Characterization of Anti-Pichinde Virus Monoclonal Antibodies for the Directed Delivery of Antiviral Drugs and Toxins

Noah Jefferson Burns III
Utah State University

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CHARACTERIZATION OF ANTI-PICHINDE VIRUS MONOCLONAL ANTIBODIES FOR THE DIRECTED DELIVERY OF ANTIVIRAL DRUGS AND TOXINS

by

Noah Jefferson Burns, III

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biology

(Virology and Immunology)

UTAH STATE UNIVERSITY
Logan, Utah

1989
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ABSTRACT

Characterization of Anti-Pichinde Virus Monoclonal Antibodies for the Directed Delivery of Antiviral Drugs and Toxins

by

Noah Jefferson Burns, III, Doctor of Philosophy
Utah State University, 1989

Major Professor: Dr. Robert W. Sidwell
Dissertation Advisor: Dr. Bill B. Barnett
Department: Biology

Mouse monoclonal antibodies directed against Pichinde virus (PCV) were produced to evaluate their application as vehicles for the delivery of antiviral drugs or toxins to virus-infected cells. Four monoclonal antibodies, PC4.9A6, PC4.9D3, PC4.7C2, and PC4.8D3, were of the IgG2a subisotype and reacted with acetone-fixed and live PCV-infected Vero-76 cells. In vivo stained splenic macrophages derived from PCV-infected hamsters that had been injected with fluorescein-labeled PC4.9A6 (FITC-9A6) demonstrated a 400% increase in total fluorescence over similarly treated, non-infected cells when analyzed by flow cytometry. This is an indication that FITC-9A6 does have some ability to specifically target PCV-infected cells in vivo. Radioimmunoprecipitation of viral proteins showed that all the antibodies precipitated two different PCV proteins, one of 64,000 daltons and another of 38,000 daltons. These proteins are, respectively, PCV
nucleoprotein (NP) and a breakdown product of NP that is present in PCV-infected cells.

An immunofluorescent assay (IFA) for PCV was developed. This IFA was used for antiviral drug assays against PCV. The assay was performed by adding fluorescein-labeled anti-PCV monoclonal antibody to fixed, virus-infected cells at 24 h after infection and counting the fluorescent cells. The 50% effective dose (ED$_{50}$) for ribavirin against PCV using this IFA was 6.0 µg/ml. The ED$_{50}$ of ribavirin using inhibition of marginal PCV cytopathogenic effect after 12 days was 6.0 µg/ml and using plaque reduction after 5 days is 2.5 µg/ml, indicating that this IFA was of comparable sensitivity.

An immunotoxin (IT) was produced by the conjugation of gelonin to PC4.9A6. This IT was tested in vivo in PCV-infected MHA hamsters. It was not active against the disease at the dosage tested and by the intraperitoneal (i.p.) treatment route employed in this study. The positive control, ribavirin, administered i.p. for 14 days at a dosage of 40 mg/ml significantly increased the number of survivors. Three of 5 IT toxicity control animals developed some humoral response that inhibited PC4.9A6 binding to infected cells. They did not show any humoral response to the gelonin moiety of the IT.
CHAPTER I
INTRODUCTION

The Arenaviridae family contains a group of viruses that have proven to be serious human health threats. Some members of this family of viruses, Junin, Machupo, and Lassa (the etiological agents of Argentine hemorrhagic fever, Bolivian hemorrhagic fever, and Lassa fever, respectively), are endemic in some areas of Africa and South America. Mortality rates vary from 3 to 30% of infected individuals during outbreaks of these viral diseases (2).

Currently available therapies, such as treatment of patients with immune plasma from previously infected individuals, have not effectively controlled outbreaks of any of the above-mentioned viral diseases. Treatment of these diseases with ribavirin, the antiviral drug that is currently the most effective drug against Lassa fever, does not ensure 100% survival of the infected individuals (2). Also, it is very difficult to ensure an adequate supply of immune plasma in many of the areas where the outbreaks may occur because of the remoteness of the locations and inadequate public health facilities available. Because these diseases are carried by rodents, it is difficult to determine where the next outbreak might occur. There are few effective preventative measures to protect against these diseases because of continuing difficulties in developing suitable vaccines against the hemorrhagic fever viruses. Thus, the arenaviruses do represent a severe health threat in countries where these viruses are endemic.

Pichinde virus (PCV) is an arenavirus from South America that is much less pathogenic to humans but still causes a very severe hemorrhagic disease in guinea pigs (1) and hamsters (3). Because of the potential for human
disease associated with the more virulent arenaviruses, PCV is a good in vivo model for the study of the pathogenesis of hemorrhagic diseases as well as for the evaluation of the efficacy of novel antiviral therapies to combat the illnesses.

Because of the dependence of viruses on cellular enzymes and ribosomes for protein synthesis and replication, it is very difficult to target a therapeutic agent to inhibit only virus-specific biochemical events. Compounds that interfere directly with the replication of the viral genome or with the synthesis of viral proteins will often inhibit similar events in the noninfected host cell. Thus, new means of targeting antiviral drugs specifically to infected host cells have the potential to significantly increase the therapeutic index of an antiviral compound. Theoretically, the targeting of antiviral agents to infected cells should bring about a significant decrease in overall host toxicity and an accompanying increase in the specificity of action of the antiviral agent.

In this research project, the effectiveness of a new means of targeting antiviral drugs or toxins using monoclonal antibodies directed against Pichinde virus (PCV) proteins was studied. Monoclonal antibodies produced against PCV were characterized as to their isotype and subisotype, reactivity with PCV-infected cells in vitro and in vivo, and reactivity with PCV-specific proteins. Also, because of the anticipated low yields of immunoconjugate production and the insufficient development of PCV cytopathogenic effect (CPE) in cell culture, an antiviral assay that used a minimum of compound but that still had a readily determined endpoint was developed. A promising anti-PCV monoclonal antibody was conjugated with the plant toxin gelonin. The resulting immunotoxin was tested in vivo to determine its antiviral effectiveness in preventing the development of hemorrhagic
disease in MHA hamsters infected with lethal doses of PCV. The humoral responses of the hamsters to the monoclonal antibody and the toxin components of the immunotoxin were also studied.

This study provided a basis for the evaluation of the use of immunotoxin conjugates for the treatment of arenaviral hemorrhagic disease. Insights were gained into the development of more adequate screening procedures for the determination of the suitability of individual monoclonal antibodies for use as vehicles for the targeted delivery of antiviral drugs and toxins. This study provided an opportunity to test the predictive nature of the surface binding and viral peptide binding characteristics of monoclonal antibodies in determining the adequacy of screening procedures used to select monoclonal antibodies for use in this drug/toxin delivery system.
LITERATURE CITED


CHAPTER II
REVIEW OF THE LITERATURE

PICHINDE VIRUS

Initial isolation and characterization of Pichinde virus. Pichinde virus was first reported as a new member of the Tacaribe group of viruses by Trapido and Sanmartin in 1970 (64). It was isolated from a chronically infected South American rodent, Oryzomys albigularis, found in the Pichindé valley of Columbia, hence the name Pichinde virus. Pichinde virus was recovered from the brain, heart, lung, liver, spleen, kidney, adrenal gland, serum, and urine of naturally infected animals. It was included in the Tacaribe group of viruses because of its reactivity in complement fixation (CF) tests with Tacaribe-group immune fluids. Its distinctiveness was also established on the basis of CF and plaque neutralization tests using Pichinde immune ascites fluids, normal serum, and homologous serum (43).

In 1971, the lymphocytic choriomeningitis virus (LCM)-like viruses were all classified in the group arenaviruses (53). This included the newly discovered Pichinde virus (43). The group was named from the Latin arenosus, or sandy, on the basis of the grainy appearance of the interior of the virion when viewed by ultra-thin section (45). The graininess is due to the incorporation of host cell ribosomes into the arenavirus virion. This is a feature that appears to be specific for arenaviruses. The virus group is now the family Arenaviridae.

Mifune et al. (43) characterized the virus as to its size, thermal stability, pH stability, density in sucrose, ether lability, and sensitivity to nucleic acid
inhibitors such as 5-iodo-2'-deoxyuridine (IdU), 1-β-D-arabinofuranosylcytosine (ara-C), actinomycin D, and 6-azauridine. Their results indicated that Pichinde virus was indeed similar to LCM in its physical properties, demonstrating a size of 50-100 μm, relative heat stability, stability over a pH range of 6.0 to 9.0, a buoyant density of 1.18 g/cm³, and an ether-soluble lipid envelope. Moreover, it was inhibited by actinomycin D and 6-azauridine, known RNA polymerase inhibitors, at concentrations that did not affect the replication of DNA viruses. It was also resistant to inhibition by known DNA antagonists IdU and ara-C. Arenaviruses have also been shown to be inhibited by the known antiviral agent ribavirin (1-2).

Since the primary isolation of Pichinde virus by Trapido and Sanmartín (64) and the initial characterization of the virus by Mifune et al. (43), much work has been done in describing both its genetic and antigenic structure. Pichinde virus has been shown to contain several species of RNA. The Pichinde virion contains large (L) and small (S) segments of RNA that have been shown to be the genomic material of the virus (50, 70). The virion also contains host cell ribosomal RNAs of 28 S, 18 S, and 1.6 S (13). Size estimates for the L RNA segment range from 2.63 x 10⁶ to 2.83 x 10⁶ daltons and for the S RNA segment from 1.26 x 10⁶ to 1.31 x 10⁶ daltons, as determined by Ramsingh et al. (50).

Historically, the arenaviruses have been classified as either Old World (e.g., LCM and Lassa viruses) or New World (e.g., Pichinde, Machupo, and Tacaribe viruses) depending upon where, geographically, the virus isolates were obtained. Because LCM has been isolated in both the Old and New World, a second classification based on serological reactivity was developed. This classification divides the Arenaviridae into two complexes: i) the LCM complex containing Ippy, LCM, Lassa, Mabala, and Mopeia viruses and ii) the
Tacaribe complex containing Amapari, Flexal, Junin, Latino, Machupo, Parana, Pichinde, Tacaribe, and Tamiami viruses. The latter classification is based on serological cross-reactivity of the members with both polyclonal and monoclonal antibody preparations. Cross-reactive epitopes within each complex and also between the two complexes have been demonstrated (71). Direct analysis of sequence data from the genomic regions that code for the nucleoprotein (NP) and the glycoprotein precursor protein (GPC) confirm that Pichinde, Lassa, and LCM are indeed all closely related (7, 58, 68).

Synthesis of both the NP (molecular mass [m.m.] = 64,000-66,000 daltons) and GPC (m.m. = ~80,000 daltons) has been mapped to the S segment of the Pichinde virus genome (4, 26). Sequence analysis, combined with appropriate S RNA segment DNA probes, has shown that Pichinde-virus-infected cells contain full length viral and viral-complementary S RNAs. Two distinct subgenomic S RNAs were also detected in infected cells. The subgenomic viral-complementary mRNA coded for NP and the subgenomic virus-sense mRNA codes for the GPC. These observations have resulted in the proposal of an ambisense coding strategy to explain how these proteins are synthesized from the same viral RNA (4, 7). This ambisense strategy denotes only that both the viral-sense and the viral-complementary mRNA transcripts can be used to synthesize viral gene products from the viral genomic S RNA.

Pichinde virus has been shown to code for three major proteins. The NP makes up an estimated 70% of the entire protein mass of the virus. The cleavage products of GPC, GP1, and GP2 make up a total of about 25% of the viral protein mass. There are approximately 400 molecules of each glycoprotein on the surface of the virion and approximately 1500 molecules of NP on the inside of the virion (9, 69).
The m.m.s of the different viral glycoprotein species are disputed in the literature (6, 9, 27, 69). Vezza et al. (69) reported the m.m. for NP to be 66,000 daltons and the m.m. for GP1 and GP2 to be 64,000 and 38,000 daltons, respectively. Harnish et al. (27) reported the m.m. for NP to be 64,000 daltons and the m.m. for GP1 and GP2 to be 52,000 daltons and 36,000 daltons, respectively. The difference in the m.m. of GP1 between the two groups is difficult to explain. Both experiments were done in a baby hamster kidney cell line (BHK-21), suggesting that simple differences in the availability of cellular glycosylases will not suffice as the explanation for the observed difference.

Other minor proteins have been reported for Pichinde virus. This includes a protein, coded on the L segment of the genome, that is thought to be the viral RNA polymerase. It has a m.m. of 200,000 daltons. There are also several other minor proteins, ranging from 12,000 to 77,000 daltons, whose functions have not yet been identified (9).

**Animal models of Pichinde virus-mediated hemorrhagic disease.** Since the initial isolation of Pichinde virus in 1971, it has become the prototype virus for studying the in vivo pathogenesis of arenaviral hemorrhagic disease in small animals such as the Syrian hamster strain MHA (21, 44) and the guinea pig strain 13 (32). Pichinde virus is preferred because of its extremely low potential for infectivity and disease in humans and also because of the reproducibility of the viral infection in these small animals. In addition, because it has been adapted to cause a lethal disease in guinea pigs, it is considered to be an alternative model to Lassa fever in these animals. Lassa fever virus does not produce clinical disease in MHA hamsters (32).
Pathogenesis of viral hemorrhagic fevers. The comparative study of the pathogenesis of Pichinde virus in two different hamster strains reported by Murphy et al. (44) showed that only the MHA strain of adult hamsters was susceptible to lethal infection. The LVG strain of Syrian hamsters, though showing some signs of disease early in the infection, was able to clear the virus completely from its system by day 10 post-infection.

Infection of MHA hamsters with Pichinde virus resulted in a severe hemorrhagic disease in the animals, grossly characterized by edema around the eyes, bleeding from the eyes and nose, and diarrhea, as well as a lethargy as the disease progressed. Internally, infection of the macrophages in the spleen resulted in the depopulation of the organ with destruction of both the red pulp and the white pulp regions. In moribund animals, antigenic and necrotic tissue was observed to be held in place by only an intact splenic capsule.

In the liver, the Kupffer cells and hepatocytes were the targets of viral infection. Necrotic Kupffer cells and hepatocytes were present as foci in infected livers by day 10. A large amount of cellular debris was present in the sinusoids of the organ. The lesions observed in the infected livers have been reported to be very similar to the lesions caused by Lassa fever virus in humans (44, 72).

