjugated, goat anti-mouse IgG (Cooper Biomedical Inc., Malvern, PA) which had been diluted in PBHS. After 1 h at 37°C, sections were washed in PBHS and mounted in carbonate-buffered glycerol containing 1 mg/ml p-phenylenediamine (Sigma, St. Louis, MO).<sup>30</sup> Controls included tissues from uninfected, age-matched mice and infected tissues processed with normal mouse ascitic fluid as the primary antibody.

*Enzyme-linked immunosorbent assay.* Antiviral antibody in individual serum samples was titrated by enzyme-linked immunosorbent assays (ELISA). Gradient-purified PTV (400 ng of protein) in 50  $\mu\text{l}$  of borate-buffered saline, pH 8.4 (BBS), was added to each well of 96-well polyvinylchloride microtiter plates (Dynatech, Alexandria, VA), and allowed to dry overnight at room temperature. Residual binding capacity was blocked by preincubation with BBS containing 10% fetal bovine serum for 1 h at room temperature. The plates were then washed with BBS containing 0.4% Tween 80 (BBST). Fifty microliters of appropriately diluted mouse sera were added and allowed to react for 1 h at 37°C. The plates were then washed in BBST and 100  $\mu\text{l}$  of affinity-purified, alkaline phosphatase-labelled, goat anti-mouse IgM or IgG (Kirkegaard and Perry, Gaithersburg, MD) were added and reacted for 1 h at 37°C. The plates were again washed in BBST and developed using disodium p-nitrophenyl-phosphate as substrate. After 30 mins the optical densities were determined with a Dynatech Microelisa spectrophotometer (Model MR580). The titers were expressed as the reciprocal of the highest dilution which yielded a reading two standard deviations above that obtained with normal mouse serum at comparable dilution.

*Neutralization assays.* Plaque-reduction neutralization tests were performed essentially as described by Earley *et al.*<sup>31</sup> Briefly, 4-fold serial dilutions of sera in HBSS containing 2% fetal bovine serum were mixed with an equal volume of EMEM containing 50-100 PFU of PTV and were incubated overnight at 4°C. Fifty-microliter samples were then inoculated in duplicate Vero cell monolayers in 24-well plates. After 1 h at 37°C, the monolayers were overlaid essentially as described for virus assays. Before performing neutralization tests on viremic sera, samples were irradiated with  $2.4 \times 10^6$  rads from a Cobalt 60 source (Gamma cell 40, Atomic Energy of Canada, Ottawa) to eliminate infectious virus. (This procedure did not alter the ELISA titers of these sera as measured by the standard assay described above.)

*Interferon assays.* L929 cell monolayers in 24-well plates were pretreated with 0.3 ml of serially diluted mouse sera overnight at 37°C. The monolayers were then infected with 100 PFU of the Indiana strain of vesicular stomatitis virus (VSV) for 1 h at 37°C, and subsequently overlaid with 0.5 ml of EMEM containing 2% fetal bovine serum, 200 U/ml of penicillin, 200  $\mu\text{g}/\text{ml}$  of streptomycin, and 0.5% agarose (Marine Colloids, Rockland, ME). After a 24-h incubation period at 37°C, a second overlay containing 0.5% agarose and 0.01% neutral red in EMEM, was added and plaques were counted. Dilutions of the NIH mouse interferon standard (12 000 U/ml) were tested concurrently with the mouse sera. Interferon titers are expressed as the reciprocal of the highest dilution of serum which caused an 80% reduction in VSV plaques

and arithmetic means for each treatment group were calculated. In this assay, 0.5 units of NIH interferon standard was found to be required for an 80% plaque reduction.

*Treatment with interferon or anti-interferon.* Mouse interferon ( $\alpha$  and  $\beta$ ) and rabbit anti-mouse interferon ( $\alpha$  and  $\beta$ ) were obtained from Enzo Biochemicals, Inc. (New York, NY). The interferon preparation had a specific activity of  $2 \times 10^7$  units/mg and was prepared in C243 cells induced by Newcastle disease virus and theophylline. The rabbit anti-interferon serum had a titer of  $2.8 \times 10^{-4}$  U/ml when assayed against 10 units of mouse interferon. Four-week-old mice were inoculated intraperitoneally with either 1000, 5000, or 10000 units of mouse interferon ( $\alpha$  and  $\beta$ ), 4 h prior to a virus challenge consisting of 4000 PFU of PTV (200 LD<sub>50</sub>) given subcutaneously. An identical challenge was carried out on groups of 8-week-old mice, 4 h after injection of the indicated amounts of rabbit anti-mouse interferon serum.

