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DEVELOPMENT OF METHODS FOR CARRIER-MEDIATED TARGETED DELIVERY OF ANTIVIRAL COMPOUNDS USING MONOCLONAL ANTIBODIES

ANNUAL REPORT

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SRI International and Utah State University are developing methodology to assess the effectiveness of antiviral drug-monoclonal antibody conjugate therapy for the treatment of viral infections. Specifically, monoclonal antibodies to Pichinde virus surface antigens were prepared and conjugated to the antiviral drug ribavirin, and the resulting conjugates will be screened for antiviral activity both in vitro and in animal model systems.

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FOREWORD

For the protection of human subjects, the investigator(s) adhered to the policies of applicable Federal Law 45CFR56.

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RESEARCH SUMMARY

Research was conducted to: 1) synthesize modified analogs of the antiviral drug ribavirin that would permit attachment of this drug to monoclonal antibodies, 2) covalently link these ribavirin derivatives containing tether groups to monoclonal antibodies directed against the viral antigens expressed on the surface of Pichinde virus-infected cells, 3) develop *in vitro* and *in vivo* Pichinde virus models for use as test systems for evaluating the targeted delivery of antiviral compounds using monoclonal antibodies, and 4) derive, produce, and evaluate monoclonal antibodies against Pichinde virus-coded antigens expressed on the surface of the virally infected cells.

1. Synthesis of Ribavirin Derivatives Having Tether Group Functionality and Their Conjugation to Anti-Pichinde Virus Monoclonal Antibodies

The ammonium salts of the 5'-hemisuccinate, 5'-(2,3-dimethyl)-hemisuccinate, and 5'-(5-carboxypentyl)phosphate esters of ribavirin were prepared and characterized. The 5'-hemisuccinate had one-tenth the antiviral activity of ribavirin against Pichinde virus, whereas the 5'-(2,3-dimethyl)hemisuccinate had one-hundredth the activity.

The phosphate diester has been submitted for antiviral testing. Two anti-Pichinde virus monoclonal antibody PC 4.9A6 conjugates with ribavirin-5'-hemisuccinate were synthesized, characterized, and submitted for biological evaluation. A study was conducted to determine the optimum method of determining drug loading on the immunoconjugate. A direct method using radiolabeled ribavirin derivatives was found to be the most accurate but also the most labor-intensive. An indirect method that measured the release of a chromophoric species from the activated 4-nitrophenyl ester of the 5'-hemisuccinate was also reliable.

2. In Vitro Antiviral Evaluations. I. Development and Application of an Immunofluorescence Assay Suitable for in vitro Antiviral Drug Screening Against Pichinde Virus

Pichinde virus (PCV) produces only a marginal cytopathogenic effect (CPE) in vitro. This CPE does not develop until 10-14 days postinoculation (p.i.). Because of these limitations on assays based upon CPE, antiviral assays against PCV often utilize plaque reduction (PR) that requires relatively large amounts of drug and is slow to reach an acceptable endpoint. We have developed an immunofluorescence assay (IFA) that can be used in antiviral drug assays against PCV. The antiviral drug activity is determined by measuring the inhibition of PCV infectivity in Vero 76 cells. The assay is performed using an immunofluorescent cell count assay to detect Pichinde virus-infected cells in 96-well cell culture plates. This

assay has the following advantages: the presence or absence of fluorescing cells is easy to score, the assay is read after only 24 h, the amount of drug required to perform an assay is reduced by over 80%, and the sensitivity and reproducibility are comparable with that achieved with assays based on plaque reduction. This section describes the immunofluorescence antiviral assay and demonstrates the performance of the assay in the evaluation of the antiviral drugs selenazofurin, ribavirin, 3-deazauridine, and (S)-DHPA against PCV. The antiviral drug assay based on inhibition of immunofluorescence was found to be as sensitive as antiviral assays based on inhibition of CPE or PR, with the added advantages of economy of reagents, rapidity, and a less ambiguous endpoint. The PCV immunofluorescence assay was then used to determine the in vitro antiviral activity of several ribavirin derivatives. The ED_{50} of ribavirin using inhibition of immunofluorescence as the parameter for scoring antiviral activity was consistently between 10 and 32 μg/ml, which is in the same range as the antiviral activity measured using inhibition of CPE, reading a marginal endpoint, or using PR. A candidate derivative for drug tethering, ribavirin-5'-hemisuccinate, was found to have an ED_{50} of 10-32 μg/ml and an MTD of 1,000 μg/ml, values suggesting that ribavirin-5'-hemisuccinate may be a suitable derivative for tethering to antibodies. A more stable derivative, ribavirin-5'-dimethylhemisuccinate, had an ED_{50} of 100-320 μg/ml and an MTD of 32 μg/ml.

3. Immunofluorescence Assay Suitable for Evaluating Antiviral Immunoconjugates. Development and Application for Antiviral Evaluation of Ribavirin-Immunoconjugates

In this section we describe an antiviral assay utilizing inhibition of immunofluorescent foci formation for the evaluation of antibody-targeted immunodrugs. A small amount of an immunoconjugate consisting of ribavirin attached to monoclonal antibody (MAb) PC 4.9A6 was evaluated using this modified anitiviral assay. In a traditional in vitro antiviral assay, the test compound is added within an hour of the virus inoculum. The test compound then has the opportunity to exert an antiviral effect prior to the first round of viral replication. Whereas this type of antiviral assay is effective for screening traditional antiviral drugs, it is not suitable for evaluating antiviral immunoconjugates. An antibody-targeted antiviral drug is produced by conjugating an antiviral drug to an antibody that targets a marker antigen defining the population of target cells. The targeted antivirals being produced in this project utilize MAbs specific for viral antigens expressed on the surface of virus-infected cells. Thus, the targeting potential of these immunoconjugates cannot be realized until virus-infected cells have begun to express viral antigens at the cell surface. The initial multiplicity of infection (MOI), the time of addition of the test compound, and the time at which the test is read take on a more critical role in an assay with antiviral immunoconjugates.

The antiviral activity (ED_{50}) of ribavirin when added 20 h p.i., as measured in the assay described in this section, was 32 μg/ml compared with an ED_{50} of 10 to 32 μg/ml when ribavirin was added concurrently with PCV. Because there was a limited amount of the ribavirin-5'-hemisuccinate-immunoconjugate available at present, only one dose was used for screening. There was a slight indication of a reduction in the number of cells infected following treatment at the 1-μg/ml dose of ribavirin-immunoconjugate (the maximum concentration evaluated based on ribavirin content). There was no indication of activity at the corresponding level of antibody alone. Because the ED_{50} for ribavirin was 32 μg/ml, the lack of antiviral activity with the immunoconjugate at 1 μg/ml was not surprising. The obvious problem was that the concentration of ribavirin in the immunoconjugate was 32-fold less than the ED_{50} of nonconjugated ribavirin.

4. Derivation of Hybridoma Cell Lines that Secrete Monoclonal Antibodies toward Pichinde Virus Antigens Expressed on the Surface of Infected Cells. I. Fusion, Selection, and Expansion

The fusion procedure described in this section was used to derive more than 50 antibody-producing hybridoma cell lines. Four of these cell lines (PC 4.7C2, PC 4.8D2, PC 4.9D3, and PC 4.9A6) produced antibody toward PCV antigens expressed on the surface of Pichinde virus-infected cells. These were each cloned twice. The

hybridization technique included several modifications over previous procedures that failed to yield sufficient numbers of hybridoma cell lines. The hybridization procedure was modified to include peritoneal macrophages in the fusion wells. These macrophages serve as feeder cells, they condition the media, and they phagocytize dead cells and debris, thereby reducing the toxicity of the fusion culture media. Other modifications included an antigen boost administered to the spleen donor mouse five days prior to the fusion, the avoidance of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) when dimethyl sulfoxide was present, more gentle manipulation of the fusion cell mixture during the critical fusion steps, and, finally, not adding the selection agent aminopterin to the fusion cell cultures until 24 h postfusion.