An observation of interest regarding the natural resistance of the LVG hamsters to Pichinde virus infection is that this resistance can be abrogated by immunosuppression with cyclohexamide. Also, immunosuppression of the MHA hamsters with cyclohexamide did not prevent lethal infection with the virus. Kenyan et al. (35) reported that immunosuppression of guinea pigs that were later infected with virulent Junin virus resulted in no apparent effect on the development of the disease in these animals. They
also demonstrated that immunosuppressed animals infected with an attenuated strain of Junin developed fatal Argentine hemorrhagic fever, whereas the immunocompetent, infected animals did not. Immunosuppression prevented the development of antibody-dependent virus-specific cytotoxic spleen cell activity. Also, no harmful effects induced by the immune response were observed in any of the infected, immunocompetent animals. This evidence suggests that, unlike the immune complex-mediated disease observed in LCM-infected animals (10), there is a direct role for the arenaviruses of the Tacaribe complex and Lassa fever virus in the pathogenesis of the hemorrhagic fevers (44).

The specific mechanisms of viral pathogenesis due to arenaviral infection have been difficult to elucidate. Liu (40) has described the effect of PCV on the hearts of infected guinea pigs. The beating hearts, observed in situ on days 11 to 19 post-infection, showed three types of cardiac abnormalities: i) engorgement of the superior and inferior vena cavae with an accompanying dilation of the right side of the heart, ii) sluggish cardiac contractions and diminished heart rate, and iii) a systolic bulge at the apex of the right ventricle. Cardiac and other tissues sampled immediately after the death of the animals did not manifest any obvious histopathological lesions. Jahrling et al. (32) have previously reported that no virus was recovered from the hearts of infected guinea pigs. Thus, there appears to be no direct histopathological evidence for an involvement of Pichinde virus in causing cardiac dysfunction.

An earlier study by Liu et al. does shed some light on how Pichinde virus infection may be indirectly involved in producing the observed cardiac dysfunction (41). Infection of guinea pigs with Pichinde virus caused an increase in the serum levels of the leukotriene LTD4. Leukotrienes are
eicosanoids and are related to the prostaglandins. They are synthesized from arachidonic acid and have been shown to act as vasoconstrictors and to also increase vascular permeability. The leukotrienes LTC4 and LTD4 are thought to be the most important components of Slow Releasing Substance of Anaphylaxis (SRS-A), which causes smooth muscle contraction often seen in allergic asthma. Treatment of infected animals with the LTD4 antagonist FPL-55712 led to an increase in mean survival time and food intake as well as a decrease in weight loss. Treatment did not mitigate a persistent fever in the guinea pigs. These studies propose that infection with Pichinde virus induced the overproduction of these leukotrienes and, indirectly, that these biochemical "lesions" disturbed the chemical homeostasis of the heart muscle, eventually leading to the death of the animal.

**MONOCLONAL ANTIBODIES**

**Monoclonal antibody technology.** With the introduction of monoclonal antibody technology by Kohler and Milstein in 1975 (37), the scientific research community was provided a very powerful tool for probing, potentially, the molecular secrets of any immunogen. This technology has proven itself far superior over conventional polyvalent sera in that: i) antibody of a single specificity can be obtained; ii) hybridomas secrete an almost unlimited amount of the desired antibody; iii) impure or partially purified antigen can be used to produce monospecific antibody; iv) many different specificities can be isolated from a single mouse; v) specific B cells are enriched during hybridization; vi) low-producing B cells, when fused to myeloma cells, become much higher producers; vii) the hybridoma can be genetically manipulated to produce non-naturally occurring antibody; and
viii) the technique of fusing immortalized cells to normal cells will bestow the immortalized phenotype to a variety of cells such as T cell clones (3, 36).

In 1975, Kohler and Milstein (37) published a paper in which they described a technique for fusing antibody-producing B cells with immortalized myeloma cells. This technique created a hybridoma that was immortalized and that retained the antibody-secreting phenotype of the B cell fusion partner. It was quickly recognized that this approach to producing antibody of a single, defined specificity would revolutionize nearly all fields in biology, biochemistry, and molecular biology. The advent of monoclonal antibody technology has not only led to great strides in researching antigen-antibody interactions, but it has had tremendous impact on such diverse fields as the diagnosis and treatment of disease (5, 51); purification of proteins and other biomolecules; the development of new, safer vaccines (1, 34, 60); and the development of customized enzymes (48, 62, 63). This technology has also revolutionized the field of immunology, allowing the elucidation of the biosynthesis of immunoglobulins, immunoglobulin class switching, and the characterization of many of the subgroups of the T cell family of immune cells. These subsets of T cells have now been shown to have specific, critical roles in the development of both humoral and cellular immunity.

The production of monoclonal antibodies is accomplished as described below. Spleen cells are taken from a donor that has been previously immunized with the antigen of interest. These cells are teased from the splenic capsule and collected. Myeloma cells that are deficient in hypoxanthine or adenosine phosphoribosyl transferase (HPRT or APRT) are then mixed with the immune spleen cells, and the cells are then fused together via an agent such as polyethylene glycol. The fused cells are then
gently suspended in growth media and plated out. The plates are then fed with media containing hypoxanthine or adenosine, aminopterin, and thymidine. This is done in order to select only cells that have been fused together successfully. The selection is based upon the deficiencies in the purine nucleotide salvage pathways of the myeloma cells and the inhibition of de novo purine nucleotide biosynthesis by aminopterin. Only hybridomas that contain a functional salvage pathway, HPRT- or APRT-containing cells, are capable of growing in the selection media. Theoretically, only the hybridomas that meet these requirements can grow, because of the presence of the salvage pathway enzymes HPRT and APRT from the immune B cell fusion partner. Unfused B cells and myelomas die after a few days of exposure to the selection media (22).

There are problems associated with the production of monoclonal antibodies. Because hybridomas contain 2-10 times the normal complement of genetic material, they often throw out the extra chromosomal baggage. This can result in a change in the phenotype of the cell. Occasionally, the antibody secretory genes are deleted from an unstable hybridoma and monoclonal antibody production is lost. Other times, the salvage pathway genes are lost and the hybridoma dies. Hybridomas that survive selection and that are positive antibody producers are cloned by limiting dilution to isolate clones derived from individual hybridomas and retested for antibody production. It is not unusual for a hybridoma to be recloned and rescreened three or four times prior to using it for producing large quantities of antibody.

**Therapeutic conjugates employing monoclonal antibodies for directed drug or toxin delivery.** The concept of using antibodies for the delivery of
drugs to infected or malignant tissues can be traced back to Paul Ehrlich who postulated the "magic bullet" idea during the late nineteenth century (30). With the advent of monoclonal antibody technology in 1975 (37), it became possible to develop antibodies that react specifically with tumor-associated antigens. Monoclonal antibodies were made against a variety of different tumor cell antigens and were then used experimentally as therapeutic agents with the hope of exploiting the exquisite specificity of these molecules in destroying malignant tissues (28). It soon became evident that, even though monoclonal antibodies were directed against tumor-specific antigens, they were much better suited for use as diagnostic reagents, rather than as therapeutic agents for the treatment of cancer.

As clinicians were seeking to develop monoclonal antibodies for use as therapeutic agents, they were also developing new chemotherapeutic agents to specifically inhibit and kill cancer cells. These agents were designed to inhibit specific biochemical reactions, such as the methylation of DNA by dihydrofolate reductase, that occur at much higher than normal levels in rapidly replicating tumor cells. Many of the newly developed chemotherapeutic agents were promising against tumor cells in vitro, but later proved to be too toxic when tested in vivo. Host toxicity is still the single most significant problem associated with cancer chemotherapeutic agents.

As these events took place, other scientists were purifying and characterizing potent toxins derived from both bacteria and plants. The earliest plant toxins to be purified and characterized were ricin and abrin. These were isolated from *Ricinus communis* (Castor bean) and *Abrus precatorius* (Jequirity bean), respectively (24, 19). At the end of the nineteenth century, Paul Ehrlich was able to demonstrate the separate
immunological identities of these two toxins by demonstrating that mice immunized with either toxin were not protected against lethal challenge with the other (19). Several other plant and bacterial toxins, such as gelonin (59), pokeweed antiviral protein, modeccin, diphtheria toxin, and Pseudomonas exotoxin A (14), have since been isolated and characterized for use in the construction of therapeutic immunoconjugates for use in cancer and antiviral immunotoxin therapy (2, 8).

Plant and bacterial toxins are composed of either a single A chain protein monomer or a heterodimer composed of an A and a B chain. The A chain of a heterodimeric toxin contains the catalytic (toxin) domain, whereas the B chain usually contains a lectin or lectin-like molecule that mediates binding of the entire toxin molecule to sugar residues expressed on the surface of cells. The B chain domain also may be important in the translocation of the toxin across the cell membrane (2).

The catalytic domains of A chains of either the monomeric or heterodimeric toxins require entry into the intact cell before toxic effects are observed. Once inside the cell, the toxins exert their effects on the eukaryotic 28 S ribosomal RNA. Endo et al. (16) have demonstrated that ricin, abrin, ricin A chain, and modeccin inactivate the 28 S ribosomal subunit by modifying both or either the guanosine4323 or adenosine4324 nucleoside residues. This effectively shuts down the protein synthesis machinery of the cell, resulting in cell death.

Because of the dissatisfying results obtained in using monoclonal antibodies as therapeutic agents for cancer, oncologists soon realized that what monoclonal antibodies lacked was the "punch" associated with the often too-toxic chemotherapeutic agents (24). They theorized that by combining the specificity of monoclonal antibodies with the potency of
chemotherapeutic agents or the newly discovered toxins, the "magic bullets" originally put forth by Ehrlich, could finally be produced. Thus, the components necessary for the production of the immunoconjugates were in place, and the synthesis and evaluation of these agents could begin (38).

Chemotherapeutic agents, such as methotrexate (15, 20, 33, 46, 55), daunomycin, (31, 47, 66), daunorubicin (65, 67), and adriamycin (doxorubicin), (56) were covalently attached to monoclonal antibodies directed against various tumor antigens. Work was undertaken comparing the biodistribution of two distinct chemotherapeutic agents, methotrexate and daunorubicin, that were conjugated separately to a monoclonal antibody that reacts specifically with the osteosarcoma 791T. These experiments demonstrated that the conjugates were stable over the course of the three-day experiment. They also demonstrated a 100-fold increase in tumor levels of the drugs in the conjugated treated animals over those seen in animals treated with free drug. Further, an unrelated monoclonal antibody against colon carcinoma HCT8 was conjugated with daunomycin and did not show any targeting efficacy in the osteosarcoma system (47). Controls in which methotrexate was conjugated to normal mouse IgG did not target the chemotherapeutic agent to the tumor xenograft (46).

Monoclonal antibody-toxin conjugates have been used in attempts to inhibit tumor growth in vitro and in vivo. A variety of monoclonal antibodies directed against a wide range of mouse and human tumor antigens have been conjugated with ricin A chain (25, 49, 52, 57), diphtheria toxin (11), gelonin (39, 54, 57), saporin (61), Pseudomonas exotoxin A (17), and pokeweed antiviral protein (39, 49). Immunotoxins such as these exert their effect on cells by binding to the antigen of interest, moving over the surface of the cell until it is over a clatherin-coated pit, being endocytosed
into an endosome, and eventually forming a lysosome and releasing the toxin from the antibody inside of the cell (12). These immunotoxins have for the most part been evaluated in vivo only against human tumor transplants in mice or in mouse tumor models. Results from these tests have yielded results from no increase in mean survival time to 100% protection from tumor development (18, 23).

Recently, one of the most promising immunotoxins, T101-ricin A chain T101-RTA, composed of a monoclonal antibody directed against human CD5 antigen conjugated with ricin A chain, underwent phase I trials in humans. The study showed that the immunotoxin was reaching and binding to the cells of interest, but that there was no sustained benefit in any of the patients (n=4) tested. This was demonstrated to be due to the natural resistance of the patients' leukemic cells that were refractile to in vitro treatment with T101-RTA at levels >2000 ng/ml (29).

A similar approach to the development of a possible AIDS treatment, using a portion of *Pseudomonas* exotoxin A hybridized to recombinant human CD4, was shown to selectively kill HIV-infected cells in vitro (14). This report reinforces the concept that the immunotoxins can be used for the treatment of viral disease. Though this study was done in vitro, it is still significant in that it demonstrates that targeted delivery of toxin molecules to virally infected cells can take place and achieve the desired results.

The potential for using both drug-monoclonal-antibody and toxin-monoclonal-antibody conjugates for the treatment of cancer and viral disease is exciting and full of possibilities. Essentially no work has been done in the field of treatment of viral diseases with immunoconjugates. Researchers have only now begun to investigate the possible application of this approach to the treatment of viral disease, particularly with respect to the
AIDS epidemic. The gains that are currently being made in this field are small but significant and are seminal in providing the groundwork for new therapies for the treatment of viral, neoplastic, and genetic diseases.
LITERATURE CITED


65. Tromet, A., M. Masquelier, R. Baurain, and S. D.-D. Campeneere. 1982. A covalent linkage between daunorubicin and proteins that is stable in serum and reversible by lysosomal hydrolases, as required for a


A NEWLY DEVELOPED IMMUNOFLUORESCENT ASSAY
FOR DETERMINING THE PICHINDE VIRUS-INHIBITORY EFFECTS OF SELECTED NUCLEOSIDE ANALOGUES

An immunofluorescent assay (IFA) for Pichinde virus (PCV), a member of the family Arenaviridae, was developed for antiviral drug assays against the virus. The assay was performed by adding fluorescein-labeled anti-PCV monoclonal antibody to virus-infected cells at 24 h after the initial infection and counting the infected cells with an epifluorescence microscope. The 50% effective dose (ED₅₀) for a series of nucleoside analogues tested against PCV using this IFA was: 2-β-D-ribofuranosylselenazole-4-carboxamide (selenazofurin), <1.0 µg/ml; 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin), 6.0 µg/ml; ammonium 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5'-phosphate hydrate (ribavirin-5'-monophosphate), 15.8 µg/ml; ammonium 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5'-hemisuccinate (ribavirin-5'-hemisuccinate), 14.7 µg/ml; ammonium 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5'-(2,3-dimethyl)hemisuccinate [ribavirin-5'-(2,3-dimethyl)hemisuccinate], 213.5 µg/ml; 4-hydroxy-1-β-D-ribofuranosyl-2-pyridone (3-deazauridine), 5.2 µg/ml; and (S)-9-(2,3-dihydroxypropyl)adenine, ([S]-DHPA), 471.0 µg/ml. In comparison, the ED₅₀ of ribavirin using inhibition of marginal PCV-induced cytopathogenic effect after 12 days was 6.0 µg/ml and using plaque reduction after 5 days was 2.5 µg/ml, indicating that this IFA was of comparable sensitivity to these other tests.
The Arenaviridae family contains a group of viruses that have proven to be serious human pathogens, with clinical signs of disease presenting as hemorrhagic fevers. Some members of this family of viruses, Junin, Machupo, and Lassa (the etiological agents of Argentine hemorrhagic fever, Bolivian hemorrhagic fever, and Lassa fever, respectively), are endemic in some areas of Africa and South America. Pichinde virus (PCV) is an arenavirus from South America that is much less pathogenic to humans but causes a very severe hemorrhagic disease in guinea pigs (8) and hamsters (12). Ribavirin has previously been shown to have in vitro and in vivo antiviral activity against several of the arenaviruses (11, 16).