*Irradiation of animals.* Animals were subjected to a lethal dose of radiation (950 rads = 1 LD<sub>00</sub>) as an immunosuppressive measure, with a Gammacell 40 Cesium-137 source (Atomic Energy of Canada, Ottawa). The dose rate was calculated to be 115.5 rads/min. Following this level of radiation, the mean survival time for 8-week-old C57BL/6J mice was 10 days.

*Passive transfer of antibody.* Four-week-old C57BL/6J mice in groups of 10 were given a single dose of 0.2 ml of unfractionated hyperimmune mouse ascitic fluid, 4 h before infection, or 24 or 48 h after infection. This ascitic fluid possessed neutralization and ELISA titers of 640 and 2000, respectively.

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APPENDIX 2

Antigenic Analysis of Punta Toro Virus and Identification of  
Protective Determinants with Monoclonal Antibodies

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PROTEINS

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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.

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

Hybridoma cell lines 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. These 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 vivo in a murine model. Of 36 independently derived hybridomas obtained, 23 monoclonal antibodies reacted with the G2 glycoprotein, five with the G1 glycoprotein, and nine with the nucleocapsid protein. Both G1- and G2-specific antibodies were obtained which neutralized virus infectivity in vitro, indicating that determinants on both of these antigens are exposed at the virion surface under physiological conditions. Monoclones to both surface proteins also inhibited hemagglutination, whereas nucleocapsid-specific antibodies were inactive in both neutralization and hemagglutination inhibition assays. 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. The monoclonal antibodies were assessed for their ability to protect in vivo, both alone and in the context of an impaired immune response. Five G1-reactive monoclonal antibodies which bound to epitopes in two distinct topological sites, as determined by competitive binding assays, were found to be capable of providing efficient

protection to both immunocompetent and immunosuppressed mice. In contrast, of eight G2-reactive antibodies which protected immunocompetent mice, only one was able to protect immunosuppressed animals. The degree of protection achieved in vivo did not correlate directly with in vitro neutralization titers.

## 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 worlds, 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 virion particle is enveloped and contains the genomic RNA complexed with a nucleocapsid protein of 26,000 daltons. Associated with the envelope are two glycoproteins, G1 and G2, of 66,000 and 56,000 daltons, 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 daltons) 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; Ihara et al., 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 non-structural protein (NS<sub>m</sub>), was found to be of negative polarity, the S segment, which codes for the nucleocapsid protein and a presumptive non-structural protein (NS<sub>s</sub>), 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 phleboviruses. We have recently described an animal model in C57Bl/6J mice for PTV infections (Pifat and Smith, 1987), and this model system was used to identify those monoclonal antibodies 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

Vero cell monolayers were infected with the Adames strain of Punta Toro virus (Pifat and Smith, 1987) at a multiplicity of infection (MOI) of 0.1, and incubated for 48 hr in EMEM containing 2% fetal bovine serum (FBS) at 36°. Virus particles in media fractions were concentrated by ammonium sulfate precipitation and purified by gradient centrifugation as described previously (Ihara et al., 1985a). <sup>35</sup>S-methionine-labeled virions were prepared similarly by the addition of 50 uCi/ml of <sup>35</sup>S-methionine to EMEM otherwise free of methionine. Protein concentrations were determined spectrophotometrically with a dye-binding assay (BioRad Laboratories, Richmond, California).

### 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. Adult Balb/cJ mice undergo an acute infection with the Adames strain of PTV, but greater than 90% of the mice survive (Pifat and Smith, 1987). 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, gamma-irradiated PTV or purified

glycoproteins. The glycoprotein preparation was obtained by the disruption of 2.8 mg of purified virus with 2% octylglucoside (Calbiochem, La Jolla, California) in TNE (0.1M NaCl, 0.01M Tris, 0.001M EDTA; pH 7.2). This lysate was layered over a discontinuous CsCl gradient consisting 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 to remove the detergent and residual CsCl and to effect precipitation of the glycoproteins. This glycoprotein preparation was 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/O-Ag14 myeloma cells (Shulman et al., 1978) was essentially that described by Kennett et al. (1980) and Earley and Osterling (1986). Briefly, spleen cells and Sp2/O-Ag14 cells were washed in Optimem medium (Gibco, Grand Island, New York), mixed in a 1.5:1 ratio, collected by centrifugation, and the

pellet gently resuspended at 42° in 2 ml of DMEM containing 50% PEG 1500 (Hybridoma Science Inc., Atlanta, Georgia). This cell suspension was centrifuged at 275 X g at 37° for 30 sec, slowly diluted to 20 ml with Optimem over 4 min, and then diluted to 50 ml. The cell suspension was centrifuged for 7 min at 275 X g, resuspended in 50 ml of HAT medium, and distributed into 96-well plates over feeder layers of mouse peritoneal macrophages. These 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. HAT medium was replaced by HT medium 14 days after plating.