5. Derivation of Hybridoma Cell Lines that Secrete Monoclonal Antibodies toward Pichinde Virus Antigens Expressed on the Surface of Infected Cells. II. The Use of Macrophage Feeder Cells to Increase Hybridoma Yields

Hybridoma cells derived by the procedures described in this annual report died after only a few divisions unless macrophages were included in the fusion cultures. The inclusion of macrophages in the fusion cultures significantly increased the yield of viable hybridoma cell lines. This section describes the procedures for using macrophages to enhance hybridoma cell recovery. An experiment comparing the hybridoma cell yields with and without macrophages is

described. At seven days postplating, hybridoma cell colonies in wells cultured with macrophages had an average population of 98 ± 90 cells, whereas those in wells without macrophages averaged 24 ± 28 cells/colony. The percentage of wells yielding hybridoma colonies large enough to warrant antibody screening (at least 200 cells) was 9% in plates without macrophages and 22% in plates with macrophages. All four of the hybridoma cell lines that produced MAb toward PCV antigens that were expressed on the surface of infected cells were obtained from the fusion mixtures that had been cultured with macrophages.

6. Derivation of Hybridoma Cell Lines that Secrete Monoclonal Antibodies toward Pichinde Virus Antigens Expressed on the Surface of Infected Cells. III. Cloning and Storage of Hybridoma Cell Lines that Produce Monoclonal Antibodies toward Pichinde Virus

Representative doubly cloned hybridoma cell lines from each of the four anti-PCV MAb secretor hybridoma lines were expanded to cell densities of 1-2 x 10^6 cells/ml in T75 cell culture flasks, and then 1.0-ml aliquots were frozen. It was determined that cloning in RPMI medium without feeder cells was generally satisfactory. Cloning in fresh RPMI medium prepared with fetal bovine serum (FBS) that had not been heat-inactivated resulted in a higher yield of mature clones than did cloning in conditioned medium.

7. Derivation of Hybridoma Cell Lines that Secrete Monoclonal Antibodies toward Pichinde Virus Antigens Expressed on the Surface of Infected Cells

A cell-freezing procedure suitable for the FOX-NY myeloma cell lines is described in this section. MAb-secreting hybridomas were frozen and stored in liquid nitrogen. Frozen FOX-NY myeloma cell lines were thawed and cultured. After three cell doublings, over 98% of the cells in the recovered cultures were viable.

8. Ascites Tumor Formation Using Hybridomas Derived from the Fusion of FOX-NY Myeloma Cells with Spleen Cells from RBF/Dn Mice

Hybridoma cell lines derived from the fusion of FOX-NY myeloma cells with spleen cells from RBF/Dn mice are more stable in cell cuture than are conventionally derived hybridoma cell lines (Taggart and Samloff, 1983). Taking advantage of this increased genetic stability, we derived a series of (FOX-NY x RBF/Dn) hybridoma cell lines that secreted MAbs toward PCV antigens. Four of these MAbs recognized PCV antigens expressed on the surface of Pichinde virus-infected cells *in vitro* and *in vivo*. Because large quantities of these MAbs were required for *in vivo* antiviral studies, the corresponding hybridoma cell lines were injected into mice for ascites production. However, we found that the usual protocols for the production of ascites tumors applied to these hybridoma cell lines

resulted in erratic, inefficient ascites tumor production. Attempts to propagate these (FOX-NY x RBF/Dn) hybridoma cell lines by generally accepted protocols in recipient mice of the strains RBF/Dn and BALB/c, and the (RBF/Dn x BALB/c)F_1 hybrid of the two resulted in limited ascites production. However, more aggressive immunosuppression of the recipient mice did increase the success rate for ascites tumor production. By increasing the pristane dose or by including total body gamma irradiation in the immunosuppression protocols, ascites production was greatly enhanced.

9. Purification of Anti-Pichinde Virus Monoclonal Antibodies

MAbs PC 4.9A6 and PC 4.9D3 were purified from murine ascites fluids using ammonium sulphate precipitation followed by affinity chromatography on protein A-Sepharose. The purification procedure and yields at each step are described in this section. The immunoglobulins following ammonium sulfate precipitation were approximately 75% pure IgG based on examination by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The purity after the affinity-chromatography step was above 95%. The yield of IgG after the ammonium sulfate precipitation was about 85% for PC 4.9A6 and 60% for PC 4.9D3. The yield for the affinity-chromatography step was 15%-40%. The overall yields for the purification of PC 4.9A6 and PC 4.9D3 were 34% and 9%, respectively. The purified MAbs retained complete antigen-binding activity.

10. The Effect of Lyophilization on the Binding Activity of Anti-Pichinde Virus Monoclonal Antibodies PC 4.9A6 and PC 4.9D3

Lyophilization usually does not grossly affect the activity of polyclonal antisera, but some MAbs lose binding activity upon lyophilization. In this study, the effect of lyophilization on anti-Pichinde virus MAbs PC 4.9A6 and PC 4.9D3 was examined. MAbs PC 4.9A6 and PC 4.9D3, either as crude ascites preparations or as highly purified immunoglobulins, were not adversely affected by lyophilization.

11. Preparation of Fluorescein-Labeled Monoclonal Antibodies

MAbs PC 4.9A6 and PC 4.9D3 specific for PCV antigens expressed on the surface of Pichinde virus-infected cells were conjugated with fluorescein isothiocyanate (FITC). All three conjugations resulted in fluorescein-labeled antibody preparations with excellent immunostaining characteristics. The immunofluorescent conjugates described in this section have proved to be very useful tools, several of the applications of which are described in other sections of this annual report. The fluorescein-labeled MAbs were subsequently used in direct immunofluorescent cell count assays for PCV. The fluorescein-labeled MAbs were also used to determine the in vivo distribution of MAbs PC 4.9A6 and PC 4.9D3 in infected and noninfected hamsters (see Section XIII of this annual report). The

fluorescein-labeled MAbs were also used in place of radiolabeled antibodies in competitive binding assays to determine the epitope-binding specificity of several anti-Pichinde virus MAbs (see Section XII).

