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David H.L. Bishop and Richard W. Compans, eds.  
The Replication of Negative Strand Viruses

## Antigenic Components of Punta Toro Virus

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The sandfly fever agents or Phlebotomus Fever Serogroup viruses have been recently proposed as members of a *Phlebovirus* genus of the family Bunyaviridae. Together with the most prominent member of this proposed genus, Rift Valley Fever virus, these agents share antigenic characteristics and common molecular characteristics. All members so far studied contain a negative-stranded, tripartite, RNA genome, two surface envelope glycoproteins and a smaller, nonglycosylated nucleocapsid or core protein. The participation of these structural proteins in the serological reactivities of these viruses has not yet been described.

Our research has been primarily concerned with developing models that will help to explain the diversity that exists between members of this group in their biology, antigenicity and molecular characteristics such as oligonucleotide fingerprints. We suspect that genetic change in nature may be a continual process and that dual infection of arthropod vectors resulting in natural reassortants is a real possibility. If these genetic changes are phenotypically reflected as changes in the antigens of these viruses, it becomes difficult to pursue vaccine development, interpret serological responses or predict immunity without defining specific antigenic determinants as markers. Thus, describing some of these antigens for a single virus was the purpose of this preliminary study.

Punta Toro virus was selected as a starting point because of its antigenic relationship to Rift Valley Fever virus, a major pathogen that we plan to

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ultimately investigate; however, Punta Toro virus does not require the strict laboratory containment of the Rift Valley Fever agent. Other reasons for the selection of this agent included the recent observation that isolates of Punta Toro virus from different geographic regions of Panama differ markedly in their virulence for laboratory animals (Table 1). Such variation appears quite common among this group of agents. An Egyptian strain of Rift Valley Fever virus produces a benign infection of Lewis rats, a fulminant infection of Wistar-Furth animals and a late encephalitis of ACI strain rats. Different strains of Rift Valley Fever virus can be readily distinguished by the disease produced in the Wistar-Furth rat. Lunyo, an agent antigenically indistinguishable from Rift Valley Fever virus, produces no detectable disease in mice. Avirulent variants can be selected from virulent isolates of RVFV that produce no disease in rat, hamster, or mouse and, as mentioned previously, different geographic isolates of Punta Toro virus differ markedly in virulence for the hamster.

Lymphocyte hybridomas producing monoclonal antibody were investigated as a possible method that would provide: (1) identification of the major virus antigens that contribute to immunity or protection from disease, (2) an adjunct to the classical biochemical and biophysical antigen separation procedures that classically result in a certain amount of degradation, and (3) the serological tools necessary to investigate the antigenic relationships that these viruses share. Infected suckling mouse brain suspensions were selected for animal immunization in anticipation of a preparation containing all virus-specified proteins and a minimum number of foreign host cell contaminants. Young adult BALB/C mice were given a primary immunizing injection and a booster intravenous injection at eight weeks. Three days after the second injection, spleens were harvested from the immunized mice and fused with the P3 plasmacytoma IgG<sub>1</sub> secreting cell line using polyethylene-glycol. Cultures of the fused products or lymphocyte hybridomas were screened for Punta Toro virus antibody with a solid phase, radioimmune assay (RIA) using nonionic detergent disrupted infected cell lysates as anti-

**Table 1. Evidence of Genetic Variation in Various Phlebotomous Fever Serogroup Viruses as Measured by the Disease Produced in Laboratory Animals.**

Genetic variable	Constant	Disease		
		Benign	Fulminant	Late encephalitis
Inbred rats	Egyptian RVF virus	Lewis	Wistar-Furth	ACI
RVF virus	Wistar-Furth rat	South African	Egyptian	—
RVF virus	Mouse	Lunyo	Other	—
RVF virus	Mouse, hamster, rat	Selected clones	Egyptian	
Punta Toro	Hamster	Western Panama	Eastern Panama	

gen which were attached directly to polyvinyl chloride microtiter plates (Table 2). Of the 864 cultures inoculated with the products of the cell fusion, 675 or 78% produced actively growing hybridomas. A total of these growing cultures yielded RIA detectable antibody in supernatant fluids. Over 300 of these culture fluids were also examined by fluorescent antibody (FA) procedures and obtained good correlation with the preceding RIA data. Because of the physical limitations of the laboratory, only 105 of these positive cultures were subsequently cloned on soft agar over feeder layers. Representative clones, presumably originating from a single hybridoma cell, were selected, again on the basis of an RIA positive supernatant, passed to larger cultures and frozen in liquid nitrogen. Only 19 of these have been removed from nitrogen, passed in culture to produce antibody containing cell culture fluids and injected into Pristane primed BALB/C mice for the production of ascitic fluids. These 19 clones are presented in some detail and include all cultures of current serological interest.

To determine if any of these monoclonal antibodies were directed at nonstructural virus proteins, the 105 positive cell supernatants were examined by RIA using both infected cell lysate antigens and purified virion antigens. All of these 105 hybridomas reacted with both infected cells and purified virions.