Selected hybridoma cultures were cloned by limiting dilution using Balb/cJ mouse peritoneal macrophages as a feeder layer. 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".

#### **Production of ascitic fluid**

Two weeks after "priming" adult Balb/cJ mice by intraperitoneal injection of Pristane (2,6,10,14-tetramethyl

pentadecane),  $10^7$  viable hybridoma cells were injected intraperitoneally. After development of ascites, fluid was removed, pooled, and clarified by centrifugation, and stored in 1 ml aliquots at  $-20^\circ$ .

#### **Enzyme-linked immunoassay**

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

#### **Plaque reduction neutralization test**

This test was performed essentially as described by Earley et al. (1967). Dilutions of ascitic fluids were incubated overnight at  $4^\circ$  with approximately 50 PFU of PTV, and used to infect duplicate monolayers of confluent Vero cells in 24-well plates. After 1 hr at  $37^\circ$ , each well was overlaid with 0.5% agarose in EMEM, incubated for an additional 5 days at  $37^\circ$ , and subsequently stained by the addition of 0.01% neutral red in EMEM containing 0.5% agarose. Titers are expressed as the highest antibody dilution giving either 80% or 50% plaque reduction.

#### **Hemagglutination inhibition test**

This test was 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, Illinois) and detected with a peroxidase-labeled goat-anti-rabbit IgG.

#### **Specificity determination by immunoprecipitation**

Radio-labeling of viral antigens, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis were carried out as previously described (Smith and Pifat, 1982). Briefly, Vero cell monolayers were infected with PTV at a MOI of 5, incubated for 16 hr, and then labeled with <sup>35</sup>S methionine (50 uCi per ml) for 2 hours in methionine-deficient medium. The monolayers were then disrupted in lysis buffer, and aliquots of this lysate were incubated with 50 ul of each ascitic fluid for 4 hr at 4°. Immune complexes were collected on protein A-sepharose, washed and solubilized in 100 ul of SDS-sample buffer, and resolved on 13% DATD-crosslinked polyacrylamide gels. Immunoprecipitates

with monoclonal antibodies which were not bound by protein A were collected on agarose beads containing goat anti-mouse immunoglobulins (Cappel Laboratories, Malvern, Pennsylvania). Following electrophoresis, the gels were fixed in 20% trichloroacetic acid and fluorographed as described by Bonner and Laskey (1974).

#### **Indirect immunofluorescence assay**

Confluent Vero cell monolayers were infected with heterologous phleboviruses, incubated for 48 hr at 37°, harvested, and mixed with an equal number of uninfected cells, which served as controls. The mixtures of infected and uninfected cells were placed on teflon-coated glass slides, air dried, and fixed with acetone at 4°. 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 (PBS), pH 7.4 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<sub>50</sub>) 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, Missouri). Four-week old C57BL/6J mice were passively immunized with 1 to 100 ul of purified monoclonal antibody, 24 hr after a subcutaneous virus challenge, as above. All mice were monitored for illness or death for 30 days.

#### **Labeling of monoclonal antibodies with <sup>35</sup>S-methionine**

Selected monoclonal antibodies which were found to be protective were metabolically labeled with <sup>35</sup>S-methionine essentially as described by Yelton et al. (1981). Hybrid cells (10<sup>7</sup>) in logarithmic growth phase were resuspended in 10 ml of methionine-free EMEM containing 5% FBS, 1% non-essential amino acids, 3.5 mg/ml of glucose, and <sup>35</sup>S-methionine (100 uCi/ml). After a 24 hr incubation, the cells were removed by centrifugation and replaced by a second aliquot of 10<sup>7</sup> log-

phase cells. Twenty four hours later this process was repeated. The culture medium was then collected and dialyzed against PBS containing 2 mM unlabeled methionine.