There has been an interest in developing a simple arenavirus antiviral assay. Standard antiviral assays that depend upon plaque reduction (PR) or inhibition of viral cytopathogenic effect (CPE) are not suitable for working with PCV because this virus does not cause consistently quantifiable CPE (13). This report describes an immunofluorescence assay for use in evaluating potential antiviral compounds against PCV. The assay is based on enumeration of virus infected cells utilizing fluorescein-labeled anti-PCV monoclonal antibody 24 h after exposure of cells to the virus. Utilization of this assay as an antiviral test appears reproducible and provides the means to rapidly screen many compounds while reducing both the sample size and assay time.
MATERIALS AND METHODS

Cells. The African green monkey kidney cells, Vero-76 cells, used in this study were obtained from the American Type Culture Collection (Rockville, MD). Cells were passaged in minimum essential medium (MEM, GIBCO, Grand Island, NY) supplemented with 9% fetal bovine serum (FBS, HyClone Labs, Logan, UT) and 50 µg gentamicin/ml. For antiviral assays, cells were seeded at 4 x 10^4 cells/well in 96-well polystyrene cell culture plates (Corning, Corning, NY) and incubated at 37°C in humidified 5% CO₂ in air. The plates were used 20-24 h after seeding.

Virus. PCV strain An 4763 was provided by Dr. Joseph D. Gangemi, University of South Carolina School of Medicine, Columbia, SC. Virus stocks were prepared in Vero cells from twice plaque-purified PCV. The virus was grown at 37°C and was harvested 2-3 days post-virus exposure, aliquoted into ampules, and stored at -90°C until used. The virus stock used in these experiments had a titer of 4 x 10^5 plaque-forming units (pfu)/ml. The virus was also grown in the following cell lines: KB, human oral epidermoid carcinoma, HeLa markers, ATCC CCL 17; BHK, BHK-21, kidney cells derived from one-day-old Syrian hamsters, ATCC CCL 10; MDBK, Madin-Darby bovine kidney cells, ATCC CCL 22; L, L929 cells, in growth medium containing 10% fetal bovine serum rather than 10% horse serum; L929, NCTC clone 929, mouse connective tissue cell line grown in media supplemented with 10% horse serum, ATCC CCL 1; Vero, Vero-76, African green monkey kidney, ATCC CRL 1587; MDCK, Madin-Darby canine kidney cells, ATCC CCL 34; MRC-5, fetal male human lung diploid cells, ATCC CCL 171; WI-38, fetal female human lung diploid cells, ATCC CCL 75; LLC-MK₂D, rhesus monkey kidney, ATCC 7.1; HaK, adult Syrian hamster kidney.
cells, ATCC CCL 15; MA-104, embryonic African green monkey kidney cells, passage 52 from Dr. Mary Estes, Baylor University, Houston, TX. This was done in an attempt to find a cell line in which PCV would produce consistently quantifiable CPE.

**Antiviral compounds.** Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) and 3-deazauridine (4-hydroxy-1-β-D-ribofuranosyl-2-pyridone) were provided by ICN Pharmaceuticals (Costa Mesa, CA). Selenazofurin (2-β-D-ribofuranosylselenazole-4-carboxamide) was provided by Dr. P. D. Cook, Warner Lambert/Parke Davis, Inc. (Ann Arbor, MI). Ribavirin-5'-monophosphate (ammonium 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5'-phosphate monohydrate), ribavirin-5'-hemisuccinate (ammonium 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5'-hemisuccinate), and ribavirin-5'-(2,3-dimethyl)hemisuccinate (ammonium 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5'-(2,3-dimethyl)hemisuccinate) were synthesized in one of our laboratories (M.D.). The (S)-DHPA [(S)-9-(2,3-dihydroxypropyl)adenine] was provided by Dr. Erik De Clercq, Rega Institute for Medical Research, Leuven, Belgium. All compounds were stored in sealed vials at room temperature in the presence of dessicant. For antiviral testing, the compounds were solubilized in MEM and then stored at 4°C until used.

**Monoclonal antibodies.** The hybridoma cell lines PC4.9A6 and PC4.9D3 were derived through the polyethylene-mediated fusion of FOX-NY myeloma cells with the splenocytes from RBF/Dn mice. The spleen donor mice had been hyperimmunized with homogenized brain tissue in Freund's adjuvant (Sigma, St. Louis, MO) from suckling RBF/Dn mice that had been infected intracranially with PCV. The adenosine phosphoribosyltransferase procedure as described by Taggart and Samloff (17) was used to select for hybridoma cell lines. The PC4.9A6 and PC4.9D3 hybridoma cell lines
secreted anti-PCV monoclonal antibodies of the IgG2a subisotype. The two monoclonal antibodies bind different epitopes of a PCV protein on PCV-infected cells. The hybridoma lines had been subcloned twice and the cells were in the exponential growth phase when injected into recipient (BALB/c x RBF/Dn) F1 mice that had been primed by intraperitoneal injection with 1.0 ml of 2,6,10,14-tetramethylpentadecane (pristane). The resulting ascites fluids contained anti-PCV monoclonal antibodies at concentrations ranging from 5-15 mg/ml.

**Preparation of fluorescein-labeled monoclonal antibodies.** Immunoglobulins, predominantly monoclonal antibodies, were isolated from ascites fluids by precipitation with ammonium sulphate and further purified by affinity chromatography on protein A using the Bio-Rad Affi-Gel Protein A MAPS II Kit (BIO-RAD Laboratories, Richmond, CA). Immunoglobulin samples were dialyzed against 0.1 M Na₂HP0₄, pH 9.0. Following dialysis, precipitates were removed by centrifugation for 15 min at 550 x g. Sufficient fluorescein isothiocyanate (FITC, Isomer 1 from Sigma Chemical Co., St. Louis, MO) was added to result in a protein to FITC wt:wt ratio of 50:1 in the conjugation reaction mixture. The FITC was added as a solution containing 1.0 mg/ml of FITC dissolved in 0.2 M Na₂HP0₄, pH 9.0; this FITC solution was prepared just before addition to the immunoglobulin. The conjugation of fluorescein to protein was then accomplished by bringing the pH of the immunoglobulin-FITC mixture to 9.5 by dropwise addition of 0.2 M Na₃PO₄ and allowing the reaction to proceed at 25°C for 2.5 h. The conjugation conditions were selected to yield a fluorescein to protein ratio in the final conjugate of approximately 10 fluorescein molecules per molecule of immunoglobulin (4). The unconjugated FITC was removed by dialysis against Dulbecco's phosphate-buffered saline: 0.5 mM MgCl₂, 2.7 mM KCl, 1.5
mM KH₂PO₄, 8 mM Na₂HPO₄ and 140 mM NaCl, pH 7.2 (PBS), containing 0.1% sodium azide. The fluorescein-labeled monoclonal antibody preparations were then further purified by gel exclusion chromatography on Sephadex G-75 (Pharmacia Fine Chemicals, Piscataway, NJ). The resulting fluorescein labeled monoclonal antibody preparations were titrated in a direct immunofluorescent cell assay using PCV-infected Vero cells. There was only the slightest nonspecific staining at dilutions of 1:100 or greater, while the specific staining was very clear and intense out to dilutions of at least 1:1,600.

**Immunofluorescence assay for PCV-infected cells.** The IFA for PCV-infected cells used a cocktail of fluorescein-conjugated murine monoclonal antibodies towards PCV designated PC4.9A6 and PC4.9D3. These were diluted to approximately 0.01 to 0.03 mg protein/ml and then combined in equal volumes in PBS prior to use. Unused antibody was kept frozen at -20°C until thawed immediately prior to use. At 20-24 h postinfection (p.i.), the medium was removed from the wells of the 96-well plates and the cells were allowed to dry thoroughly at room temperature. If the cell sheets were not allowed to dry thoroughly prior to adding the 80% acetone fixative, many of the cells would detach from the well during the fixation and staining procedures. The cells were then fixed in 80% acetone by adding cold distilled water (50 μl/well) followed by immediate addition of cold (-15°C) acetone (200 μl/well) to the water in each well. If the acetone and water were mixed prior to addition to the wells, or if the acetone was added first, the plastic was etched, thus spoiling the specimens for microscopic examination. The fixation was allowed to proceed for 5 minutes and then the fixative was poured from the plate and the cell sheets were allowed to dry thoroughly.
Cells were either immunostained immediately after drying or stored at -15°C.

Fluorescein-labeled monoclonal antibody towards Pichinde virus was used at a dilution of 1:500 to provide intense specific staining with only minimal nonspecific background. For immunostaining, 50 µl of the fluorescein labeled antibody was added to each well and immunostaining was allowed to proceed at 37°C for 1-2 h before the immunostain was poured from the plate. The plate was inverted on absorbent paper and allowed to drain, a drop of mounting medium (5) was added, and immunostained cells were viewed through the bottom of the plate using a 16X objective and 10X eyepieces on a Zeiss epifluorescence microscope. The number of fluorescent cells in one strip across the center of each well was counted and the total fluorescent cells per well determined as described by Barnett et al. (2) and Tu et al. (18). The multiplication factor calculated for the one strip counts in this study was 6.36.

Immunofluorescence antiviral assay (IFA) and toxicity evaluation.

Growth medium was aspirated from confluent monolayers of Vero cells growing in 96-well culture plates and 100 µl of PCV diluted in MEM with 2% FBS was added to the appropriate wells. The virus inoculum was such that there were approximately 10 fluorescing cells per microscope field in virus control wells, the multiplicity of infection (MOI, the ratio of infectious virus to cells) was approximately 0.002. The virus was allowed to adsorb for 30 minutes at room temperature after which time 100 µl of test compound diluted in MEM was added without removing the virus inoculum. Seven concentrations of test compound were used: 1000, 320, 100, 32, 10, 3.2 and 1 µg/ml. The plates were then sealed with plastic wrap (Saran Wrap™, Dow Chemical Corp.) and incubated for 20-24 h at 37°C. The cells were fixed and
immunostained to detect PCV-infected cells as described above. The number of fluorescing cells in treated wells was determined and compared to the number of fluorescing cells in wells without drug. Each assay included toxicity controls as well as virus and normal cell controls similar to a virus reduction assay described previously (15). Three virus-containing wells were used for each dose, with one additional well being used for toxicity controls (cells + sterile virus diluent + drug). Six wells on each plate were used for normal cell controls (cells + sterile virus diluent + drug diluent). Six wells on each plate were used for virus controls (cells + virus + drug diluent). The antiviral activity was expressed as the 50% effective dose (ED$_{50}$), which was the concentration of drug required to reduce the number of fluorescing infected cells by 50%. The ED$_{50}$ was determined by plotting the percent inhibition versus compound concentration on a semilogarithmic chart.

Drug toxicity was evaluated by examining the toxicity control cells microscopically for morphological changes, when compared with normal cell controls. The 50% cytotoxic dose (CD$_{50}$) was defined as that concentration which caused approximately a 50% change in cellular morphology of toxicity control cells. This toxicity determination, while subjective and approximate, was based on graded morphological changes observed at increasing levels of the test compounds, with 0% representing no morphological change and 100% representing total cell destruction. The therapeutic index (TI) is defined as the CD$_{50}$/ED$_{50}$.

Inhibition of cytopathogenic effect and plaque reduction assays. Ribavirin was run in side by side assays in order to compare the sensitivity of the immunofluorescence antiviral assay to the more standard antiviral assays such as inhibition of viral CPE (15) and the PR (6) assays. In the CPE assay, the drug was added in triplicate to wells of a 96 well plate in one-half
log₁₀ dilutions from 1000 μg/ml to 1.0 μg/ml. The plates were then scored when virus controls reached a maximum level of CPE. In these studies using PCV at an MOI of 0.001 this did not occur until approximately 12 days after virus infection and the maximum CPE was considered to be 1+. In the PR assay the test compound was incorporated into the overlay media and was then placed onto infected cell monolayers in 6-well plates. The monolayers in this assay were infected at an MOI of 0.0001. After 5 days of incubation, the plaques in the treated wells were counted and compared with those of the virus control wells.

RESULTS

Attempts to find a cell line in which PCV would produce consistent and quantifiable CPE. Several cell lines, noted in Materials and Methods, were used in an attempt to find a cell line that produced consistently quantifiable CPE when infected with PCV. This was done in order to use the inhibition of CPE assay (15) that is used as a standard antiviral screening test in this laboratory. Only one cell line, Vero-76, showed slight viral CPE, in this case appearing 12 days p.i. PCV did not produce adequate CPE in any of the other cell lines that were tested.

Antiviral activity of ribavirin, 3-deazauridine, (S)-DHPA, and ribavirin derivatives against PCV. An example of an antiviral test using the IFA procedure is seen in Table 1. Ribavirin was used as the test compound. Toxicity controls exhibited morphologic changes only at 1000 and 320 μg/ml dose levels; the CD₅₀ was determined to be 1000 μg/ml. Exposure of the infected cells to ribavirin resulted in total inhibition of fluorescent cells at 10
through 1000 μg/ml dosage levels; a 43% inhibition of infected cells was seen at 3.2 μg/ml. The TI for ribavirin was calculated to be 217.4 in this test.

Using the IFA procedure, the compounds described earlier were evaluated against PCV. The virus was found to be most sensitive to selenazofurin (Table 2). The data also indicate that selenazofurin had the greatest TI of the compounds tested. Ribavirin-5'-hemisuccinate and, as described above, ribavirin were very effective against PCV using this assay. In contrast, methyl groups incorporated at the 2- and 3- positions of the hemisuccinate side chain reduced the TI 21-fold. Ribavirin-5'-monophosphate and 3-deazauridine were also active against the virus. Very weak activity against PCV was seen with (S)-DHPA.