12. Anti-Pichinde Virus Monoclonal Antibodies. Characterization: Binding to Pichinde Virus Antigens Expressed on the Surface of Living Cells, Isotype Analysis, Epitope Specificity, and Viral Protein Specificity

This section describes the characterization of several MAbs derived against PCV. It was determined that MAbs PC 4.7C2, PC 4.8D2, PC 4.9D3, and PC 4.9A6 bind to antigens expressed on the surface of viable Pichinde virus-infected cells. This observation indicated that these MAbs may be suitable candidates as drug delivery vectors for the antibody-mediated targeted delivery of antiviral drugs. Twenty-five anti-Pichinde virus MAbs resulting from the PC4 fusion experiment were examined by ELISA using isotype-specific reagents. The majority, 22 of 25, were of the IgG_{2a} isotype; two were of the IgG_1 isotype; and one was of the IgG_{2b} isotype. MAbs PC 4.7C2, PC 4.8D2, PC 4.9D3, and PC 4.9A6, which reacted with PCV antigens expressed on the surface of infected cells, were of the IgG_{2a} isotype. These four MAbs were selected for further characterization. The epitope specificities of the four MAbs were examined using competitive binding assays. MAbs PC 4.9A6 and PC 4.7C2 recognized the same epitope, whereas MAb PC 4.9D3

recognized a second, nonoverlapping epitope. Competitive binding assays with anti-LCMV MAb WE 33.6, which cross-reacts with the GP2 glycoprotein of PCV, resulted in partial competition with MAbs PC 4.9A6 and PC 4.9D3. This was one of the indications that anti-Pichinde virus MAbs PC 4.9A6 and PC 4.9D3 bind to epitopes on the GP2 glycoprotein of PCV. In this section we describe the binding specificities of MAbs PC 4.7C2, PC 4.8D2, PC 4.9D3, and PC 4.9A6 as revealed by applying radioimmunoprecipitation techniques using ^{35}S-labeled lysates from Pichinde virus-infected cells. All four precipitated a 38,000-dalton polypeptide that was the major viral polypeptide in the cytosols. At this time it appears that the four candidate MAbs bind to Pichinde virus glycoprotein GP2, which is displayed on the surface of Pichinde virus-infected cells.

13. <u>In Vivo Targeting with Anti-Pichinde Virus Monoclonal Antibodies PC 4.9A6 and PC 4.9D3</u>

Fluorescein conjugates of MAbs PC 4.9A6 and PC 4.9D3, which bind to antigens expressed on the surface of Pichinde virus-infected cells, were injected into infected and noninfected MHA hamsters and the biodistribution of the labeled antibodies was examined by fluorescence microscopy of thin-sections prepared from spleen, liver, lung, heart, kidney, and brain. There was a marked distribution of the labeled MAbs to the liver, kidney, spleen, and lung tissues of the infected animals as compared with the noninfected animals. The difference was particularly dramatic for the livers.

The virus titer in the livers was a thousandfold higher than titers observed in any other organs. The results of this in vivo targeting study indicate that MAbs PC 4.9A6 and PC 4.9D3, when administered i.p. to Pichinde virus-infected hamsters, concentrate in the livers of the infected animals, reaching at least 10-fold greater levels in the livers of infected animals than in livers of noninfected animals. This is an indication that these MAbs have potential as a means for evaluating the targeted delivery of antiviral drugs to virus target organs.

STATEMENT OF PROBLEM UNDER STUDY

Under USAMRIID Contract No. DAMD17-86-C-6120, SRI International and Utah State University are developing methodology to assess the effectiveness of antiviral drug-monoclonal antibody conjugate therapy for the treatment of viral infections. Specifically, we are undertaking the preparation of monoclonal antibodies directed against viral antigens expressed on the surface of Pichinde virus, the conjugation of the antiviral drug ribavirin to these monoclonal antibodies, and the biological assessment of the antiviral activity of these conjugates compared with that of free drug in virally infected cells in culture and in hamsters.

BACKGROUND

Approximately 60% of human illnesses are caused by viral infections (Horsfall, 1965). In particular, military personnel may be exposed to many different virulent pathogens when deployed abroad. The spread of these pathogens is exacerbated by inadequate sanitation, poor health conditions, and crowded living and working conditions. The arenaviruses (Junin, Lassa, and Machupa) cause hemorrhagic fevers that have high mortality rates in humans. These diseases are characterized by high fever, leucopenia, gastrointestinal hemorrhagic manifestations, shock, and, in some instances, a neurologic syndrome (Johnson et al., 1967). The duration of illnesses is generally two to three weeks, and complete recovery requires a long convalescence. The mechanism of pathogenesis for these viruses in humans appears to be direct damage to the cells. The viruses cause extensive capillary damage (either directly or indirectly), which has been proposed as the causative factor of the organ damage resulting from these diseases (Casals, 1982).

Because vaccination therapy or drug therapy for many of the infections caused by members of the Arenaviridae is not available or effective, new methods of treatment must be developed. In addition,

in those cases where antiviral drugs have been shown to inhibit the virus *in vitro*, effective therapy *in vivo* may require the administration of such high drug doses that toxic side effects occur. Therefore, by targeting the delivery of antiviral drugs to the virus or the virally infected cell, lower concentrations of drugs could be administered, thereby reducing their toxic side effects.

RATIONALE

Targeted drug therapy using antiviral drug-monoclonal antibody conjugates has potential value for the treatment of viral infections. Monoclonal antibodies (MAbs) to specific antigens expressed on the viral surface or on the surface of virally infected cells would specifically deliver the conjugated drug to the virus or the infected cell. Therefore, drug efficacy would be enhanced and systemic side effects would be reduced relative to standard treatment modalities using unbound drugs.

The drug-monoclonal antibody method of drug delivery is currently being investigated as a new therapeutic approach for the treatment of cancer (Arnon and Sela, 1982; Bjorn et al., 1986; Dillman et al., 1986; Embleton et al., 1986; Forrester et al., 1984; Gilliland et al., 1980; Hurwitz, 1982; Hurwitz et al., 1983, 1985; Kato et al., 1984; Kulkarni et al., 1981; Pimm et al., 1982; Seeger et al., 1982; Tsukada et al., 1982, 1983; Youle and Neville, 1980). Monoclonal antibodies that are specific for antigenic determinants on tumor cells have been developed (Hurwitz et al., 1985; Seeger et al., 1982). The chemotherapeutic drugs doxorubicin (Dillman et al., 1986; Hurwitz et al., 1983; Pimm et al., 1982), daunomycin (Kato et al., 1984; Tsukada et al., 1982, 1983), and

methotrexate (Kulkarni et al., 1981), the toxin ricin (Forrester et al., 1984; Youle and Neville, 1980), other toxins (Bjorn et al., 1986), and the protein A chain of ricin (Embleton et al., 1986; Gilliland et al., 1980) have been covalently linked to antibodies against antigens on tumor cell lines. The resultant conjugates have been screened in both in vitro and animal model systems. Conjugation of both drugs and toxins did not interfere with the binding of antibodies to their antigenic determinants. The conjugates had increased specific cytotoxicity to cancer cells in culture compared with control cells lacking the antigens (Kato et al., 1984; Tsukada et al., 1982). Most important, the conjugates prolonged the survival times of animals infected with the cancer cells over those of infected animals treated with the unbound drugs or antibodies alone or with combinations of free drugs with the antibodies (Tsukada et al., 1983).

TASK REPORTS

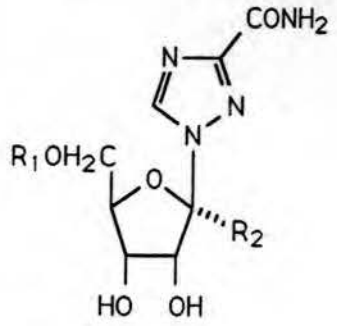
This annual progress report describes research accomplished in the second year of the contract (February 16, 1987 to March 15, 1988) of USAMRIID Contract No. DAMD17-86-C-6120. Because of the wide scope and diversity of this multidisciplinary project, the following report has been subdivided into particular task reports, which are presented in the order set forth in the Research Summary.