Having selected 105 clones on the basis of their RIA and FA reactions, it was next of interest to determine the specific viral proteins that contained the antigenic determinants recognized by these monoclonal antibodies. This was accomplished by an immunoprecipitation assay in which Punta Toro virus infected cells served as antigen. Infected cells were labeled for 5 hours with  $^3\text{H}$ -leucine 16 hours after infection. These cells were subsequently solubilized in buffer containing detergents, and aliquots of this lysate were reacted with media supernatants of each of the selected clones. The resulting immune complexes were precipitated with solid phase protein A, washed repeatedly in lysis buffer and prepared for discontinuous electrophoresis and fluorography. The precipitates generated by anti-Punta Toro hyperimmune mouse ascitic fluids served to establish the electrophoretic migration of each of the known viral proteins.

**Table 2. Fusion Efficiency in Lymphocyte Hybridoma Production and Selection of Representative Clones.**

Production of lymphocyte hybridomas producing monoclonal antibody to Punta Toro virus	
Hybridomas produced	675/864—78%
Positive for Punta Toro antibody	435/675—64%
Selected for cloning and freezing	105/435—24%
Selected for intensive study	19/105—18%

Using these procedures, the majority of the selected clones (83) were shown to react specifically with the nucleocapsid protein (27K), four reacted with GP2 (56K) and one with GP1 (66K). Unreacting clones were further tested in a modified indirect assay utilizing rabbit antisera reactive against mouse IgM, IgA and IgG<sub>1</sub>. This procedure demonstrated that seven additional clones contained activity against the nucleocapsid protein. The specificity of the remaining clones could not be determined and presumably represents clones that no longer produce antibody or do so at undetectable levels.

Each of the monoclonal antibody containing ascitic fluids were examined for their ability to inhibit virus hemagglutination (Table 3). The anti-GP1 and all four of the anti-GP2 clones reacted to high titer with the homologous Punta Toro antigen. None of the anti-nucleocapsid reactors exhibited any evidence of inhibition. All ascitic fluids were similarly tested for inhibition of Rift Valley Fever virus hemagglutinin but were uniformly negative.

Neutralizing activity of these antibodies was examined using the plaque reduction neutralization test (Table 4). Antibody to the GP1 determinant neutralized Punta Toro virus to high titer as did one of the reactors to GP2. The other three anti-GP2 reactors must be considered suspect because these low titers do not reflect the higher hemagglutination-inhibition titers of these same clones. There were surviving plaques in the titration of these three ascitic fluids, making the precise estimation of a titer difficult and perhaps suggesting a different antibody avidity or a separate determinant. Again the anti-nucleocapsid ascitic fluids were negative and all monoclonal antibodies failed to neutralize Rift Valley Fever virus.

Even though the ability of serum neutralizing antibody is often associated with immunity, the ultimate test for protection involves challenge of an

**Table 3. Reaction of Ascitic Fluids Containing Monoclonal Antibody in the Hemagglutination-Inhibition Test.**

Antibody	Antigen	
	Punta Toro	Rift Valley Fever
Anti-GP1	320	<10
Anti-GP2		
(A)	640	<10
(B)	320	<10
(C)	160	<10
(D)	1280	<10
Anti-nucleocapsid (N = 14)	all < 10	all < 10
Punta Toro HMAF	320	40
Rift Valley Fever HMAF	10	160

**Table 4. Evidence of Plaque Reduction Neutralizing Antibody in Ascitic Fluid from Hybridomas Reactive to the Envelope Glycoproteins.**

Antibody	Virus	
	Punta Toro	Rift Valley Fever
Anti-GP1	2560	<10
Anti-GP2		
(A)	40	<10
(B)	40	<10
(C)	±10	<10
(D)	2560	<10
Anti-nucleocapsid (N = 14)	<10	—
Punta Toro HMAF	>2560	40
Rift Valley Fever HMAF	±10	2560
Karimabad HMAF	10	40

animal with the infectious agent. We attempted to determine the protective capacity of these monoclonal antibodies by injecting hamsters with ascitic fluids and challenging with virulent Punta Toro virus (Table 5). The prototype Punta Toro virus used to immunize the mice for the preparation of the hybridomas does not normally kill hamsters. Consequently, the challenge virus consisted of the virulent isolate from eastern Panama previously described. The single anti-GP1 and an additional anti-GP2 were capable of protecting hamsters from a lethal challenge. It is not surprising that these are the same antibody preparations that contained high titered neutralizing antibody. One of the anti-GP2 ascitic fluids remains to be examined.

**Table 5. Passive Protection of Hamsters Using Monoclonal Antibodies to Punta Toro Envelope Glycoproteins.**

Hybridoma	Protection
Anti-GP1	Pos
Anti-GP2	
(A)	Neg
(B)	Neg
(C)	NT
(D)	Pos
Anti-nucleocapsid (N = 11)	Neg
Punta Toro HMAF	Pos

In summary, we have produced a number of lymphocyte hybridomas producing monoclonal antibodies to Punta Toro virus. The majority of these antibodies reacted exclusively with the nucleocapsid protein; however, four clones produced antibody to envelope glycoprotein GP2 and a single hybridoma produced antibody to GP1. All five of the monoclonal antibodies to envelope glycoproteins inhibited Punta Toro virus hemagglutination. The anti-GP1 and a single anti-GP2 clone had high virus neutralization titers and each of these were capable of passively protecting hamsters from lethal virus challenge. None of these monoclonal antibodies cross reacted with Rift Valley Fever virus in the limited number of experiments conducted. It is anticipated that further study of these viruses with monoclonal antibody will ultimately lead to a better picture of their antigenic composition and the biological function of these proteins.

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