The infected cells were easily distinguished from uninfected cells by the bright apple green fluorescence of the immunostain. There was virtually no background staining of the uninfected cells. The stain appeared to bind to the membrane and cytoplasmic components of the fixed, infected cells. The nuclei of the infected cells did not appear to bind any immunostain. This pattern would be consistent with the replication and maturation of PCV particles in the cytoplasm and their budding through of the cytoplasmic membrane of infected cells.

Comparison of IFA with inhibition of viral CPE and PR antiviral assays. A comparison of the IFA to the inhibition of viral CPE and PR assays is shown in Table 3. The IFA sensitivity appeared comparable to that of the other two assays used although the PR assay appears to be slightly more sensitive than the IFA or the inhibition of viral CPE assay. This may be, in part, due to the relative MOI's used in the different assays, with the PR assay being infected with 10-20-fold less virus than the inhibition of viral CPE or IFA assays, respectively. Also, the PR assay required larger amounts of the
compound than either IFA or CPE procedure and the time to endpoint was 4 days longer that that required in the IFA test.

DISCUSSION

The IFA procedure described here appears to be an acceptable tool for antiviral drug testing. By comparing the results obtained from evaluating the antiviral efficacy of ribavirin using several different antiviral assays, the IFA demonstrates several important advantages: 1) The IFA is essentially as sensitive as either of the other assays used in this study, 2) it has the advantage of requiring much less compound than the PR assay, 3) the endpoint is much less ambiguous than that of the CPE assay in which reproducible, readily discernible PCV-induced CPE was difficult to achieve, and 4) the IFA assay can be performed in 24 h instead of the 5-12 days required for PR testing or CPE inhibition, respectively. The procedure worked well for evaluation of all nucleoside analogues used in this study.

The relative costs of the three assays used in this study are comparable, if the production costs of the monoclonal antibodies are not taken into consideration. This procedure can be adapted for use as an indirect method using virus-specific polyclonal sera and FITC-labeled secondary antibody. The monoclonal antibodies to PCV produced in our laboratories that were used for this study are not available commercially.

The results reported here indicate that PCV is reasonably sensitive to selenazofurin, ribavirin, ribavirin-5'-hemisuccinate, ribavirin-5'-monophosphate, and 3-deazauridine. Selenazofurin and ribavirin have previously been reported active against PCV using PR tests (10), and ribavirin has also exhibited in vivo efficacy against this virus (7). 3-
Deazauridine is known to inhibit a number of other RNA viruses, including rhino, influenza A and B, parainfluenza 1, and vesicular stomatitis viruses (9), as well as Gross and Rauscher murine leukemia viruses (14). Ribavirin-5'-monophosphate exhibited both in vitro and in vivo efficacy against influenza A and B and murine hepatitis viruses (1). The aliphatic nucleoside (S)-DHPA has been reported to have a broad-spectrum antiviral activity (3) although prior to these experiments it had never been tested against PCV. The results of testing (S)-DHPA against PCV indicate that the compound was active only at the highest concentrations. This is the first report of antiviral testing with the other ribavirin derivatives described here. The loss of activity associated with the incorporation of methyl groups at the 2- and 3- positions of the 5'-hemisuccinate of ribavirin indicates that steric hindrance may inhibit either the transport of the molecule into the infected cell or, more likely, the hydrolysis to free ribavirin.
TABLE III-1. Effect of ribavirin against Pichinde virus infection in Vero cells as determined by IFA procedure.

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>Cytotoxicity&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>Fluorescent cells (No. of cells/well)</th>
<th>Mean fluorescent cells/well</th>
<th>Inhibition fluorescent cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>50</td>
<td>0, 0, 0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>320</td>
<td>25</td>
<td>0, 0, 0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>0, 0, 0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>32</td>
<td>0</td>
<td>0, 0, 0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0, 0, 0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>3.2</td>
<td>0</td>
<td>133, 89, 57</td>
<td>93</td>
<td>43</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>127, 146, 147</td>
<td>140</td>
<td>14</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>171, 235, 178, 114, 173, 102</td>
<td>162</td>
<td>0</td>
</tr>
</tbody>
</table>

ED<sub>50</sub><sup>b</sup>: 4.6 µg/ml  
CD<sub>50</sub><sup>c</sup>: 1000 µg/ml  
TI<sup>d</sup>: 217.4

<sup>a</sup> As determined by microscopic examination of toxicity control wells, expressed as enlarged, flattened cells. The percent indicated is the approximate degree of enlargement and flattening that occurred.

<sup>b</sup> ED<sub>50</sub>: 50% effective dose, determined by plotting concentration of drug versus percent inhibition on a semilogarithmic chart.

<sup>c</sup> CD<sub>50</sub>: 50% cytotoxic dose as determined by microscopic examination of toxicity control wells.

<sup>d</sup> Therapeutic index = CD<sub>50</sub>/ED<sub>50</sub>. 
TABLE III-2. Activity of nucleoside analogues against PCV as measured by IFA procedure.

<table>
<thead>
<tr>
<th>Drug</th>
<th>ED$_{50}^a$ (µg/ml)</th>
<th>CD$_{50}^b$ (µg/ml)</th>
<th>TI$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selenazofurin</td>
<td>&lt;1.0</td>
<td>320</td>
<td>&gt;320.0</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>6.0</td>
<td>1000</td>
<td>166.7</td>
</tr>
<tr>
<td>Ribavirin-5'-hemisuccinate</td>
<td>14.7</td>
<td>&gt;1470</td>
<td>&gt;100.0</td>
</tr>
<tr>
<td>Ribavirin-5'-monophosphate</td>
<td>15.8</td>
<td>&gt;1000</td>
<td>&gt;63.3</td>
</tr>
<tr>
<td>3-Deazauridine</td>
<td>5.2</td>
<td>320</td>
<td>61.5</td>
</tr>
<tr>
<td>Ribavirin-5'-(2,3-dimethyl)</td>
<td>213.5</td>
<td>&gt;1000</td>
<td>&gt;4.7</td>
</tr>
<tr>
<td>hemisuccinate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S)-DHPA</td>
<td>471.0</td>
<td>&gt;1000</td>
<td>&gt;2.1</td>
</tr>
</tbody>
</table>

$^a$ ED$_{50}$: 50% effective dose, determined by plotting concentration of drug versus percent inhibition on a semilogarithmic chart; each is an average of 1 to 4 experiments.

$^b$ CD$_{50}$: 50% cytotoxic dose.

$^c$ TI = CD$_{50}$/ED$_{50}$
TABLE III-3. Activity of ribavirin against PCV as measured by different antiviral assays in Vero cells.

<table>
<thead>
<tr>
<th>Antiviral Assay&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ED&lt;sub&gt;50&lt;/sub&gt; (µg/ml)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Amt. required/ assay (mg)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Time to endpoint (days)&lt;sup&gt;d&lt;/sup&gt;</th>
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<tr>
<td>CPE</td>
<td>3.2-10</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>PR</td>
<td>1.0-3.2</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
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<sup>a</sup> CPE = inhibition of cytopathogenic effect; PR = plaque reduction; IFA = immunofluorescence assay.

<sup>b</sup> ED<sub>50</sub>'s expressed as ranges, from multiple experiments.

<sup>c</sup> Amount of compound required to make initial stock of 2000 µg/ml.

<sup>d</sup> Time at which results could be determined.


CHAPTER IV
CHARACTERIZATION OF MONOCLONAL ANTIBODIES PRODUCED AGAINST PICHINDE VIRUS

Mouse monoclonal antibodies directed against Pichinde virus (PCV) were produced to evaluate their possible application as vehicles for the directed delivery of antiviral drugs or toxins to virus infected cells. Monoclonal antibodies PC4.9A6, PC4.9D3, PC4.7C2, and PC4.8D2 were of the IgG2a subisotype and reacted with both acetone-fixed and live PCV-infected Vero-76 cells. Radioimmunoprecipitation of proteins from $^{35}$S-methionine-labeled PCV-infected cell lysates with PC4.9A6, PC4.9D3, PC4.7C2, PC4.8D2, and hyperimmune hamster serum showed that all the antibodies precipitated two different PCV proteins, one of 64,000 daltons, and another of 38,000 daltons. These proteins were, respectively, PCV nucleoprotein (NP) and a breakdown product of NP that was present in PCV-infected cells. Fluorescein-labeled PC4.9A6 (FITC-9A6) reacted optimally with PCV-infected acetone-fixed cells and did not stain noninfected cells. The staining of live PCV-infected cells with FITC-9A6 revealed that >90% fewer infected cells were stained than were observed in similarly infected cells that were acetone-fixed prior to staining. Live PCV-infected cells stained with FITC-9A6 appeared more rounded up and refractile than acetone-fixed PCV-infected cells. Both PCV-infected and noninfected live cells demonstrated some non-specific staining with FITC-9A6, although this staining was readily discernible from the virus-specific staining. In vivo stained splenic macrophages derived from PCV-infected hamsters that had been injected with FITC-9A6 demonstrated a 400% increase in total fluorescence over similarly treated, non-infected cells when
analyzed by flow cytometry. This was an indication that FITC-9A6 does have some ability to specifically target PCV-infected cells in vivo and that it warranted further investigation into its use as a delivery vehicle for the directed delivery of either antiviral compounds or toxins.

Arenaviruses are the etiological agents of several significant hemorrhagic diseases in humans, namely, Lassa fever, Argentine hemorrhagic fever, and Bolivian hemorrhagic fever. These diseases are spread by rodents that are asymptomatic carriers of the virus. These rodents shed large amounts of virus in their urine. Disease outbreaks most often occur in lesser developed areas of Africa and South America where it is often difficult to limit the contact between rural households and the rodents that harbor these pathogenic viruses.

Although ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) has been shown to be effective in reducing the mortality of patients hospitalized with severe, acute Lassa fever there still exists a significant number of patients who are not responsive to ribavirin therapy (10). Similar observations have been made using animal models of Argentine and Bolivian hemorrhagic fevers (7). Treatment of similarly infected individuals with immune plasma from patients with Lassa fever resulted in only slightly diminished mortality rates (7). Currently, more effective means of clinical intervention in arenaviral hemorrhagic diseases are needed in the areas of the world where these diseases are endemic.

One method of enhancing the effectiveness of a drug or toxin is to target its delivery to the cell population of interest, whether in neoplastic or in virally induced diseases (2, 4, 12). Monoclonal antibodies were produced that were reactive with PCV-infected cells (1). This study reports on the
suitability of these monoclonal antibodies as possible vehicles for the in vivo delivery of antiviral compounds or toxins to PCV-infected cells in MHA hamsters.

**MATERIALS AND METHODS**

**Cells.** The Vero-76 cells used in this study were obtained from the American Type Culture Collection (Rockville, MD). The cells were passaged in minimum essential media (MEM, GIBCO, Grand Island, NY) supplemented with 9% fetal bovine serum (FBS, Hyclone Labs, Logan, UT). For immunofluorescent assays in 96-well polystyrene plates (Corning, Corning, NY), 0.1 ml of cells diluted to a density of $4 \times 10^5$ cells/ml in MEM with 9% FBS was added to each well. Similar immunofluorescent assays were done using glass cover slips seeded with 0.3 ml of the same density of Vero-76 cells.

**Virus.** PCV strain An 4763 was provided by Dr. Joseph D. Gangemi, University of South Carolina School of Medicine, Columbia, SC. Virus stocks were prepared in Vero cells from twice plaque-purified PCV. The virus was grown at 37°C and was harvested 2-3 days after virus exposure, aliquoted into ampules, and stored at -90°C until used. The virus stock had a titer of $4 \times 10^5$ plaque-forming units (pfu)/ml and an immunofluorescent titer of $5 \times 10^6$ fluorescent foci-forming units/ml when stained with a PC4.9A6-fluorescein conjugate.

**Animals.** The MHA hamsters used in this experiment were supplied by Charles River Laboratories, Inc. (Wilmington, MA). The hamsters were females of >10 weeks of age and weighed approximately 60-70 g at the onset of the experiment. The animals were quarantined for at least 24-48 h prior to
use. The hamsters were housed five per cage in polycarbonate cages with corn cob bedding and were provided with Wayne Lab Blox and tap water ad libitum.

**Monoclonal antibodies.** Monoclonal antibodies PC4.9A6, PC4.9D3, PC4.7C2, and PC4.8D2 were produced against PCV as previously described (1). Monoclonal antibody 33.6, reactive with an epitope on glycoprotein 2 (GP2) that is conserved amongst all the arenaviruses, was provided by Micheal J. Buchmeier, Scripps Institute of Immunology, La Jolla, CA (18).

**Isotyping of monoclonal antibodies.** The monoclonal antibodies PC4.9A6, PC4.9D3, PC4.7C2, and PC4.8D2 were isotyped and subisotyped using a mouse antibody isotyping kit from Hyclone Laboratories (Logan, UT), as per manufacturer’s instructions. Briefly, the kit was a sandwich ELISA done in a 96-well microtiter plate using o-phenylenediamine as the horseradish peroxidase (HRPO) enzyme substrate. The microtiter plates were read at 490 nm on a Bio-Tek (Winooski, VT) microplate reader. Normal mouse serum (NMS) was used as a positive control.

**Radioimmunoprecipitation of Pichinde virus antigens.** Pichinde virus-infected Vero-76 cells were labeled with 25-50 μCi/ml of Tran35S-label® (ICN Biomedicals, Inc., Irvine, California), a cellular hydrolysate of *Escherichia coli* grown in the presence of carrier-free 35SO4²⁻. This product contained 70% L-methionine, 15% L-cysteine, and various non-labeled amino acids as described in the accompanying product literature. Infected and noninfected cells were methionine-starved with methionine-deficient media for 2 h prior to addition of the radiolabel. The cells were labeled at various times, typically at 36 h post-infection (p. i.), for 2-18 h, after which they were chased with media containing a 100-fold excess of cold methionine.
Infected and noninfected cells were harvested by scraping them from the flasks and pelleting them by centrifugation at 7500 x g. The cell pellets were resuspended in lysis buffer (0.1 M Tris, pH 8.0, 0.14 M NaCl, and 0.025% sodium azide [TSA] with 1% Triton X-100, 1% bovine hemoglobin, 1 mM iodoacetamide, 0.2 U aprotinin and 1 mM phenylmethylsulfonyl fluoride, [PMSF]) and were kept at 4°C for 1 h. After lysis of the cytoplasmic membranes the nuclei were pelleted by centrifugation and the supernatant containing solubilized membrane proteins and cytoplasmic components was collected and frozen in aliquots at -90°C.