I. Synthesis of Ribavirin Derivatives Having Tether Group Functionality and Their Conjugation to Anti-Pichinde Virus Monoclonal Antibodies

A. Introduction

Because of its high antiviral potency, ribavirin (1, Chart I-1) (Witkowski et al., 1972; Witkowski et al., 1973) was selected as the optimum drug to determine the potential of targeted drug delivery using monoclonal antibodies (MAbs). The 5'-position on the ribofuranose ring of 1 was used to introduce the tether functionality by which the drug would be attached to the MAb. The tether functionality had to be so designed that the covalent linkage of ribavirin to the MAb would be sufficiently stable to enable the conjugate to

Chart I-1. Ribavirin and Its Functionalized Derivatives



	R_1	R_2
1	H-	H-
2	H-	^{3}H-
3	$NH_4O_2CCH_2CH_2CO$-	H-
4	$NH_4O_2CCH_2CH_2CO$-	^{3}H-
5	$NH_4O_2CCHMeCHMeCO$-	H-
6	4-NO_2-C_6H_4-$O_2CCH_2CH_2CO$-	H-
7	$NH_4O_2C(CH_2)_5OP(=O)(ONH_4)$-	H-

arrive intact at the surface of the virally infected cell. Once at the cell surface, drug release from the conjugate could occur by one of two pathways: 1) chemical or enzymic hydrolysis of the conjugate at the cell surface to afford a high local concentration of the drug, which could then enter the cell by its usual active or passive transport process, or 2) invagination of the conjugate by the cell followed by lysozomal cleavage of the conjugate to release the free drug within the cell. Ester and phosphate groups were initially proposed for linking the 5'-position of ribavirin to MAbs. Therefore, the initial synthetic targets were the ammonium salt of the 5'-hemisuccinate ester 3 of ribavirin, the related 5'-(2,3-dimethyl)hemisuccinate 5, and the 5'-phosphate diester of ribavirin with the ammonium salt of 5-carboxypentanol (7).

To accurately access the biological activity of the MAb-drug conjugates, it is necessary to determine the number of drug molecules attached to each antibody. Because ribavirin has a very weak ultraviolet chromophore, conventional loading determinations using spectroscopic absorption were not possible in the presence of protein and so we investigated alternative methods: 1) direct determination using radiolabeled ribavirin (2), 2) indirect determination by assaying the number of lysine ε-amino groups reacted with the drug, 3) indirect determination by assaying the amount of 4-nitrophenoxide anion released from an active ester intermediate of the drug, and 4) direct determination using a radioimmunoassay (RIA) for ribavirin.

B. Experimental Methods

General Procedures and Instrumentation. When required, reactions and purifications were conducted with deoxygenated solvents and under inert gas (argon) and subdued light. Solvents were dried or distilled before use. Melting points were uncorrected. TLC analyses were performed on Analtech silica gel analytical plates, using detection by UV or anisaldehyde spray (0.5 ml of anisaldehyde in 0.5 ml of concentrated sulfuric acid added to 9 ml of 95% ethanol containing a few drops of acetic acid, with the sprayed plate heated to 100-110°C for 20 to 30 min). Silica gel 60 (E. Merck No. 9385) for flash-column chromatography was obtained from Brinkman. A Pharmacia Ultrarac fraction collector equipped with a UV detector (280 nm) and a peristaltic pump was used to collect column fractions where required. Lyophilizations were performed using a Labconoco lyophilizer. HPLC analyses were performed on a Spectra Physics SP8100 instrument fitted with a Waters radial compression C18 Nova-Pak reverse-phase column with a SP8440 UV/vis detector set at 254 nm and a SP4200 computing integrator. Injection samples (20 µl) were prepared at a compound concentration of 1 mg/ml. Elution was performed using the following linear gradient system: 1) 98% 0.1 M sodium acetate/2% methanol (0 to 5 min); and 2) 98-80% 0.1 M sodium acetate/2-20% methanol (5 to 16 min) at a flow rate of 2.0 ml/min. IR spectra were recorded with a Perkin-Elmer 710B infrared spectrometer. NMR spectra were obtained with a JEOL FX90Q or Varian 400 MHz

spectrometer, using tetramethylsilane as an internal standard (δ 0). UV spectra were taken on a Perkin-Elmer 575 spectrophotometer. Optical rotations were performed on a Perkin-Elmer 141 Polarimeter. Elemental analyses were conducted by MicAnal, Tucson, Arizona. Mass spectral analyses were performed by Dr. David Thomas, SRI International, using a Ribermag Model R10-10C mass spectrometer. Samples were introduced using the desorption probe for both electron-impact and chemical-ionization studies. The number in parentheses appearing at the end of each experimental procedure refers to the SRI notebook number and page number for the start of that experiment. Only procedures for those synthetic steps in the successful routes to the target compounds are described.

2,3-Dimethylsuccinic anhydride (10). An 8.00-g (54.8-mmol) portion of meso-2,3-dimethylsuccinic acid (8) was dissolved in 12.8 g (164 mmol) of acetyl chloride (9). The resultant solution was stirred at room temperature under argon for 1 h and then heated at reflux for 12 h. The solution was concentrated by evaporation at reduced pressure and the residue was diluted with 30 ml of ether. Upon cooling in a dry ice/acetone bath, the product (10) precipitated. It was collected (3 x 10 ml of ether rinse) and dried at reduced pressure. The ether rinses were cooled in the bath to obtain a second crop of crystals. The combined yield of 10 was 6.23 g (89%), mp 38-39.5°C [reported: 36-37.2°C (Cason and Schmitz, 1963; Dozen and Hatta, 1975; Ragab and Butler, 1981), and 39-40°C

(Linstead and Whalley, 1954; Bode and Brockmann, 1972)]; 90 MHz ^1H NMR ($CDCl_3$) δ 1.31 (d, 6 H, CH_3), 3.19 (m, 2 H, CH).

(7784-31)

2'-Trimethylsilylethyl 2,3-Dimethylhemisuccinate (12). To a solution of 5.45 g (42.2 mmol) of 10 in 40 ml of anhydrous ethyl acetate was added 5.08 g (42.99 mmol) of 2-trimethylsilylethanol (11) and 4.17 g of triethylamine. This solution was stirred at room temperature under argon for 1 h and then at reflux for 1.5 h. After concentration at reduced pressure, the reaction mixture was taken up into 5% aqueous sodium bicarbonate. This solution was washed with ether (2 x 30 ml), acidified to pH 2 with 2 N sulfuric acid, and extracted with ether (3 x 40 ml). These ether extracts were dried (Na_2SO_4) and concentrated to afford 8.64 g (82%) of 12 as an oil: IR ($CHCl_3$) 2960, 2945, 2875, 2740, 1720, 1705, 1460, 1385, 1250, 1165, 1075, 935, 855, 835 cm^{-1}; 90 MHz ^1H NMR ($CDCl_3$) δ 0.08 (s, 9 H, $SiMe_3$), 0.95 (m, 2 H, $SiCH_2$), 1.24 (d, 3 H, $CHCH_3$), 1.28 (d, 3 H, $CHCH_3$), 2.79 (m, 2 H, $CHCH_3$), 4.19 (t, 2 H, CO_2CH_2).

(7794-33)

t-Butyldimethylsilyl 2'-Trimethylsilylethyl 2,3-Dimethylsuccinate (14). To a solution of 8.64 g (35.0 mmol) of 12 in 18 ml of anhydrous dimethylformamide was added 5.80 g (38.4 mmol) of t-butyldimethylsilyl chloride (13) and 4.80 g (70.5 mmol) of imidazole.