#### **Competitive binding assays**

<sup>35</sup>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<sup>-1</sup> to 10<sup>-7</sup> and allowed to react for 1 hr at 37°. Negative controls consisted of equivalent dilutions of normal ascitic fluid prepared with SP2/O cells. Plates were drained and subsequently incubated with 50 ul of the appropriate dilution of <sup>35</sup>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, placed in a scintillation vial, and counted in a scintillation counter. The competing titer of each monoclonal was calculated as the last dilution of unlabeled ascitic fluid to cause a 50% reduction in bound radioactivity. The radioactivity bound with <sup>35</sup>S-labeled PTV-specific monoclonal antibodies 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

Of 20 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  $^{35}\text{S}$ -methionine-labeled, PTV-infected cell lysates revealed that 23 of the monoclonal antibodies reacted with the G2 (56 K) surface glycoprotein, while four reacted with the G1 (66 K) glycoprotein. Nine hybridomas with demonstrable anti-nucleocapsid activity in screening assays were subcloned and their expected specificity confirmed by immunoprecipitation as above. Monoclonal antibodies were not

obtained which reacted with the large, 200,000 dalton protein, nor were any monoclonal antibodies obtained which reacted with presumptive non-structural proteins. Similarly, none of these monoclonal antibodies precipitated polypeptides of higher molecular weight than the 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 monoclonal antibodies as determined in ELISA assays with isotype and class-specific rabbit antisera.

The ELISA titers obtained under conditions of antigen excess varied between  $1 \times 10^3$  and  $1.6 \times 10^5$ , and were independent of the antigen specificity or the isotype of the monoclonal antibodies (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 ml of ascitic fluid. The much wider variation in endpoint ELISA titers of these same ascites suggests that a substantial range of affinities is represented by this panel of antibodies.

All monoclonal antibodies 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, suggesting that each antibody possessed a unique binding specificity. Based on these experiments, at least three distinct binding sites on the nucleocapsid protein, and at least 21 distinct antibody binding sites on the G2 protein were recognized by this panel of monoclonal antibodies. One monoclonal (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 monoclonal antibodies also exhibited 80% endpoint titers (Table 2), whereas 17 additional G2-specific monoclonal antibodies 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 demonstrable 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 (Table 1-3). The HI titers of the G1-reactive monoclonal antibodies varied between 640 and 2560 (Table 1), while the titers for the G2-reactive monoclonal antibodies varied between 10 and 10,240 (Table 2,3). None of the nucleocapsid-reactive monoclonal antibodies 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. Post-infection administration ensured that target organs were infected. All of the G1-reactive monoclonal antibodies provided efficient protection (Table 5). In addition, most of the neutralizing G2-specific monoclonal antibodies also provided significant protection (Table 6). However, the IgM monoclonal antibody, 4G11, which possessed a high in-vitro neutralization titer, 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 monoclonal antibody 6E8, which had very low neutralization activity, protected all mice from an otherwise lethal virus challenge.

Although these studies were conducted with unfractionated ascitic fluids, there was no indication that non-immunoglobulin components contributed to the protection observed. Twenty-three other ascitic fluids containing non-neutralizing monoclonal antibodies reactive either with G2 or the nucleocapsid proteins, or normal ascitic fluids produced with Sp2/O 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 and, were found to elute in a single peak by low pressure cation exchange

chromatography. Passive immunization with either of these purified immunoglobulins protected all mice in doses of less than 1 ug 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 non-neutralizing, G2-reactive

monoclonal antibodies described previously, and several selected nucleocapsid-reactive monoclonal antibodies, were tested in these studies and were found to provide no protection (data not shown).

#### Competitive binding assays

Ten monoclonal antibodies 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 clones 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.

## DISCUSSION

In this study we produced and characterized 36 monoclonal antibodies to the 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 monoclonal antibodies 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.

In a previous effort to generate monoclonal antibodies to PTV antigens, spleen donor mice were immunized and boosted with infected mouse brain suspensions and hybridoma culture supernatants were screened against unfractionated, infected cell lysates (Dalrymple et al., 1981). This study yielded 99 PTV-reactive monoclonal antibodies of which 94 reacted with the nucleocapsid polypeptide, four with the G2 glycoprotein, and a single antibody was found to be G1-specific. In the present study, immunization and screening procedures were designed to increase the yield of glycoprotein-reactive monoclonal antibodies. Although this was successful in that 27 glycoprotein-specific monoclonal antibodies were obtained, most of the positive hybridomas which were not further characterized, nonetheless secreted antibodies directed at the nucleocapsid polypeptide. This has also been

found to be the case with the related phlebovirus, Rift Valley Fever virus, and presumably reflects the immunodominance of this structurally repetitive immunogen.