Prior to the immunoprecipitation of the radiolabeled antigens with the relevant monoclonal antibodies, the cell lysates were cleared with an irrelevant ascites fluid containing an IgG2a monoclonal antibody produced against an unrelated bacterial antigen. This reduced the nonspecific binding of the components of the cell lystate to the relevant monoclonal antibodies. The irrelevant ascites fluid was added to the cell lysates and incubated for 1 h at 4°C. The nonspecific complexes were precipitated by addition of 50-100 μl of 10% suspension of Staphylococcus aureus Cowan strain II (Staph A) cells to the pretreated cell lysates. These cells express protein A on their surfaces. Protein A has a high affinity for the Fc portion of mouse IgG2a immunoglobulin molecules and can be used to precipitate antigen-antibody (Ag-Ab) complexes. The hybridoma cell culture fluids that contained the relevant monoclonal antibodies, PC4.9A6, PC4.9D3, PC4.7C2, and PC4.8D2 were added to the precleared cell lysates, containing 10^6 counts per minute of radiolabel, and were incubated for 1-2 h at 4°C. The Ag-monoclonal antibody complexes were then precipitated by adding 50 μl of Staph A cells that had been washed twice with lysis buffer. The complexes were pelleted by centrifugation for 5 seconds in a microfuge (3000 x g, Hermle model
Z230A, National Labnet Co., Woodbridge, NJ). The Staph A-monoclonal antibody-antigen complexes were washed twice in cold dilution buffer (0.1% Triton X-100, and 0.1% bovine hemoglobin in TSA), once in cold TSA buffer, and once in cold 0.0625 M Tris-Cl, pH 6.8. The pelleted complexes were resuspended in 2x sample buffer (0.125 M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 1 mM dithiothretoiol [DTT], and 0.001% bromophenyl blue) and boiled for 3 min to dissociate the radiolabeled antigen from the complex. The Staph A cells were again pelleted by centrifugation and the supernatant, containing the now dissociated protein of interest, was immediately loaded onto a 10% polyacrylamide gel for electrophoresis (13).

**Polyacrylamide gel electrophoresis.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (8). Samples for electrophoresis were solublized by boiling them for 3 min in 2x sample buffer or in 2x nonreducing sample buffer that did not contain any reducing agent such as DTT or β-mercaptoethanol. Samples were loaded on to a discontinuous polyacrylamide gel consisting of a 4% stacking gel (final concentrations: 4% total [T] acrylamide 2.7% crosslinked [C] acrylamide, 0.1% SDS, and 0.125 M Tris-Cl, pH 6.8) poured over a 10% separating gel (final concentration: 10%T 2.7%C acrylamide, 0.1% SDS, and 0.375 M Tris-Cl, pH 8.8). The gel apparatus was filled with tank buffer (0.025 M Tris-Cl, pH 8.3, 0.192 M glycine, and 0.1% SDS) and the solublized samples were loaded into the appropriate wells. A constant current of 20 mA per gel was applied until the bromphenol blue tracking dye reached the bottom edge of the gel. Molecular mass markers (Diversified Biotech, Newton Centre, MA, molecular mass [m.m.] range 12,700 - 95,000 daltons) suitable for silver staining were included on the gels that were silver stained. $^{14}$C-Radiolabeled m.m. standards (Bethesda
Research Laboratories Life Technologies, Inc., Gaithersburg, MD, m.m. range 14,300 - 200,000 daltons) were included in the gels that were used for fluorography. The gels were fixed in 10% (v/v) glacial acetic acid and 30% (v/v) methanol, treated with FluroHance (Research Products International Corp., Prospect, IL) according to manufacturer's instructions, dried and used to expose Kodak X-O-Mat X-Ray film, silver stained as described below, or used for electrophoretic transfer of the separated proteins to nitrocellulose for use in immunodetection.

**Silver staining of polyacrylamide gels.** Polyacrylamide gels (0.75 mm thick) were fixed in 10% (v/v) glacial acetic acid and 30% (v/v) methanol overnight and washed (3 x 10 min) in 5% (v/v) glacial acetic acid and 10% (v/v) methanol to further remove any residual SDS. The gels were soaked in 0.3 mM potassium dichromate and 0.0032 N nitric acid for 5 min. The gels were washed (4 x 30 sec) in deionized water. The gels were placed in a solution of 0.12 M silver nitrate for 25 min under ambient light. The silver nitrate solution was decanted and the gels were washed quickly twice with 0.28 M sodium carbonate and 0.05% (v/v) formalin. The gels were developed in a third wash of this mixture until the bands reached the desired intensity. Development of the gels was stopped by immersing the gel in a solution of 1% acetic acid. The silver stained gels were dried onto paper backing.

**Immunodetection of proteins in cell extracts.** Extracts of cells infected with PCV at an multiplicity of infection (MOI) of 0.1 and noninfected cells were prepared by the addition of lysis buffer containing 0.1 M Tris, pH 8.0, 0.5% Triton X-100, 1 mM iodoacetamide, aprotinin (0.2 U/ml), and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis, MO) at 4°C for 1
The nuclei were then pelleted by centrifugation and supernatants were aliquoted into vials and stored at -90°C until used.

PCV-infected and noninfected cell lysates were subjected to SDS-PAGE prior to being transferred onto nitrocellulose employing the procedure of Towbin et al. (14). Proteins reactive to the monoclonal antibodies were detected using Auroprobe BLplus® (Janssen Biotech N. V., Piscataway, NJ) according to manufacturer's instructions. Total cell lysates were stained with AuroDye Forte® (Janssen Biotech) as per manufacturer's instructions.

**Detection of viral and cellular glycoproteins.** Viral and cellular glycoproteins were detected on the electrophoretic nitrocellulose transfers of the PCV-infected and noninfected cell lysates using a method described by Clegg (3). Briefly, the blots were blocked with a solution of 2.5% bovine serum albumin (BSA) in PBS for 1 h at room temperature with agitation after which they were incubated in a solution of concanavalin A (Con A, 10 μg/ml, Sigma, St. Louis, MO) in phosphate-buffered saline (PBS, Sigma, St. Louis, MO) with 0.5% Triton X-100 (Sigma, St. Louis, MO) for 1 h at room temperature with agitation. The transfers were washed (5 x 5 min) with washing buffer (PBS with 0.5% Triton X-100). The blots were incubated in a solution of HRPO (50 μg/ml, Sigma, St. Louis, MO) for 1 h at room temperature with agitation. The blots were again washed (5 x 5 min) with washing buffer. The viral and cellular glycoproteins were detected by incubating the transfers in a solution of 3,3'-diaminobenzidine (DAB, 0.5 mg/ml, Sigma, St. Louis, MO) in Tris-Cl buffer, pH 7.6, with 0.04% NiCl₂ and 0.004% H₂O₂. Development was stopped by immersion of the blots in deionized water.

**Comparison of the binding of FITC-labeled PC4.9A6 monoclonal antibody to fixed and non-fixed PCV-infected cells.** Vero-76 cells were
seeded onto glass cover slips in Petri dishes at a density of 1.2 x 10^5 cells/cover slip. At 24 h after the cover slips were seeded, the confluent cultures were infected with PCV diluted in maintenance medium (MEM with 2% FBS, 0.1% NaHCO_3, and 50 μg gentamycin/ml). At 48 h p.i., the cells were either fixed with acetone for 5 min and stained with 6 μg/ml of FITC-labeled PC4.9A6 conjugate or stained alive by diluting the PC4.9A6-FITC conjugate directly into the maintenance medium at the same concentration. All cells, acetone-fixed or alive, were stained for 3 h with the conjugate after which they were rinsed with Evan's Blue stain in PBS and mounted onto a glass slide with a drop of elvanol mounting medium (6). The cells were then observed and photographed immediately under 400x magnification employing both Hoffman contrast and epifluorescent optics using a Nikon Diaphot microscope.

**In vitro targeting of PCV-infected spleen cells by FITC-labeled PC4.9A6 monoclonal antibodies** Two groups of MHA hamsters, PCV-infected (n=4) and noninfected (n=5), were used to investigate the in vitro targeting of the FITC-labeled PC4.9A6 conjugate to PCV-infected spleen cells. Infected hamsters used for in vitro targeting were infected subcutaneously (s.c.) with 10 LD_{50} units of PCV. Noninfected hamsters were included as controls.

Both infected and noninfected hamsters in the in vitro targeting study were sacrificed on day 9 p.i. by cervical dislocation and their spleens were removed. The spleens were placed into 10 ml of Puck's balanced salts solution and a spleen cell suspension was made using a Stomacher laboratory apparatus (Tekmar Co., Cincinnati, OH). The cell suspension was pelleted by gentle centrifugation and the contaminating red blood cells were lysed by hypotonic shock by suspending the pellet in 9 ml of water for 10 seconds after which 1 ml of 10x PBS was added to the suspension. The
remaining cells were pelleted and washed twice in RPMI 1640 with 15% FBS. Prior to staining, 10^6 cells were aliquoted into a test tube and the samples were pelleted by centrifugation and resuspended in 1 ml of RPMI 1640. Cells were stained for 1 h at room temperature with 60 µg/ml of FITC-labeled PC4.9A6 after which the macrophage population of the spleen cell suspension was subjected to flow cytometry analysis using a Coulter Electronics (Hialeah, FL) EPICS flow cytometer.

In vivo targeting of PCV-infected spleen cells by FITC-labeled PC4.9A6 monoclonal antibodies in MHA hamsters. PCV-infected (n=4) and noninfected (n=5) hamsters from the in vivo targeting groups were injected i.p. with 0.5 ml of 60 µg/ml FITC-PC4.9A6 conjugate on day 9 p.i., 8 h prior to being sacrificed. Hamsters in both groups were sacrificed by cervical dislocation, the spleens were removed, and spleen cell suspensions were prepared for flow cytometry as described above except that the suspensions were not subjected to any further staining at anytime during the experiment. Spleen cell suspensions were then subjected to flow cytometry analysis as described below.

Flow cytometry analysis of in vivo and in vitro stained hamster spleen cell suspensions. Flow cytometry was performed with an EPICS-C flow cytometer (Coulter Electronics Inc., Hialeah, FL), equipped with an argon laser tuned to 400 mW at the 488 nm line. Cells were carried in Isoflow sheath fluid (Coulter) through a 76 micron tip. Fluorescence data for FITC were obtained using a bit-map format, gated on the forward light scatter versus right-angle light scatter histogram. The bit map was used to isolate cells of interest, splenic macrophages, and to exclude debris and doublets. Fluorescent signals were processed through a three-decade log amplifier and displayed on a 256-channel scale. Fluorescence intensity was standardized
using 10 μm latex beads (Immunocheck, Coulter) and adjusting the laser power to place the log-green histogram in channel 119. Other settings remained constant for all samples. Data were stored and later analyzed using the Cytologic® software package (Coulter).

RESULTS

Subisotype of anti-PCV monoclonal antibodies. Monoclonal antibodies PC4.9A6, PC4.9D3, PC4.7C2, and PC4.8D2 were found to be of the IgG2a subisotype (Table 1).

Staining of acetone-fixed and live PCV-infected and noninfected Vero-76 cells using FITC-labeled PC4.9A6 monoclonal antibody. When acetone fixed PCV-infected Vero-76 cells were stained with FITC-conjugated PC4.9A6 and viewed under epifluorescence optics there was intense, apple-green virus-specific fluorescent staining observed. Usually 30-50 infected cells were present in areas of fluorescence which appeared to overlap in many of the regions of the coverslips (Fig. 1A). The acetone-fixed noninfected cell controls showed no virus specific staining and very little background staining (Fig. 1B). Using Hoffman contrast optics, no morphological or pathological distinctions were observed between the acetone-fixed PCV-infected cells and the noninfected cells (Figs. 1C and 1D, respectively).

When live, unfixed, PCV-infected cells that had been stained with FITC-labeled PC4.9A6 were observed using epifluorescence optics virus-specific as well as some nonspecific staining was observed. The virus-specific staining observed was similar to that seen with the acetone-fixed cells, except that the specifically stained areas contained only a few (1-5) fluorescent cells (Fig.
2A). When the same field was viewed using Hoffman optics, the cells that were fluorescing appeared much more rounded-up and more refractile (Fig. 2C) than the cells that had been acetone-fixed prior to staining (Fig. 1C).

Nonspecific staining occurred in both the infected and noninfected cells and was characterized by an even, diffuse, light green fluorescence of entire individual cells. There was only one cell per foci that took up the stain in a nonspecific manner (Fig. 2B). This staining faded rapidly under the UV light, and no longer fluoresced within 30 to 90 seconds of being placed in the UV beam of the microscope. There were no morphological differences observed in the cells that demonstrated nonspecific staining in either the infected or noninfected groups when observed under Hoffman optics (Figures 2C and 2D, respectively).

Characterization of the PCV peptide binding specificity of the anti-PCV monoclonal antibodies. The monoclonal antibodies PC4.9A6, PC4.9D3, PC4.7C2, and PC4.8D2 were used to immunoprecipitate $^{35}$S-methionine-labeled peptides from PCV-infected cell lysates. Monoclonal antibody 33.6, hyperimmune hamster sera (HHS), and an unrelated hybridoma ascites fluid (FOX-NY) were included as positive (33.6 and HHS) and negative (FOX-NY) controls, respectively. Monoclonal antibodies PC4.9A6, PC4.9D3, PC4.7C2, and PC4.8D2 all immunoprecipitated the same viral peptides, a 64,000 dalton protein, and a 38,000 dalton protein (Fig. 3). HHS reacted with the same two viral peptides. Monoclonal antibody 33.6, specific for an epitope on GP2 that is conserved amongst all arenaviruses (17), did not precipitate any virus proteins in this assay. The negative control FOX-NY also did not precipitate any arenavirus proteins. None of the antibody preparations precipitated proteins from noninfected Vero-76 cell lysates.
Detection and localization of PCV glycoproteins on nitrocellulose from electrophoretic transfers of PCV-infected and noninfected cell lysates. Glycoproteins present in both PCV-infected cells and noninfected cells were detected using a Con A-HRPO detection system (3). This system is based on the ability of the divalent lectin Con-A to bind to the glycoproteins immobilized on the nitrocellulose membrane and also to the HRPO detection enzyme that is also a glycoprotein. Using this system, only the glycoproteins present in the lysates were detected and localized on the nitrocellulose membrane. In the lane containing the PCV-infected cell lysate (lane 3 of Fig. 4) there were two bands that do not appear in the lane containing the noninfected cell lysate (lane 4 of Fig. 4). These two bands that were unique to the PCV-infected cell lysate are glycoproteins (GP1 and GP2) of 57,000 daltons and 33,000 daltons in size. Monoclonal antibody 33.6 reacted with the 33,000 dalton glycoprotein (GP2) in the PCV-infected cells lysate (lane 2 of Fig. 4) and did not react with any proteins found in the noninfected cell lysate lane (lane 1 of Fig. 4). Monoclonal antibodies PC4.9A6, PC4.9D3, PC4.7C2, and PC4.8D2 did not react consistently in this assay giving spurious results that included high background in both the infected and uninfected cell lysates or not reacting at all.