The reaction mixture was stirred at room temperature for 24 h under argon, poured into 60 ml of 2.5% aqueous sodium bicarbonate, and extracted with hexane (3 x 50 ml). The combined organic extracts were dried and concentrated to afford an oil, which was distilled (104-107°C, 0.30 mm) to give 9.62 g (76%) of 14 as a colorless oil: IR ($CHCl_3$) 2950, 2930, 2860, 2750, 1730, 1715, 1460, 1250, 1160, 835 cm^{-1}; 90 MHz ^1H NMR ($CDCl_3$) δ 0.026 (s, 9 H, $SiMe_3$), 0.25 (s, 6 H, $SiMe_2$), 0.87-0.94 (m, 2 H, $SiCH_2$), 0.91 (s, 9 H, CMe_3), 1.13 (d, 3 H, $CHC\underline{H}_3$), 1.21 (d, 3 H, $CHC\underline{H}_3$), 2.7 (m, 2 H, CHCH), 4.15 (t, 2 H, CO_2CH_2).

(7794-35)

2'-Trimethylsilylethyl 2,3-Dimethylsuccinyl Chloride (16). A literature procedure (Wissner and Grudzinskas, 1978) was adapted. To a solution of 2.3 g (8.8 mmol) of 14 in 15 ml of dry methylene chloride was added 1 ml of dimethylformamide followed slowly by 1.05 g (8.27 mmol) of oxalyl chloride (15), via a syringe. This mixture was stirred at 0°C for 2 h and then concentrated at reduced pressure (14 mm followed by 0.25 mm for 1 h) to remove t-butyldimethylsilyl chloride, giving 1.4 g (83%) of crude acyl chloride 16 as a colorless oil, which was used immediately: 90 MHz ^1H NMR ($CDCl_3$) δ 0.06 (s, 9 H, $SiMe_3$), 0.98 (m, 2 H, $SiCH_2$), 1.14 (d, 3 H, $CHC\underline{H}_3$), 1.22 (d, 3 H, $CHCH_3$), 2.61 (m, 2 H, $C\underline{H}CH_3$), 4.16 (m, 2 H, CO_2CH_2).

(7794-25)

1-[5-[3-(2-Trimethylsilyl)carbethoxy-2,3-dimethylpropionyl]-β-D-ribofuranosyl}-1,2,4-triazole-3-carboxamide (19). To a solution of 1.1 g (4.5 mmol) of ribavirin (1) and 0.1 g (0.52 mmol) of 2,5-di(t-butyl)pyridine (17) in 10 ml of anhydrous N-methylpyrrolidinone (18) was added dropwise, via an addition funnel, a solution of 1.4 g (5.3 mmol) of acyl chloride 16 in 15 ml of 18 at room temperature under argon. The resulting yellow solution was stirred for 16 h before the solvent was removed by vacuum distillation at 0.5 mm. The residue was purified by flash-column chromatography on silica gel using 3-8% methanol/chloroform as the eluant to give 0.77 g (36%) of 19, which by HPLC (reverse-phase Radialpak A column, methanol) was mostly one peak (90%); IR ($CHCl_3$) 3660, 3590, 2990, 2960, 2880, 2390, 1715, 1705, 1580, 1515, 1470, 1420, 1220, 1040, 925 cm^{-1}; 400 MHz ^1H NMR ($CDCl_3$) δ 0.02 (s, 9 H, $SiMe_3$), 0.92 (m, 2 H, $SiCH_2$), 0.96 (d, J = 3 Hz, 3 H, $CHCH_3$), 1.04 (d, J = 3 Hz, 3 H, $CHCH_3$), 2.02 (m, 1 H, $CHCH_3$), 2.35 (m, 1 H, $CHCH_3$), 4.13 (q, J = 7 Hz, 2 H, 5'-CH_2), 4.3-4.7 (m, 3 H, 2',3',4'-CH), 5.81 (br d, 1 H, 1'-CH), 7.63 and 7.81 (2 s, 2 H, $CONH_2$), 8.82 (s, 1 H, 5-CH).

(7794-27)

1-[5-(3-Carboxy-2,3-dimethylpropionyl)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide Ammonium Salt [Ribavirin 5'-(2,3-Dimethylhemisuccinate) Ammonium Salt] (5). A solution of 0.76 g (1.6 mmol) of silyl ester 19 in 4 ml of 1.0 M tetra(n-butyl)ammonium

fluoride (20, 4.0 mmol) in tetrahydrofuran was stirred at room temperature under argon for 13 h, at which time TLC (20% methanol/-chloroform) showed no spot (R_f 0.57) corresponding to 19. The reaction mixture was concentrated at reduced pressure to give 0.9 g of residue, which was dissolved in the minimum amount of water and chromatographed on a DEAE-Sephadex A-25 column (HCO_3^- form). Elution by a linear gradient of 0.0 to 0.25 M ammonium bicarbonate gave, after lyophilization, 0.51 g (85%) of 5 as a hygroscopic, fluffy, white solid: 400 MHz ^{1}H NMR (Me_2CO-d_6) δ 0.95 (d, 3 H, $CHC\underline{H}_3$), 1.02 (d, 3 H, $CHC\underline{H}_3$), 2.24 (m, 1 H, $C\underline{H}CH_3$), 2.36 (m, 1 H, $C\underline{H}CH_3$), 4.05-5.9 (ms, 5 H, 2',3',4'-CH, CO_2CH_2), 7.62 and 7.85 (s, 2 H, $CONH_2$), 8.82 (s, 1 H, 5-H); ^{13}C NMR (Me_2SO-d_6) 13.6, 14.7, 42.3, 43.6, 63.9, 70.5, 74.1, 81.8, 91.8, 145.2, 157.4, 160.4, 174.8, 176.6 ppm.

(7794-53)

[1-^{3}H]Ribavirin-5'-hemisuccinate Ammonium Salt (4). To a solution of 8.00 mg (0.032 mmol) of [1-^{3}H]ribavirin (2) having specific activity of 0.778 mCi/mmol in 300 μl of anhydrous N-methylpyrollidinone (18) at 23°C under argon was added 15 mg (0.063 mmol) of 2-trimethylsilylethyl succinyl chloride (21). This mixture was stirred for 17 h. The solvent was removed at reduced pressure and the residue was chromatographed on 2.5 g of Merck silica gel using 25-ml volumes of 2%, 4%, 6%, 8%, and 10% MeOH/$CHCl_3$ to give 7.0 mg

(48%) of [1-^{3}H]-1-[5-[3-carbo-(2-trimethylsilyl)ethoxypropionyl]-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide (22), which had R_f 0.18 (10% MeOH/$CHCl_3$), corresponding to that of unlabeled 22.

Silyl ester 22 was dissolved in 300 µl of dry THF and then 100 µl (0.1 mmol) of 1.0 M (n-Bu)$_4$NF (20) in THF was added. The reaction mixture was stirred at room temperature for 16 h. The solvent was removed at reduced pressure. The residue was chromatographed on DEAE A-25 Sephadex, which had been preswollen in 1 M NH_4HCO_3 and then loaded onto a 1.0 x 10.0-cm column. The column was eluted with 1.0 L of a linear gradient of 0.0-0.5 M NH_4HCO_3. The product eluted in Fractions 23 to 26 (fraction volume, 3.3 ml). The fractions were combined. A 50-µl aliquot was counted in 10 ml of Ecolite scintillation fluid (WestChem), giving 11,632 cpm at 0.35% efficiency. If the specific activity remained at 0.778 mCi/mmol, then 2.03 mg (18%) of 4 was obtained. An additional 1.06 mg (10%) of 4, based on radioactive counting, was recovered from Fractions 1 to 8. This material eluted early because of column overloading of the product mixture in 0.5 M NH_4HCO_3. The combined fractions were lyophilized to dryness at -70°C and 100 µ Hg and then lyophilized twice more from 10 ml of water to remove salts. The overall yield of 4 was 3.6 mg (28%). ^{1}H NMR (Me_2SO-d_6) δ 2.43 (m, 4 H, CH_2CO), 4.1-5.0 (m, 5 H, 2',3',4'-CH, 5'-CH_2), 5.81 (d, 1 H, 1'-CH), 7.55 and 7.75 (broad s, 1 H, NH), 8.95 (s, 1 H, 5-H).