The antigenic analysis of PTV using these monoclonals confirmed that both surface glycoproteins possess determinants capable of eliciting neutralizing antibodies as monitored in vitro. As the plaque-reduction assays are carried out under physiological conditions, these data indicate that both glycoproteins carry determinants which are accessible at the virion surface. *Although not related to both glycoproteins also inhibit them* Such predictions are not readily made from results of HI assays, which are carried out at reduced pH *something detected* may therefore induce conformational alterations. Therefore both the serological assay which is used to group isolates to the Phlebovirus genus (HI) and the assay used to define individual viruses (neutralization) monitor determinants on both glycoproteins.

Studies which have analyzed the binding of these PTV-reactive monoclonals to heterologous phlebovirus antigens have shown that all of the G1 monoclonals were PTV-specific and the anti-nucleocapsid monoclonals showed very limited cross-reaction. In contrast, 20 of the 27 anti-G2 monoclonals cross-reacted with at least one and as many as seventeen other phleboviruses. The observation that each of these monoclonals had a unique cross-reactivity pattern suggests that each recognizes a distinct determinant and therefore a minimum of 21 non-identical antigenic sites must exist on the PTV G2 protein.

The observation that PTV G2 glycoprotein appears to be antigenically more conserved than the G1 glycoprotein is consistent with existing sequence information which has demonstrated 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 monoclonal 1E11, which neutralizes PTV and cross reacts with RVFV in ELISA or immunofluorescence assays fails to neutralize RVFV in vitro or protect mice challenged with RVFV (data not shown). The relatively few epitopes held in common between PTV and any other phlebovirus imply that none of the viruses studied here has originated recently from direct segment reassortment between PTV and a heterologous phlebovirus. A number of the monoclonals described here should nonetheless prove useful in diagnostic assays, particularly in discriminating between closely related viruses, such as those belonging to the Naples serogroup, which are often difficult to distinguish in classical assays using polyclonal sera.

The passive protection studies not only confirm that immunoglobulins alone can protect mice lethally infected with PTV, but also demonstrate that antibodies to several individual epitopes can provide efficient protection. All five of the G1-specific monoclonals studied here, which define epitopes in two topological sites, as well as a single G2-specific monoclonal, 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 monoclonal antibodies 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 monoclonal antibody (6E8) which showed very low in vitro neutralization capacity, nonetheless protected all immunocompetent mice and 90% of the immunosuppressed animals. In contrast, an IgM monoclonal antibody (II 4G11), with a relatively high neutralization titer, was able to protect only 2/10 immunocompetent and none of the immunosuppressed mice. Non-neutralizing 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 mechanism by which non-neutralizing antibodies may protect has not been determined

with certainty, but most theories have focused on the presumed ability of these antibodies to bind to viral antigens on the surface of infected cells, thereby rendering these cells susceptible to cellular or complement-mediated lysis.

Alternative mechanisms could involve direct opsonization, or possibly lysis of enveloped virus particles following activation of the classical complement pathway. In any event, 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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TABLE 1  
 CHARACTERISTICS OF G1-REACTIVE MONOCLONAL ANTIBODIES

DESIGNATOR	ELISA TITER	NEUTRALIZATION TITER		HI TITER	ISOTYPE
		80%	50%		
II 5F9-2-3	80,000	2560	>10240	1280	IgG2a, k
II 2F9-2	160,000	640	>10240	1280	IgG2a, k
A. 7F5	10,000	640	2560	1280	IgG2a, k
III 1D10-1	40,000	40	2560	640	IgG2b, k
I 3E8	160,000	2560	>10240	2560	IgG1 , k

TABLE 2  
 CHARACTERISTICS OF NEUTRALIZING<sup>a</sup> G2-REACTIVE MONOCLONAL ANTIBODIES

DESIGNATORS	ELISA TITER	NEUTRALIZATION TITER		HI TITER	ISOTYPE
		80%	50%		
I1 4G11	5,000	640	>10240	5120	IgM , k
A. 9G6	10,000	640	2560	1280	IgG2a, k
I1 3E6	15,000	160	>10240	2560	IgG2a, k
I 1E11	30,000	160	2560	10240	IgG2a, k
I1 1F3	80,000	160	2560	320	IgG2a, k
I1 1B5	40,000	160	2560	40	IgG2a, k
I 2F8-1-5	160,000	40	>10240	2560	IgG2a, k
I 2E2-3	40,000	40	640	1280	IgG2a, k
I2F6-1-1	40,000	10	640	2560	IgG2a, k
I1 6E8	40,000	10	160	1280	IgG2a, k

<sup>a</sup> Defined as 80% plaque-reduction neutralization.