In vitro and in vivo targeting of PCV-infected MHA hamster spleen cells by FITC-labeled PC4.9A6 monoclonal antibody. When FITC-labeled PC4.9A6 was used to stain both PCV-infected and noninfected spleen cells in vitro, there was only very weak reactivity with the cells as evidenced by very low fluorescent intensity and cell numbers when the stained spleen cells were analyzed by flow cytometry (data not shown). When the same antibody was used to target the infected cells in vivo the FITC-labeled PC4.9A6 reacted with the infected splenic macrophages. This is evidenced
by a 400% increase in the overall fluorescent intensity and total fluorescent cell numbers of in vivo stained splenic macrophages as opposed to the same population of cells taken from in vivo stained, noninfected animals (Table 2, Figs. 5A and 5B, Figs. 6B, and 6C). Background fluorescence of nonstained, normal spleen macrophages is shown in Figure 6A.

**DISCUSSION**

The anti-PCV monoclonal antibodies PC4.9A6, PC4.9D3, PC4.8D2, and PC4.7C2 were evaluated with regard to their isotype and subisotype, reactivity with PCV-infected and noninfected acetone-fixed and living cells, PCV peptide binding specificity, and their ability to target infected splenic macrophages in the MHA hamster model of PCV-induced hemorrhagic fever. This study was undertaken to investigate the suitability of these monoclonal antibodies as delivery vehicles for the targeted delivery of antiviral compounds. These monoclonal antibody-drug conjugates could possibly be used as therapeutic regimens for the treatment of human arenaviral hemorrhagic fevers such as Lassa fever, Argentine hemorrhagic fever, and Bolivian hemorrhagic fever for which there is still a substantial mortality rate in regions where the viruses that cause these diseases are endemic.

All four of the monoclonal antibodies were initially screened using epifluorescence microscopy first against infected, acetone-fixed cells and secondly, against living PCV-infected cells. All four demonstrated at least some ability to bind to nonfixed, infected cells. Monoclonal antibody PC4.9A6 was selected as the candidate for further, more extensive, evaluation on the basis of its ability to bind to nonfixed, living infected cells.
Hoffman contrast microscopy, used in conjunction with epifluorescence microscopy, revealed that PC4.9A6 was reacting with living PCV-infected cells that appeared to be more rounded and refractile than similar cells that had been fixed with acetone and then stained. It also showed that the PC4.9A6 was reacting with a much smaller population of cells under nonfixed conditions. Apparently, the intact, live, unfixed infected cells were more resistant to staining with PC4.9A6. The cells that were specifically stained with PC4.9A6 may have had membranes that were more porous or less resistant to penetration by the FITC-labeled conjugate. Another explanation for this occurrence was that the infected cells, at some point during the infection, had the PCV nucleoprotein on their surface as shown by Zeller et al. (19). Alternative routes of antigen presentation, such as the exocytosis of protease degradation products from previously endocytosed virus particles, may account for these observations (11).

The monoclonal antibodies that were produced in our laboratories immunoprecipitate two proteins from infected cell lysates, one of 64 kiloDaltons (kDa) and another of 38 kDa. Harnish et al. (5) have reported that proteins of these sizes correspond to PCV nucleoprotein (NP) and a breakdown product of NP, respectively. It must be noted that Vezza et al. (15) have reported molecular masses of 66 kDa for NP, 64 kDa for GP1, and 38 kDa for GP2. The molecular masses of the proteins isolated with our monoclonal antibodies tend to confirm the observations of Harnish et al. (5) with regard to molecular masses of the PCV proteins species. The monoclonal antibody 33.6 did not demonstrate any ability to bind GP2 using the radioimmunoprecipitation technique. The failure of the HHS and monoclonal antibody 33.6 to precipitate any of the viral glycoproteins in this assay may be due to the 12 h chase period used to radiolabel the infected
cells. Because the glycoprotein precursor protein (GPC) is readily cleaved into GP1 and GP2, a lengthy chase period (>9 h) could result in the cleavage and secretion into the medium of all radiolabeled viral glycoproteins. This would result in the disappearance of the radiolabeled glycoproteins from the infected cells lysates (5).

Immunodetection of viral and cellular proteins transferred to nitrocellulose (Western blotting), using PC4.9A6, PC4.9D3, PC4.7C2, and PC4.8D2 as the primary antibodies, did not yield satisfactory results due to high background when these antibodies were used (data not shown). This was in part due to the autonucleation of the silver intensifier used to enhance the signal of the gold-conjugated secondary antibody used in the immunodetection system. Another explanation for this lack of reactivity in the Western blotting system was that the antibodies were reactive with a conformational-dependent epitope that was destroyed or denatured during the processing of the gels and the blots, although measures were taken to minimize this problem. Antibody 33.6 did react with a single 33 kDa glycoprotein when it was used as the primary antibody for Western blotting. Glycoprotein detection using concanavalin A and HRPO to localize cellular and viral glycoproteins confirms that there is a major PCV glycoprotein band at 33 kDa that most likely corresponds to GP2. The other viral glycoprotein, GP1, migrated at 57 kDa. The PCV glycoprotein precursor protein (GPC, ~80 kDa) was not detected using this assay. Further, glycoprotein detection did not reveal a viral glycoprotein band at 64 kDa or at 38 kDa. The presence of viral glycoproteins in these positions would have been a strong indication that PC4.9A6, PC4.9D3, PC4.8D2, and PC4.7C2 did indeed react with viral glycoprotein antigens that may be more readily accessible to antibodies on the surface of PCV-infected cells.
Although PC4.9A6 apparently is not maximally bound to all live, virally infected cells, the antibody does have some ability to target splenic macrophages from PCV-infected hamsters when it was administered in vivo. Splenic macrophages from PCV-infected hamsters that were inoculated with FITC-labeled PC4.9A6 on day 9 p.i. 8h prior to being sacrificed demonstrated an average 400% increase in total fluorescence and fluorescent intensity over similar uninfected animals when the samples were analyzed using flow cytometry. Similar splenic macrophages, stained in vitro and run in parallel, did not demonstrate this same targeting effect. This suggests that the availability of free antigen may influence the uptake of the FITC-conjugate. It is possible that PCV may produce an over-abundance of NP and that this may be accessible to the immune system. Thus, the FITC-conjugate may be able to bind this free antigen and be carried into the macrophages. Another possibility is that the infected macrophage may express portions of NP on its surface, perhaps in the context of a class II histocompatability antigen, in its role as the antigen-presenting cell (APC) to the immune system. Recent advances in the study of antigen presentation by macrophages indicate that both antigens found on the surface of virus particles as well as proteins from the interior of the virion can be expressed on the surface of the APCs (9, 11, 16, 19). This may allow for the conjugate to bind and be taken up into the cell even though the conjugate is directed toward an internal viral protein. Lafon and Lafage have demonstrated the antiviral activity of monoclonal antibodies directed toward the internal proteins of rabies virus (9). Also, Mills et al. have demonstrated that cytotoxic T cells were induced to proliferate when exposed to APCs that expressed the internal matrix protein of influenza virus (11). Thus, the monoclonal antibodies tested here may target infected
cells although they are directed toward an internal PCV protein. At least a portion of the binding may be attributable to the presence of Fc receptors on the surface of the macrophage. This appears the most likely explanation for the binding of the FITC-labeled 9A6 to macrophages in noninfected animals. The levels seen in this case more closely parallel the levels from the in vitro targeting data. These lower levels may be attributable to low or nonexistent levels of free antigen in the animals or in the cell culture medium in which the in vitro targeting cells were stained.
FIG. IV-1. Comparison of acetone-fixed PCV-infected and noninfected Vero-76 cells stained with FITC-labeled PC4.9A6 using epifluorescence and Hoffman contrast optics. A: Infected cells viewed with epifluorescence; B: Noninfected cells viewed with epifluorescence; C: Same microscopic field as A viewed with Hoffman optics; D: Same microscopic field as B viewed with Hoffman optics (1200 x magnification).
FIG. IV-2. Comparison of live, unfixed PCV-infected and noninfected Vero-76 cells stained with FITC-labeled PC4.9A6 using epifluorescence and Hoffman contrast optics. A: Infected cells viewed with epifluorescence (note stippled staining of infected cells); B: Noninfected cells viewed with epifluorescence; C: Same microscopic field as A viewed with Hoffman optics; D: Same microscopic field as B viewed with Hoffman optics. (1200 x magnification).
FIG. IV-3. Immunoprecipitation of proteins from PCV-infected Vero-76 cells radiolabeled with $^{35}$S-methionine by monoclonal antibodies PC4.9A6, PC4.9D3, PC4.7C2, and PC4.8D2. Lanes are labeled, n = noninfected cell lysate; i = PCV-infected cell lysate.
FIG. IV-4. Western blot of PCV-infected and noninfected Vero-76 cells.

Lane 1: noninfected Vero-76 cells probed with 33.6 as primary antibody.
Lane 2: PCV-infected Vero-76 cells probed with 33.6 as primary antibody.
Lane 3: Glycoprotein detection of PCV-infected Vero-76 cells. Lane 4: Glycoprotein detection of noninfected Vero-76 cells.
FIG. IV-5. Flow cytometry histograms of MHA hamster splenic macrophages stained in vivo with FITC-labeled PC4.9A6. A: Noninfected hamster spleen macrophages (n=5); B: PCV-infected hamster spleen macrophages (n=4).
FIG. IV-6. Comparison of flow cytometry histograms of splenic macrophages from MHA hamsters. A: Unstained, normal hamster spleen macrophages; B: Noninfected hamster spleen macrophages stained with FITC-labeled PC4.9A6; C: PCV-infected hamster spleen macrophages stained with FITC-labeled PC4.9A6.
Table IV-1. Reactivity of anti-PCV monoclonal antibodies in mouse subisotype immunoassays.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>IgM</th>
<th>IgG1</th>
<th>IgG2a</th>
<th>IgG2b</th>
<th>IgG3</th>
<th>IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC4.9A6</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PC4.9D3</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PC4.7C2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PC4.8D2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NMS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> NMS = normal mouse serum.
Table IV-2. Comparison of areas and mean channels from histograms A and B of Figure 5.

<table>
<thead>
<tr>
<th>Histogram</th>
<th>Percent of histogram to the right of cursor&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean Channel&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (Noninfected hamster)</td>
<td>8.33</td>
<td>36.3</td>
</tr>
<tr>
<td>B (PCV infected hamster)</td>
<td>41.81</td>
<td>41.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentage of total area of the histogram found to the right of the cursor. The cursor was set to exclude background fluorescence as determined by the autofluorescence of nonstained MHA hamster splenic macrophages as seen in Figure 6A.

<sup>b</sup> The mean channel of the FITC-9A6 fluorescence of the PCV-infected hamsters is shifted 5.1 units to the right of the mean channel for the normal control hamsters.
LITERATURE CITED


Smith, V. Knight, and J. A. D. Smith (eds.), Clinical applications of ribavirin. Academic Press, Inc. Orlando, FL.


CHAPTER V
CHARACTERIZATION OF THE RESPONSE OF MHA HAMSTERS TO TREATMENT WITH ANTI-PICHINDE VIRUS MONOCLONAL ANTIBODY-GELOVIN IMMUNOCONJUGATE

An immunotoxin (IT) was produced by the conjugation of a phytotoxin, gelonin, to a Pichinde virus (PCV) reactive monoclonal antibody, PC4.9A6. This immunotoxin was tested in vivo in PCV-infected MHA hamsters, a model for arenaviral hemorrhagic disease. The IT was not active against the disease at the dosage tested and by the treatment route employed in this study. The positive control, ribavirin, demonstrated a highly significant ($P < 0.005$) increase in survivors. Serum titrations indicated that 3 of the 5 IT toxicity control animals had developed some humoral response that inhibited the binding of PC4.9A6 to infected target cells. The hamsters did not show any response to the gelonin moiety of the IT.

The targeting of anti-tumor drugs and phytotoxins to neoplastic and virally-infected cells using immunologically reactive macromolecules is a relatively new field of endeavor that has introduced a new degree of specificity to the pharmacological action of these compounds. By chemically linking the drugs or toxins to large protein molecules such as monoclonal antibodies (1, 9, 11) or cellular virus receptors (5), a new dimension has been added to the delivery of these compounds to specifically selected and targeted cell populations. Fifty percent inhibitory dosages ($ID_{50s}$) in the range of $10^{-11}$ M are not uncommon in determinations made using monoclonal antibody-toxin conjugates against cancer cells (8) whereas the 50% cytotoxic dosage
(CD₅₀) in antigen-negative cells is on the order of 10⁻⁷ M resulting in a therapeutic index (T. I. = CD₅₀/ID₅₀) of 10,000.

In a recent study, Chaudhary et al. (5) used the CD4 antigen as a means of targeting *Pseudomonas* exotoxin A to cells infected with the human immunodeficiency virus (HIV), this resulted in specific killing of HIV-infected cells. Human phase I trials using a mouse anti-human-CD5 monoclonal antibody conjugated with ricin A chain have been undertaken in patients with refractory chronic lymphocytic leukemia (9). This study demonstrated that the treatment was well tolerated, although no beneficial effect was observed in the patients.

In vitro and in vivo studies in other than human subjects cannot be extrapolated directly to the human patient. In vitro and in vivo therapeutic efficacy is not a guarantee that the therapy will work in humans as is demonstrated in the human phase I leukemia study by Hertler et al. (9) referred to above. Even though the IT was binding to and saturating all of the available binding sites on the lymphocytes of the patients, there was still no therapeutic efficacy in preventing the recurrence of the leukemia. Thus, surface binding is not the only criteria to be addressed in the development of monoclonal antibody-directed delivery systems. The IT must also be readily endocytosed, eventually releasing the toxin or drug into the cell (4). Antigen density is another criteria that must be considered in the development of vehicles for targeted delivery. Sufficient antigen must be readily accessible to the IT. Low antigen density will prevent the uptake and subsequent release of the toxin into the target cell. The simple fact that a molecule binds to the surface of a cell does not ensure that this molecule is well suited as a targeted delivery vehicle (8).
Prior studies indicated that FITC-labeled PC4.9A6 did target PCV-infected cells in vivo. This is significant because it indicated that an immunoconjugate did reach the target population of cells. Thus, the in vivo efficacy of a novel IT, PC4.9A6 conjugated with the ribosomal-inactivating protein gelonin, against PCV-induced arenaviral hemorrhagic fever in MHA hamsters was evaluated in this study. Currently available therapeutic treatments still have room for improvement in combating arenaviral hemorrhagic diseases in humans. By targeting the gelonin to PCV-infected cells, it was hoped that a therapeutic effect could be observed due to the selective elimination of PCV-infected cells from the still healthy cell population and the concurrent reduction in the systemic toxicity of the toxin. Although this therapeutic effect did not occur the experiment has helped to elucidate some of the problems to be considered in using IT for antiviral therapy.