(8144-5)

[1-^{3}H]Ribavirin-5'-hemisuccinate Conjugate with MAb PC 4.9A6 (25). To a gently stirred solution of 3.0 mg (2.0×10^{-5} mmol) of MAb PC 4.9A6 (24) in 2.0 ml of 0.01 M potassium phosphate, 0.5 M NaCl, pH 7.6, in a 10-ml test tube cooled in an ice bath was added a 4°C solution of 1.60 mg (0.062 mmol) of [1-^{3}H]ribavirin-5'-hemisuccinate ammonium salt (4; specific activity, 0.778 mCi/mmol) and 3.1 mg (0.01 mmol) of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (23) in 1.0 ml of 0.01 M potassium phosphate, 0.5 M NaCl, pH 7.6. The reaction mixture was slowly stirred for 1.5 h, during which period the solution remained clear and free of precipitate. The reaction mixture was chromatographed on G-75 Sephadex, preswollen in reaction buffer, and then loaded onto a 1.0 x 8.0-cm column, with elution by the reaction buffer at 4°C. The void volume (8.5 ml) was pooled and concentrated to 2.6 ml by centrifugation at 2500 rpm in a Centricell® (30,000 molecular-weight cutoff; Polysciences, Inc.). The protein concentration was 2.60×10^{-6} M by UV absorbance at 280 nm ($A = 0.360$, $\varepsilon = 1.38 \times 10^{5}$). The drug concentration, as determined by scintillation counting of a 0.5-ml aliquot (2,564 cpm, 35% efficiency, specific activity 0.778 mCi/mmol), was 1.73×10^{-5} M. Therefore, drug/MAb loading of conjugate 25 was 6.65 moles per mole.

(8144-19)

Ribavirin-5'-[3-carbo(4-nitro)phenoxypropionate] (6). Ribavirin-5'-hemisuccinate ammonium salt (3, 150 mg, approximately 0.31 mmol because some ammonium salts were also present) was stirred with 1.0 mL of anhydrous pyridine for 1 h to form the pyridinium salt and then the excess pyridine was removed at 0.05 mm Hg for 24 h. The resultant white residue was diluted with 1.2 mL of dioxane (distilled from sodium). To this stirred mixture (not all of the precipitate having dissolved) was added 0.063 mL (0.78 mmol) of pyridine, 0.124 mL (0.79 mmol) of diisopropylcarbodiimide (3), and 44 mg (0.32 mmol) of 4-nitrophenol (26). The reaction mixture was stirred at room temperature for 20 h, at which time an abundant white precipitate had formed. Dioxane (1 mL) was added and the precipitate was removed by centrifugation (1-mL dioxane rinse). The supernatant solution was concentrated to remove the dioxane, and the residue was chromatographed (14 g of silica gel, 10% MeOH/$CHCl_3$, (10-mL fractions) to give 45 mg (23%) of the 4-nitrophenyl ester 6 (R_f 0.32); IR ($CHCl_3$) 3100-3500, 3060, 2960, 1740, 1690, 1640, 1600, 1520, 1500, 1355, 1300, 1240, 1140, 1050 cm^{-1}; ^1H NMR (MeOH-d_4) δ 2.8-3.0 (m, 4 H, CH_2CO_2), 4.30-4.66 (m, 5 H, 2',3',4'-CH, 5'-CH_2), 5.94 (broad s, 1 H, 1'-CH), 7.32 (dd, J = 8 Hz, J = 2 Hz, 2 H, ArH ortho to NO_2), 8.26 (dd, J = 8 Hz, J = 2 Hz, 2 H, ArH ortho to CO_2), 8.65 (s, 1 H, 5-ArH).

(8205-15)

Conjugate of Monoclonal Antibody PC 4.9A6 with Ribavirin-5'-hemisuccinate (28). To 5 mg of lyophilized MAb PC 4.9A6 dissolved in 2.5 mL of 0.01 M potassium phosphate buffer, 0.5 M NaCl, pH 7.6, was added a solution containing 4.72 mg (7.96 μmol) of 4-nitrophenyl ester 6 in 52 μl of MeOH diluted with 2.45 mL of 0.01 M potassium phosphate buffer, 0.5 M NaCl, pH 7.6. This yellow solution was stirred at 5°C for 64 h with the absorbance monitored at 404 nm to determine release of 4-nitrophenoxide ($\varepsilon = 1.41 \times 10^4$). A control reaction containing all reagents except the MAb was run concurrently to determine the amount of 4-nitrophenoxide present in the original sample and that formed by hydrolysis in the buffer. At 64 h, the absorbance of the conjugate was 1.534 at 404 nm, whereas that of the control was 1.090, indicating that 0.444 absorbance units resulted from reaction with the MAb and, therefore, a loading of 4.77 mol of ribavirin-5'-hemisuccinate/mol of protein was obtained.

The reaction mixture was purified by chromatography on a 2.5 × 13-cm Sephadex G-75 column (22-mL void volume) using 0.01 M potassium phosphate buffer, 0.5 M NaCl, pH 7.6, as the eluant (4-mL fractions). Column fractions were monitored at 206, 280, and 404 nm (Figure I-1). Fractions 4 to 6 contained the conjugate. These fractions were pooled and had an absorbance at 280 nm of 0.384 (MAb PC 4.9A6 at 280 nm, $\varepsilon = 1.38 \times 10^5$), indicating approximately 5.2 mg of protein if no correction for the ribavirin absorbance were made. The pooled fractions were concentrated and the buffer was changed to 0.01 M potassium phosphate, 0.15 M NaCl, pH 7.6, using a