TABLE 3  
 CHARACTERISTICS OF NON-NEUTRALIZING<sup>a</sup> G2-REACTIVE MONOCLONAL ANTIBODIES

DESIGNATORS	ELISA TITER	NEUTRALIZATION TITER		HI TITER	ISOTYPE
		80%	50%		
II 5F3	10,000	<10	2560	640	IgM , k
II 2F9-3	80,000	<10	>10240	2560	IgG2a, k
II 2G3	20,000	<10	2560	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

<sup>a</sup> Defined as 80% plaque reduction neutralization

TABLE 4  
 CHARACTERISTICS OF NUCLEOCAPSID-REACTIVE MONOCLONAL ANTIBODIES

DESIGNATORS	ELISA TITER	NEUTRALIZATION TITER		HI TITER	ISOTYPE
		80%	50%		
I 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 2C7	80,000	<10	<10	<10	IgG2a, k
II 4B3	40,000	<10	<10	<10	IgG2a, k
III 1D10-2	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 <sup>a</sup>	NORMAL MICE <sup>b</sup> PROTECTED/TOTAL	MEAN DAY TO DEATH	RANGE	IRRADIATED MICE <sup>c</sup> PROTECTED/TOTAL	MEAN DAY TO DEATH	RANGE
I. 7F5	9/9	-	-	10/10	- <sup>d</sup>	-
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

<sup>a</sup> 0.2 ml of undiluted ascitic fluid was transferred 24 hr after viral challenge.

<sup>b</sup> 4-week-old C57Bl/6J mice were inoculated subcutaneously with 4000 PFU of PTV.

<sup>c</sup> 4-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.

<sup>d</sup> Survival equivalent to animals receiving radiation alone.

TABLE 6

## PASSIVE IMMUNIZATION WITH G2-REACTIVE MONOCLONAL ANTIBODIES

MONOCLONE <sup>a</sup>	NORMAL MICE <sup>b</sup> PROTECTED/TOTAL	MEAN DAY TO DEATH	RANGE	IRRADIATED MICE <sup>c</sup> 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
II 2E2-1	n.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

<sup>a</sup> 0.2 U1 of undiluted ascitic fluid was transferred 24 hr after inoculation.

<sup>b</sup> 4-week-old C57BL/6J mice were inoculated subcutaneously with 4000 PFU of PTV.

<sup>c</sup> 4-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.

TABLE 7  
 COMPETITIVE BINDING ANALYSIS<sup>a</sup>  
 WITH SELECTED PTV-REACTIVE 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 <sup>2</sup>	10 <sup>2</sup>	-	-	-	-	-	-	-	-
I 1B3(G2)	10 <sup>3</sup>	10 <sup>2</sup>	-	-	-	-	-	-	-	-
I 2F8-1-5(G2)	-	-	10 <sup>3</sup>	-	-	-	-	-	-	-
II 6E8(G2)	-	-	-	10 <sup>2</sup>	10 <sup>3</sup>	-	-	-	-	-
A. 9G6(G2)	-	-	-	10 <sup>2</sup>	10 <sup>2</sup>	-	-	-	-	-
III 1D10-1(G1)	-	-	-	-	-	10 <sup>3</sup>	10 <sup>3</sup>	10 <sup>3</sup>	10 <sup>3</sup>	-
I 3E8(G1)	-	-	-	-	-	10 <sup>2</sup>	10 <sup>2</sup>	10 <sup>2</sup>	10 <sup>2</sup>	-
II 5F9-2-3(G1)	-	-	-	-	-	10 <sup>3</sup>	10 <sup>2</sup>	10 <sup>2</sup>	10 <sup>2</sup>	-
II 2F9-2(G1)	-	-	-	-	-	10 <sup>3</sup>	10 <sup>3</sup>	10 <sup>3</sup>	10 <sup>2</sup>	-
A. 7F5(G1)	-	-	-	-	-	-	-	-	-	10 <sup>2</sup>

<sup>a</sup> The competing titer of each monoclonal was calculated as highest dilution of unlabeled ascitic fluid to cause a 50% reduction in bound radioactivity when compared to controls.

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  $^{35}\text{S}$ -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 E and H are aliquots of the same cell lysate immunoprecipitated with G1, G2, and nucleocapsid-reactive monoclonones, respectively.

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).



A B C D E F G H