MATERIALS AND METHODS

Cells. The Vero-76 cells used in this study were obtained from the American Type Culture Collection (Rockville, MD). The cells were passaged in minimum essential media (MEM, GIBCO, Grand Island, NY) supplemented with 9% fetal bovine serum (FBS, Hyclone Labs, Logan, UT). For immunofluorescent assays in 96-well polystyrene plates (Corning, Corning, NY), 0.1 ml of cells diluted to $4 \times 10^5$ cells/ml in MEM with 9% FBS was added to each well.

Animals. The MHA hamsters used in this experiment were supplied by Charles River Laboratories, Inc. (Wilmington, MA). The hamsters were females that weighed approximately 40-50 g at the onset of the experiment. The animals were quarantined for 24-48 h prior to use. The hamsters were
housed five per cage in polycarbonate cages with corn cob bedding and were provided with Wayne Lab Blox and tap water ad libitum.

**Titration of PCV An4763 6-3-86 (B) virus pool in MHA hamsters.**

Hamsters were infected subcutaneously (s.c.) with 0.2 ml of one-half log_{10} dilutions (10^{-1.5} to 10^{-6}) of PCV pool, PCV strain An 4763 of 6-3-86 (B). Animals were observed and counted on a daily basis. All days of death were recorded. LD_{50} and LD_{90} determinations were made using the method of Reed and Muench (15).

**Ribavirin and PC4.9A6-gelonin immunotoxin.**

1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) was provided by the U. S. Army Medical Research Institute for Infectious Diseases via Technoassociates, Inc. (Rockville, MD). The IT, PC4.9A6 conjugated with the A chain phytotoxin gelonin (Sigma, St. Louis, MO), was prepared by Dr. Bill B. Barnett (Utah State University, Logan, UT). The IT was shown to inhibit in vitro protein synthesis when the disulfide bridge connecting the gelonin to the monoclonal antibody was chemically reduced. The immunotoxin was 16% gelonin by weight (personal communication, B. B. Barnett).

**In vivo determination of antiviral efficacy of the PC4.9A6-gelonin immunotoxin.**

Hamsters used for the determination of antiviral effectiveness of the PC4.9A6-gelonin immunotoxin (IT) were all infected s.c. with 0.2 ml of a PCV suspension containing one LD_{90} (80 immunofluorescent cell forming units) of PCV strain An 4763. The infected hamsters were randomly divided into six treatment groups of ten animals each. The treatment groups were as follows: i) PC4.9A6 treated (0.56 mg/kg/day); ii) Gelonin treated (0.1 mg/kg/day); iii) PC4.9A6/gelonin mixture (0.56 mg/kg/day of PC4.9A6 + 0.1 mg/kg/day of gelonin); iv) PC4.9A6-gelonin IT (0.66 mg/kg/day); v) Ribavirin (40 mg/kg/day); and vi) Saline. All treatments were performed
once a day intraperitoneally (i.p.) on days 3-5 post-infection (p.i.) except for the ribavirin-treated group that was treated twice a day on days 1-14 p.i. The uninfected animals were housed completely separate from the infected animals. The toxicity control group consisted of five uninfected, PC4.9A6-gelonin IT-treated hamsters. The normal controls were five uninfected, untreated hamsters. Toxicity and normal controls were weighed individually immediately prior to the first treatment and then again prior to all subsequent IT treatments. After day 7 p.i., they were weighed on a weekly basis. Toxicity controls were bled via the orbital sinus on days 3, 10, 17, 24, and 31 p.i.

**Production of hamster anti-gelonin serum.** Four PCV-infected hamsters that had been treated with gelonin and had survived the course of the experiment were boosted with 0.1 ml i.p. of a 1 mg/ml dilution of gelonin in sterile phosphate buffered saline (PBS), 8 weeks after the initial injections of gelonin. Serum was collected from these animals and pooled 4 days after the second injection of gelonin. Anti-gelonin activity of the hamster serum was determined using an indirect enzyme-linked immunosorbent assay (ELISA) as described below.

**ELISA for the detection of the inhibitory effects of toxicity control hamster sera on the binding of PC4.9A6 to PCV-infected Vero-76 cells.** Twenty-four hour confluent monolayers of Vero-76 cells in microtiter plates were infected with PCV were fixed as previously described (3), and stored at -20°C until used. The cells were infected with a dilution of PCV that maximized the observable fluorescence of the acetone-fixed infected monolayers when stained with a PC4.9A6-FITC conjugate. Ten-fold dilutions (10⁻¹ to 10⁻⁴) of serum collected from the IT toxicity control animals were made in dilution buffer (PBS with 0.1% BSA [Jannsen, Piscataway, NJ]
and 0.1% sodium azide [Kodak, Rochester, NY]). To each dilution of hamster serum (0.9 ml) was added 0.1 ml of a 60 µg/ml dilution of purified PC4.9A6 antibody (final concentration 6 µg/ml). The dilution tubes were incubated in a waterbath at 37°C for 1 h. Triplicates of the dilutions were plated (25 µl/well) on the fixed, infected microtiter plates. Normal hamster serum, PC4.9A6 alone (6 µg/ml), rabbit anti-PC4.9A6 serum (1:100 dilution) with 6 µg/ml PC4.9A6, pooled IT toxicity serum without PC4.9A6, and nontreated blanks were included as controls. The plates were wrapped in Saran Wrap® and incubated for 1 h at 37°C. After incubation, the plates were washed three times with dilution buffer and 50 µl of a 1:1000 dilution of goat anti-mouse IgG-urease conjugate (Sigma, St. Louis, MO) was added to each well. Urease is an enzyme isolated from *Canavalia ensiformis* (Jack bean) that is not found in mammalian cells. Urease catalyzes the cleavage of ammonia from urea causing an increase in the pH of weakly buffered solutions. This increase in pH is detected, either visually or spectrophotometrically, as a change in the color from yellow to purple of the pH indicator bromcresol purple in the urease chromogen (13). The plates were incubated for 2 h at 37°C, after which the urease conjugate was decanted from the plates and the plates were washed three times with water. Urease chromogen (80 mg/l bromcresol purple, 0.15 mM NaOH, 0.1% urea, and 0.2 mM EDTA, pH 4.6, 100 µl/well) was added to the plates, and the plates were incubated until the positive controls reached an endpoint. Absorbance at 590 nm was read on a BioTek microplate reader.

**ELISA for the detection of anti-gelonin antibodies in hamster sera.**

Round bottom ELISA plates (Immulon 2, Dynatech Labs, Inc., Chantilly, VA) were coated with gelonin (10 µg/ml) by adding 0.1 ml/well of gelonin diluted in coating buffer (0.1 M carbonate, ph 9.6). The plates were incubated
for 24 h at 4°C, after which they were washed four times with water and allowed to dry. Coated plates were stored at -20°C until used.

Remaining protein binding sites on the gelonin-coated plates were blocked prior to use by adding 100 μl of blocking buffer (5% BSA in PBS) to each well and incubating at 4°C overnight. Dilutions (10^-1 to 10^-4) of hamster sera in dilution buffer were made. To each well of the microtiter plate was added 25 μl of the appropriate dilution of hamster sera and the plates were allowed to incubate for 1 h at 37°C. The plates were washed three times with dilution buffer. Fifty μl/well of the urease conjugate (Sigma) diluted 1:1000 in dilution buffer was added to the plates and they were further incubated at 37°C for 2 h. The plates were then washed three times with water and 100 μl/well of urease chromogen was added. Wells treated with normal hamster serum and hamster anti-gelonin serum, as well as nontreated wells, were included as controls. Plates were incubated until positive control wells reached an endpoint. Absorbance at 590 nm was then read on a BioTek microplate reader.

Statistical evaluation. Increases in survivors were evaluated using Chi-square analysis with Yates' correction. Mean survival time of the treated hamsters was evaluated using Student's t-test. Data from the mouse anti-PC4.9A6 assays and from the mouse anti-gelonin assays were analyzed by analysis of variance using a randomized block design.

RESULTS

Titration of PCV in MHA hamsters. Hamsters infected with PCV had an average day of death of 10.7 ± 2.1 days (Fig. 1). The LD_{50} titer of this virus pool was 1.6 x 10^6 LD_{50}/ml.
Effects of i.p. treatment with PC4.9A6-gelonin IT. The data from the experiment performed to investigate the antiviral efficacy of the IT is summarized in Table 1. There was no significant increase in survivors or in mean survival time in any of the treatment groups, except for the ribavirin group (10) that showed a highly significant ($P<0.005$) increase in survivors. Increase in mean survival time for the ribavirin-treated group would have been statistically significant except for one death that occurred on day 20 p.i. (Fig. 2). Days of death data are shown in Figure 3.

Toxicity of PC4.9A6-gelonin IT. Data from the toxicity controls collected during the first week of treatment suggests that there may have been some host toxicity associated with the administration of the IT, as indicated by the decrease in the average weights of the IT-treated animals after day 3 p.i (Fig. 4). The toxicity controls were able to overcome this initial toxicity and went on to gain normal or above amounts of weight after day 4 p.i. (Fig. 5). Normal controls did not manifest any loss of weight throughout the course of this experiment.

Humoral response of MHA h·msters to IT treatment. Analysis of the data collected from ELISA for the inhibition of PC4.9A6 binding to PCV-infected Vero-76 cell by dilutions of serum collected weekly from the IT toxicity control animals revealed that there was a highly significant ($P<0.001$) inhibition (Table 2, Figs. 6 and 7). At the $10^{-1}$ dilution of hamster serum taken at week 1 (Fig. 6), there is a significant decrease in absorption, indicating that there is a component in this dilution of serum that prevents the binding of PC4.9A6 to infected cells. When the same data was plotted with respect to the time of sample collection and holding the dilution constant ($10^{-1}$ dilution, Fig. 7), the data indicate that there were 3 of the 5 animals whose serum appeared to at least partially block PC4.9A6 binding.
The data from the $10^{-2}$ dilution of hamster serum, plotted in the same fashion, demonstrated that this effect was no longer present at this dilution (Fig. 8). The same was true for the $10^{-3}$ and $10^{-4}$ dilutions in this assay (data not shown). Positive control serum, rabbit anti-PC4.9A6, was able to completely inhibit antibody binding in this assay (Fig. 6).

The same dilutions ($10^{-1}$ to $10^{-4}$) of hamster serum were tested for their gelonin binding activity employing an indirect ELISA. There was no significant reaction of the toxicity control hamsters to the gelonin moiety of the IT as evidenced by the lack of reactivity in these assays (Table 2). Positive control serum (hamster anti-gelonin) did react in this assay (Fig. 9). Normal hamster serum used as a negative control in this assay demonstrated more reactivity with gelonin than the IT toxicity control animals serum at a $10^{-1}$ dilution, although it did not surpass the positive control serum in its reactivity with gelonin (Figs. 9 and 10). The apparent reactivity of the normal hamster serum was abolished at the $10^{-2}$ dilution (Fig. 11). It should also be noted that pre-treatment serums, collected from the IT toxicity control animals prior to IT treatment, did not demonstrate increased gelonin reactivity at the $10^{-1}$ dilution (Figs. 9 and 10).
DISCUSSION

Several recent studies using monoclonal antibodies conjugated to A chain toxins, including gelonin, have suggested that these immunotoxins are able to specifically kill either lymphoid or tumor cells in vitro and in vivo (12, 14). A similar approach using Pseudomonas exotoxin A conjugated to recombinant human CD4 has been shown to selectively kill HIV-infected cells in vitro (5).

Because of the success in using these immunotoxins against cancer and HIV infection, an application of this approach was sought for the inhibition of PCV-infected cells. Gelonin appears to be particularly attractive for this application because it is not toxic to intact cells and must be transported into the cell in order for it to inhibit protein synthesis via the enzymatic inactivation of ribosomes (6, 16). The IT used in this study is approximately 16% gelonin by weight and has been shown to inhibit in vitro protein synthesis when the disulfide bonds linking the gelonin to the antibody are chemically reduced (B. B. Barnett, Utah State University, Logan, UT, personal communication).

The data from this experiment indicate that the IT is not having the desired effect in preventing hemorrhagic disease in this model (Table 1). The positive control, ribavirin (10), confirmed that the hemorrhagic disease model could be successfully treated by conventional antiviral chemotherapy. The PC4.9A6 only, gelonin only, PC4.9A6 + gelonin, and saline treated controls also did not have a significant effect on extending the survival time or number of survivors in the infected hamsters. The lack of IT activity in this model could be due to a number of factors such as insufficient dosage of IT, the blocking of the antigen binding site of the antibody by free viral
antigen in the blood and serum, the timing of the administration of the IT, or that the IT is not taken up by the infected cells. The dosage of IT used in this experiment is similar to the dosages that have shown tumor and HIV-infected cell killing activity in other systems (5, 7). In addition, the host weight loss occurring in the toxicity control animals treated with IT suggests the maximum tolerated dose was approached in this experiment.

Serum from 3 of the 5 IT toxicity control hamsters demonstrated significant ability to inhibit the binding of PC4.9A6 during the first week after the animals were treated with IT. The apparent reaction of these hamsters to the antigen-binding portion of PC4.9A6 of the IT was indicative that there may have been an antiidiotypic response induced due to treatment with the IT. The humoral response of these hamsters was weaker than the anti-PC4.9A6 serum that was used as a positive control, although still highly significant, but these animals were treated with what would be considered a very poor immunization schedule, if induction of anti-PC4.9A6 antibody was to be the end of the experiment. Because the hamster and the mouse appear to be genetically closely related, an antiidiotypic response by a hamster to the immunoglobulin is an interesting effect. It should be noted that the appearance of the PC4.9A6-inhibitory substance in the serum of the toxicity control hamsters does not appear to develop as a classical immune response to a foreign antigen, that is with a well defined lag period that gradually increased over time to a maximum titer. The inhibition was noted immediately after treatment with the IT and dropped off within a week of the cessation of treatment. The total anti-PC4.9A6 response of the IT toxicity control hamsters was not done because of the innate cross reactivity of anti-mouse reagents with hamster antibodies. This genetic relatedness of the mouse and the hamster may be what was responsible for the development of
the "antiidiotypic" response of the hamster to the mouse immunoglobulin. It is conceivable that the only region of the immunoglobulin to which the hamster could react was the hypervariable region of the mouse immunoglobulin and that this response was what blocked that binding of PC4.9A6 to PCV-infected cells in the ELISA.