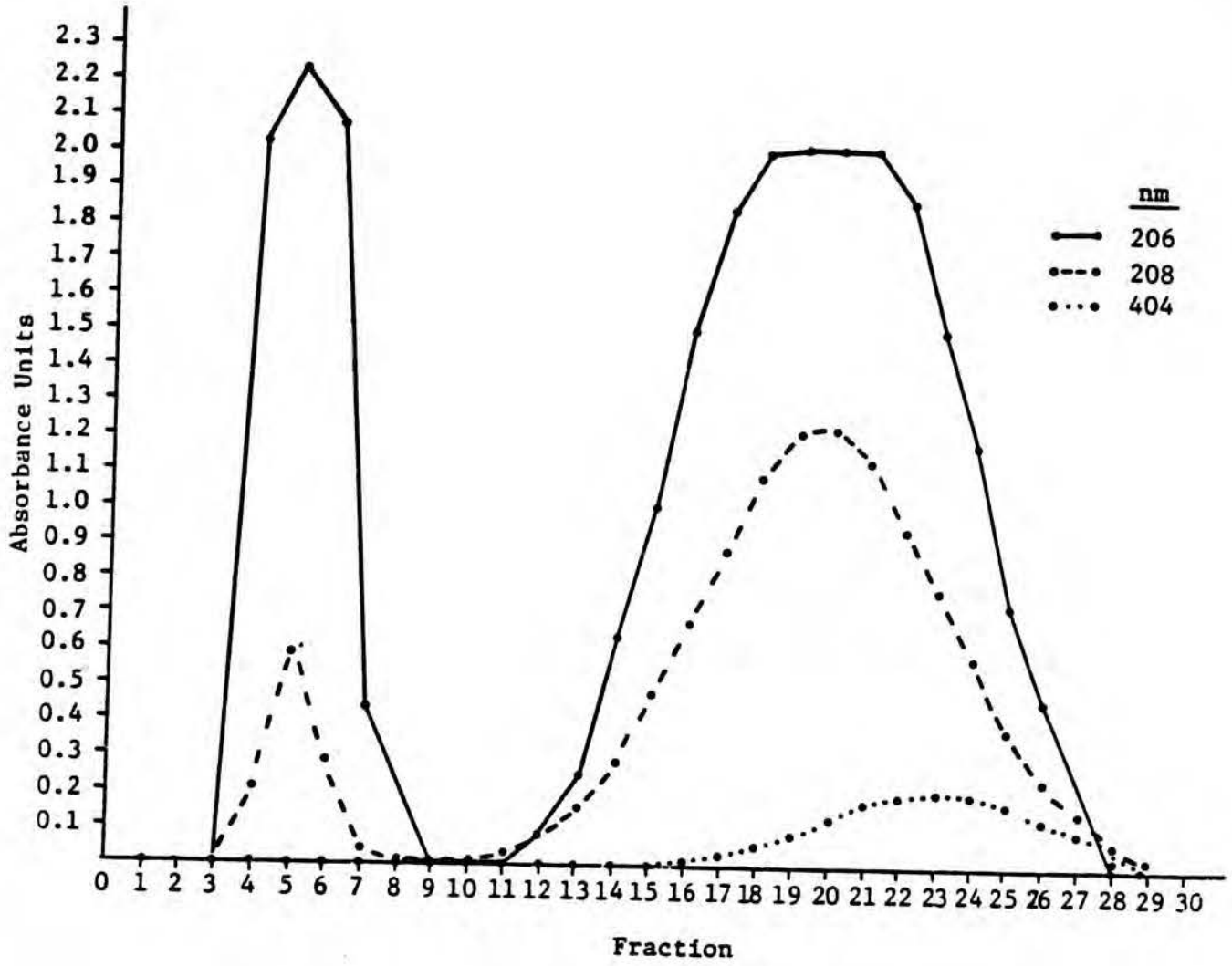


Figure I-1. Elution Profile of Conjugate at 206, 280, and 404 nm after Chromatography on Sephadex G-75.

30,000-molecular-weight-cutoff Centricell (1000 rpm). Concentration afforded 4.64 g of solution containing 3.32 mg (66% recovery) of protein. The low recovery was caused by protein sticking to the Centricell filter, because subsequent washing afforded material with a 280-nm absorbance.

(8205-17)

Ribavirin-2',3'-acetonide (32). To a magnetically stirred suspension of 1.0 g (4.1 mmol) of ribavirin (1) in 6.6 mL of triethyl orthoformate (31) was added 2.4 mL of reagent-grade acetone (30). This solution was cooled in ice under argon while 0.22 mL of ice-cold 70% perchloric acid was added dropwise over a period of 5 min. The yellow-orange reaction mixture was stirred at ice-bath temperature for 2 h and room temperature for 20 h. The deep red solution was then diluted with 50-mL portions of $CHCl_3$ and 5% $NaHCO_3$. The chloroform extraction was used to remove polymeric material. The bicarbonate layer was extracted with EtOAc (five 200-mL portions) until TLC (10% MeOH/$CHCl_3$) showed that no further product was present (R_f 0.20). The acetonide 32 (0.92 g, 92%) was obtained as a white foam; ^1H NMR ($CDCl_3$) δ 1.40 and 1.58 (2 s, 6 H, CH_3), 3.37 (s, 1 H, OH), 3.54 (m, 2 H, 5'-CH_2), 4.34 (m, 1 H, 4'-CH), 4.95 (m, 1 H, 3'-CH), 5.57 (m, 1 H, 2'-CH), 6.24 (s, 1 H, 1'-CH), 7.37 (br m, 2 H, NH_2), 8.84 (s, 1 H, 5-ArH).

(8205-11,16)

Methyl 6-Hydroxycaproate. To a stirred mixture of 150 mL of CH_2Cl_2 and 45 mL of 40% aqueous KOH at 0°C was added 15 g of N-nitrosomethylurea. The solution was cooled in an isopropyl alcohol/dry-ice bath at -50°C. After the aqueous layer had frozen, the CH_2Cl_2 layer was decanted into a second flask containing KOH pellets and allowed to dry for 1 h at -50°C. This solution was added dropwise to a solution of 4.12 g (31.0 mmol) of 6-hydroxycaproic acid (Karl Industries) at -50°C until the yellow color of diazomethane persisted. Glacial HOAc was added dropwise until the solution again became colorless. Drying ($MgSO_4$) and concentration afforded 5 mL of a yellow oil, which was evaporatively distilled (80°C, 0.25 mm Hg) to give 1.27 g (28%) of the ester as a colorless oil; ^1H NMR ($CDCl_3$) δ 1.30-1.80 (m, 6 H, CH_2), 1.90 (s, 1 H, OH), 2.35 (t, J = 9 Hz, 2 H, CH_2CO_2), 3.65 (t, J = 9 Hz, 2 H, CH_2OH), 3.70 (s, 3 H, CO_2CH_3).

(8207-13)

(5-Carbomethoxypentyl)(2-cyanoethyl)diisopropylphosphoramidite (33). A solution of 1.09 g (7.45 mmol) of methyl 6-hydroxycaproate in 1.75 mL (9.00 mmol) of distilled diisopropylamine was added to 16 mL of anhydrous THF under argon. Next, 1.63 (6.88 mmol, 1.6 mL) of N,N-diisopropylmethylphosphonamidic chloride (95%; Aldrich) was added slowly over 3 min using a syringe. The reaction mixture was stirred at room temperature for 20 min before 40 mL of EtOAc was

added to precipitate the diisopropylamine hydrochloride that formed, which was removed by filtration. The filtrate was diluted with 80 mL of EtOAc and washed with two 80-mL portions of cold 5% $NaHCO_3$, dried (Na_2SO_4), and concentrated to give 2.3 g (97%) of 33 as a colorless oil; ^1H NMR ($CDCl_3$) δ 1.2 (d, J = 10 Hz, 12 H, CH_3), 1.4-1.8 (m, 6 H, CH_2), 2.3 (t, J = 9 Hz, 2 H, CH_2CO_2), 3.3 (t, J = 7 Hz, 2 H, CH_2CN), 3.5 (t, J = 7 Hz, 2 H, OCH_2CH_2CN), 3.6 (m, 2 H, CHN), 3.7 (s, 3 H, CO_2CH_3), 4.1 (t, J = 9 Hz, 2 H, $POCH_2(CH_2)_4$); ^{31}P NMR ($CDCl_3$) -146.9 ppm relative to 85% H_3PO_4 in H_2O.