In general, the phytotoxins such as ricin, abrin, and gelonin are considered to be immunogenic in animals (1). The lack of a humoral immune response by the hamsters to the gelonin indicates that there must have been only very low amounts of gelonin on the antibody molecule. Since the IT was 16% gelonin by weight, this indicates that there was probably only one or two molecules of the toxin on the antibody molecule. This may not have been enough to induce an adequate immune response to the phytotoxin, though it would have been sufficient to induce the catalytic inactivation of the 28 S ribosomal subunits of the cells if the toxin had penetrated to the interior of the infected cells.

In conclusion, there are several possible explanations for the lack of reactivity of the monoclonal antibody toxin conjugate that was tested in this PCV-MHA hamster model of arenavirus-induced hemorrhagic disease. The immunotoxin was probably reaching at least some of the infected target cells in the animals. This was demonstrated in an earlier report (Chapter 4). The lack of reactivity in this system could be due to lack of expression of PCV nucleoprotein (NP) on the surface of the infected cells to which this antibody could bind to early in the infection process. It is possible that low levels of this antigen in the serum or other serum components such as antiidiotypic antibody directed against the paratope of PC4.9A6, would not allow this antibody to bind readily to the infected cells. There may not have been enough virus replication and infection of the target cells by days 3, 4, and 5.
p.i. for IT to be targeted. If this is the case, another antibody, directed against an antigen expressed earlier on the surface of infected cells, should be selected as the target antigen.

Another possibility is that killing the infected cells is not sufficient to stop the virus infection or that virus replication in some other way eclipses the activity of the IT. If antigen availability is required prior to the targeting of the IT to infected cells, then there may already be enough virus assembled in the infected cells to go on and infect another round of cells after the cell is inactivated by the IT. This condition may be further aggravated if the immunotoxin was taken up into the cell and the toxin portion was hydrolyzed from the IT, even though this was a desirable occurrence in order for the inactivation of ribosomes to occur. The hydrolyzed toxin would then have lost its ability to penetrate any more infected cells, due to the nature of the A chain toxin, and would be subjected to immediate clearance from the animal (2).

This experiment pointed out the large number of variables that require consideration in attempting to target toxins using monoclonal antibodies. Surface reactivity or reactivity with live cells should not be the only considerations given to screening antibodies for their potential as toxin delivery vehicles. By incorporating a step in the initial monoclonal antibody screening procedures that allows for the evaluation of many candidate monoclonals, perhaps a more critical evaluation of the specific monoclonals can be made. This step could take the form of an indirect assay using goat anti-mouse, or another such antibody, conjugated with the toxin of interest to evaluate the targeting potential of all candidate monoclonal antibodies.
Figure V-1. Day of death histogram for the titration of PCV in MHA hamsters. Titration of PCV pool 6-3-86 (B) in 6 week old female MHA hamsters. Mean day of death was $10.7 \pm 2.1$ days.
Figure v-2. Mean day of death of PCV-infected MHA hamsters by experimental treatment groups. There were no significant differences in the day of death of any of the treatment groups.

* One death at day 20 p.i.
Figure V-3. Day of death histogram of PCV-infected MHA hamsters by experimental treatment groups. The positive control ribavirin demonstrated a significant increase in survivors. No other treatment demonstrated a significant effect.
Figure V-4. Average weights of MHA hamsters from experiment days 3 to 6. IT-treated hamsters demonstrated a decrease in weight following the first day of treatment.
Figure V-5. Weekly average weights of IT toxicity and normal MHA hamster controls. Toxicity controls did not demonstrate any cumulative toxic effects over the course of the experiment.
Figure V-6. ELISA of the inhibitory effects of IT toxicity control hamster sera on PC4.9A6. Sera from 3 of 5 hamsters demonstrated significant inhibitory effects on PC4.9A6 binding to PCV-infected cells at week 1 at a 1:10 dilution.
Figure V-7. ELISA of the effects of IT toxicity control hamster sera on PC4.9A6 (1:10 dilution). Effect of IT toxicity control hamster sera on the inhibition of PC4.9A6 binding to PCV-infected cells, absorbance plotted with respect to time.
Figure V-8. ELISA of the effects of IT toxicity control hamster sera on PC4.9A6 (1:100 dilution). Effect of IT control hamster sera on the inhibition of PC4.9A6 binding to PCV-infected cells, absorbance plotted with respect to time.
Figure V-9. ELISA of the gelonin binding activity of IT toxicity control hamster sera. Hamster anti-gelonin serum was the positive control. Normal hamster serum showed slightly increased anti-gelonin activity.
Figure V-10. ELISA of the gelonin binding activity of IT toxicity control hamster sera (1:10 dilution). Normal hamster serum demonstrated slightly elevated gelonin binding activity, absorbance plotted with respect to time of sample.
Figure V-11. ELISA of the gelonin binding activity of IT toxicity control hamster sera (1:100 dilution). Normal hamster serum no longer demonstrated increased gelonin binding activity, absorbance plotted with respect to time of sample.
Table V-1. Effect of i.p. administration\(^a\) of PC4.9A6-gelonin IT on PCV-infection in 3-4 week old MHA hamsters.

<table>
<thead>
<tr>
<th>Treatment(^a)</th>
<th>Start of Treatment(^b) (mg/kg/day)</th>
<th>Dose</th>
<th>No. Survivors/ Mean survival time(^c) (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC4.9A6 only</td>
<td>72 h post</td>
<td>0.56</td>
<td>0/10</td>
</tr>
<tr>
<td>Gelonin</td>
<td>72 h post</td>
<td>0.10</td>
<td>4/10</td>
</tr>
<tr>
<td>PC4.9A6/Gelonin(^d)</td>
<td>72 h post</td>
<td>0.56/0.10</td>
<td>1/10</td>
</tr>
<tr>
<td>PC4.9A6-Gelonin IT(^e)</td>
<td>72 h post</td>
<td>0.66</td>
<td>0/10</td>
</tr>
<tr>
<td>Ribavirin(^f)</td>
<td>24 h post</td>
<td>40.0</td>
<td>9/10(^g)</td>
</tr>
<tr>
<td>Saline</td>
<td>72 h post</td>
<td>0</td>
<td>4/20</td>
</tr>
</tbody>
</table>

Toxicity controls: The IT toxicity controls all survived at a similar dose level and all lost weight after the first day of treatment, although they did begin to gain weight after the third day of treatment. Normal controls did not exhibit this loss of weight (Fig. 2).

\(^a\) Treatment once daily on days 3, 4, and 5 p.i. unless otherwise noted.
\(^b\) Relative to virus inoculation.
\(^c\) Animals dying on or before day 21.
\(^d\) A noncovalently linked mixture of the antibody PC4.9A6 and gelonin.
\(^e\) A covalently linked conjugate of PC4.9A6 and gelonin.
\(^f\) Treatment twice daily beginning 24 h p.i. on day 1 to day 14.
\(^g\) P<0.005.
Table V-2. Analysis of variance of the anti-PC4.9A6 and the anti-gelonin activity of serum collected from toxicity control MHA hamsters for experimental weeks 0, 1, and 2.

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>DF&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Anti-PC4.9A6 activity</th>
<th>Anti-gelonin activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F value</td>
<td>P value</td>
</tr>
<tr>
<td>Main Effects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hamster (H)</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dilution (D)</td>
<td>3</td>
<td>112.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Error (H x D)</td>
<td>12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Time (T)</td>
<td>2</td>
<td>9.624</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Error (H x T)</td>
<td>8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Interactions</td>
<td></td>
<td></td>
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<tr>
<td>Dilution x</td>
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<tr>
<td>Time</td>
<td>6</td>
<td>8.702</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Error (H x [D x T])</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>59</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Degrees of freedom.

<sup>b</sup> Not significant.
LITERATURE CITED


CHAPTER VI
SUMMARY AND FUTURE DIRECTIONS

Although treatment of Pichinde virus (PCV)-infected hamsters with immunotoxin (IT) did not prevent the development of disease, this model of directed delivery does demonstrate the potential for the in vivo targeting of PCV-infected cells. As is shown in chapter IV, the fluorescein-PC4.9A6 conjugate (FITC-9A6) demonstrated the ability to specifically bind to PCV-infected cells. This is an indication that the system was effective in targeting immunoconjugates and that the process of conjugation did not destroy the ability of the monoclonal antibody to bind to infected cells. The lack of antiviral activity of the IT treatment used in this study could be due to a number of different reasons such as insufficient toxin on the conjugate to effectively kill the cell, though one molecule of toxin should have been adequate if it was taken into the cell. Another possibility is that the toxin was cleaved from the monoclonal antibody and eliminated from the animal prior to the antibody reaching the target cell. Also, the surface binding of monoclonal antibodies to infected cells may not be sufficient to ensure delivery of the toxin to the interior of the cell. It should be noted that viral antigen on the surface of a cell is considered to be on its way out of the cell as it is incorporated into a viral particle and not returning to the interior of the cell. Thus, there are a number of possible reasons that this particular IT treatment regimen was not effective. The optimization of several parameters, such as the viral target antigen, the appropriate toxin, the most efficient means of covalently linking the toxin to the antibody, as well as the
treatment route and schedule, needs to be undertaken before this approach to the antiviral immunoconjugate therapy can be utilized successfully.

Initially, there is a need to demonstrate, in vitro, if possible, that virus replication and spread to other noninfected cells can be prevented by using a targeted toxin delivery system based on either polyclonal or monoclonal antibodies. This could take the form of an indirect screening procedure in which polyclonal sera or an extensive panel of monoclonal antibodies, directed against a number of viral antigens and immunologically distinct epitopes, are used as the primary antibody. This could also include the testing of several different isotypes and subisotypes of antibodies, perhaps that are directed against the same epitope, in order to determine the effect of isotype and subisotype on antibody uptake into infected cells. The secondary antibody should be directed against the immunoglobulin from the species from which the polyclonal sera or monoclonal antibodies were derived. The secondary antibody could then be conjugated with any of a number of phytotoxins or bacterial toxins such as gelonin, ricin A chain, abrin, diphtheria toxin, or *Pseudomonas* exotoxin A to name a few. Inhibition of either viral CPE or fluorescence due to viral antigen would be indicative of a successful combination of primary and secondary antibodies. An in vitro screening system for antiviral activity appears to be the only practical and cost effective alternative for screening the large numbers of potential primary and secondary antibody combinations, although it may not be as effective in ensuring the delivery of the biologically active macromolecules to the target cells.

In vitro targeting could also be investigated as a possible screening method using fluorescent microscopy. By employing secondary antibodies fluorescently tagged with a green molecule, the surface binding of the
primary antibody could be investigated in live cells. The Fc binding of the live cells could be blocked by using an unlabeled, irrelevant antibody derived from a species other than that from which the primary antibody was prepared. This would allow for the detection of virus-specific reactivity of the primary antibody on the surface of infected cells. In a parallel experiment, the secondary antibody that has been conjugated with the toxin molecule could be added to the cells. Following this step, a third or tertiary antibody, directed against the toxin moiety of the IT, bearing a red fluorescent label could be added. This would allow for the detection of toxin on the surface of infected cells when probed with the secondary antibody. This could be performed on cells that had been fixed or on live cells in order to demonstrated binding and uptake. Similar experiment could be performed using radiolabeled primary, secondary, and tertiary antibody preparations. Also, in vivo experiments could be done similar to those performed in chapter V, once the basic parameters had been optimized in vitro.

Once this system has been optimized, it would be an excellent system for the screening of antiviral drug-monomoclonal antibody conjugates in order to evaluate the efficacy of these compounds in combating viral disease. An approach similar to that described for immunotoxin evaluation could be taken in developing indirect screening procedures for the evaluation of antiviral drug-monomoclonal antibody conjugate therapy.

In conclusion, the development of monoclonal antibody-directed drug or toxin delivery systems is not simply a matter of producing antibodies and then using them for targeting infected cells. Surface binding of monoclonal antibodies to infected cells, though it is an important criterion, does not appear to be the sole criterion for the selection of an antibody for use in this targeted delivery system. The selection of antibodies that target and deliver
toxins or drugs to infected cells does appear to be an extremely complex process that requires the optimization of many parameters before optimal results can be observed. The questions with regard to which antibodies are best suited for use in this system may best be answered by developing an indirect procedure for the screening of large numbers of potential antibodies.
VITA

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Family Status: Married to Lynette Larson (1982); daughter Katherine; son Jake.

Utah State University.
Major: Biology
Area of Emphasis: Virology

M.S. Degree: 1987, Brigham Young University
Major: Microbiology
Area of Emphasis: Virology

B.S. Degree: 1983, Brigham Young University
Major: Microbiology
Minor: Chemistry

Experience

Department of Biology, Utah State University.
Graduate student, Research assistant, 1985 to present.
Graduate Professors: Robert W. Sidwell and Bill B. Barnett. Responsibilities include the development and characterization of monoclonal antibodies to Pichinde virus for use in a carrier-mediated system for directed delivery of antiviral drugs, and the development of an antiviral assay based on detection of Pichinde virus antigen using fluorescent antibodies.
Department of Microbiology, Brigham Young University. Graduate student, Research assistant, 1982 to 1985. Graduate Professors: Byron K. Murray and Roland K. Robins. Responsibilities included the routine screening of antiviral compounds against a number of different RNA and DNA viruses. Thesis project was the in vitro evaluation of a novel aminopyrimidopyrimidine antiviral agent. While working for Dr. Robins, research into the in vitro antiviral effects of combinations selenazofurin, ribavirin, tiazofurin, and 3-deazaguanosine was undertaken in order to test for possible synergistic effects of the compounds on virus inhibition.

Awards

National Research Council Research Associate, 1988
Graduate internship award, Graduate School of Brigham Young University, 1983

Scientific Society Membership

American Society for Microbiology (Intermountain Branch)
American Association for the Advancement of Science
American Society for Virology

Publications

See attached sheet
Publications


Abstracts from meetings attended