(8297-22)

Ribavirin-2',3'-acetonide-5'-(2-cyanoethyl-5-carbomethoxypentyl)phosphate (36). To a stirred mixture of 201 mg (0.71 mmol) of anhydrous ribavirin-2',3'-acetonide (32) and 44 mL (17.6 mmol) of 0.4 M tetrazole (34) in MeCN (Applied Biosystems) under argon was added dropwise 1.23 g (33.54 mmol) of (5-carbomethoxypentyl)(2-cyanoethyl)diisopropylphosphoramidite (33) in 1.5 mL of anhydrous MeCN. The solution was stirred for 1 h at room temperature. Then 6.3 mL of a solution of 0.1 M I_2 in THF/2,6-lutidine/water (2:1:1) (Applied Biosystems) was added and stirring was continued for 30 min. A 20-mL portion of 1 M $Na_2S_2O_3$ was added to reduce unreacted I_2. The mixture was extracted with CH_2Cl_2 (3 × 100 mL). The extract was washed with 5% $NaHCO_3$ (100 mL), dried (Na_2SO_4), and concentrated at reduced pressure. The residue was chromatographed (20 g of silica gel, 1% MeOH/$CHCl_3$) to give 217 mg (56%) of 36 as a

colorless oil; ^{1}H NMR ($CDCl_3$) δ 1.30-2.0 (br s, 6 H, CH_2), 1.32 and 1.60 (2 s, 6 H, CH_3), 2.32 (br t, J = 6 Hz, 2 H, CH_2CO_2), 2.81 and 2.85 (2 t, J = 6 Hz, 2 H, CH_2CN), 3.66 (s, 3 H, OCH_3), 3.85-4.70 (m, 7 H, CH', CH_2OP), 4.92-5.4 (m, 2 H, CH'), 6.2 (s, 1 H, 1'-CH), 6.90 and 7.52 (2 br s, 2 H, NH_2), 8.50 (s, 1 H, 5-ArH).

(8315-4)

Ribavirin-2'3'-acetonide-5'-(5-carboxypentyl)phosphate Bisammonium Salt (37). Ribavirin-2',3'-acetonide-5'-(2-cyanoethyl-5-carbomethoxypentyl)phosphate (36, 183 mg, 0.34 mmol) was dissolved in a solution of 10 mL of concentrated NH_4OH and 2 mL of pyridine. This solution was stirred for 3 days at 55-65°C. Concentration at reduced pressure afforded 165 mg (95%) of 37 as a pale-yellow oil; ^{1}H NMR (D_2O) δ 1.18-1.72 (br m, 6 H, CH_2), 1.43 and 1.62 (2 d, 6 H, CH_3), 2.22 (br t, J = 6 Hz, 2 H, CH_2CO_2), 3.58 (t, J = 6 Hz, 2 H, $POCH_2$), 3.96 (t, J = 5 Hz, 2 H, 5'-CH_2), 4.52-5.42 (m, 3 H, 2',3',4'-CH), 6.30 (br d, 1 H, 1'-CH), 8.73 (s, 1 H, 5-ArH).

(8315-5)

Ribavirin-5'-(5-carboxypentyl)phosphate Bisammonium Salt (7). Ribavirin-2',3'-acetonide-5'-(5-carboxypentyl)phosphate bisammonium salt (37, 165 mg, 0.32 mmol) in 10 mL of 85% aqueous HOAc was stirred for 15 h at 65-68°C. After concentration, the mixture was chromatographed on a 1 × 12-cm Sephadex DEAE A-25 (Pharmacia) column

with 100 mL of water rinse followed by a 0.0-0.5 M NH_4HCO_3 gradient (100 mL). The product eluted in fractions 4-6 (2 mL), which were heated at 65°C at 25 mm Hg and 0.1 mm Hg for 3 h to provide 92 mg (60%) of 7 as a white powder, mp 200-215°C; ^1H NMR (D_2O) δ 1.1-1.8 (broad m, 6 H, 3 CH_2), 2.22 (br t, J = 6 Hz, 2 H, CH_2CO_2), 3.70 (t, J = 6 Hz, 2 H, $POCH_2$), 4.10 (d, J = 5 Hz, 2 H, 5'-CH_2), 4.30-5.30 (m, 3 H, 2',3',4'-CH), 6.08 (d, J = 4 Hz, 1 H, 1'-CH), 8.80 (s, 1 H, 5-ArH).

(8315-5)

General preparation method for immunoconjugates. Commercially available mouse immunoglobin (Sigma, containing 80% protein by the Lowry procedure) or human immunoglobulin (Sigma) was dissolved in 1.0 ml of 0.01 M potassium phosphate, 0.5 M NaCl, pH 7.6, and the solution was cooled to 4°C in an ice bath. In a separate vessel, the drug [MTX (38) or ribavirin-5'-hemisuccinate (3)] was dissolved in 1.0 to 2.0 ml of the buffer. Heating was required to solubilize the MTX. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EIDC, Aldrich) was added to the drug solution. This solution was cooled in ice before addition to the protein solution. The reaction mixture was stirred at 4°C for 0.75 to 1.5 h and then loaded onto a prepacked Sephadex G-75 column (1.8 x 20 cm) equilibrated with the reaction buffer at 4°C. Elution with the buffer afforded the protein fraction in the void volume (13 ml), followed by a mixture of the low-molecular-weight starting materials. The

combined protein fractions were concentrated by centricell (10,000 or 30,000 MW cutoff membrane (Polysciences); centrifugation at 2500 rpm for 10 min) to a volume of 1 ml. Protein concentration was measured by the absorbance at 280 nm compared with a standard curve (mouse and human IgG ε 2.5 x 10^5) or by the Lowry method (Lowry et al., 1951).

The quantities and reaction conditions used for the preparation of immunoconjugates are presented in Table I-1.

Habeeb Primary Amino Group Estimation. A literature procedure (Habeeb, 1966) was adapted. An aliquot of protein solution in PBS, pH 7.6, containing less than 3 mg/ml protein was brought up to a volume of 1000 µl. To this solution was added 1.0 ml of 4% $NaHCO_3$, which had been brought to pH 9.0 with solid NaOH, and 1.0 ml of freshly prepared 0.1% trinitrobenzenesulfonic acid (Aldrich) in water. The solution was mixed and incubated at 40°C in a reciprocating water bath for 2 h. Then, 1.0 ml of 10% sodium dodecyl sulfate (Aldrich) in water and 0.5 ml of 1 N HCl were added sequentially. The solution was thoroughly mixed and the absorbance was read at 335 nm (ε 7.78 x $10^3/NH_2$ group) against a control containing no protein. Protein concentrations were determined from a standard calibration curve at 280 nm (ε 2.5 x 10^5).

Table I-1. Quantities and Reaction Conditions Used for the Preparation of Immunoconjugates.

Immunoconjugate	IgG (mg)	Drug (μmol)	EDIC (μmol)	Rxn time (h)	Protein recovery (%)	Experiment
mouse IgG-MTX	mouse (3.0)	MTX (8.8)	15	1.0	42	SRI-7794-81
human IgG-MTX	human (5.0)	MTX (15.1)	32	1.5	32	SRI-7794-83
mouse IgG-ribavirin-5'-hemisuccinate	mouse (2.0)	ribavirin-5'-hemisuccinate (9.1)	17	1.5	82	SRI-7794-7

Lowry Protein Assay. A literature procedure (Lowry et al., 1951) was used. An aliquot of protein solution (0 to 3 mg/ml protein) was brought up to a volume of 0.5 ml with the appropriate buffer (0.1 M potassium phosphate, 0.5 M NaCl, pH 7.6). Next, 2.5 ml of a solution prepared from 50 ml of 2% Na_2CO_2 in 0.1 M NaOH and 1.0 ml of 0.5% $CuSO_4 \cdot 5H_2O$ in 1% Na-K tartrate. The solution was mixed well and allowed to stand for 10 min before 0.25 ml of 1.0 N Folin phenol reagent was added. After mixing, the solution was allowed to stand for 30 min or longer at room temperature. The absorbance was read at 660 nm against a control containing no protein. Protein concentration was determined from a standard curve at 280 nm.

C. Results

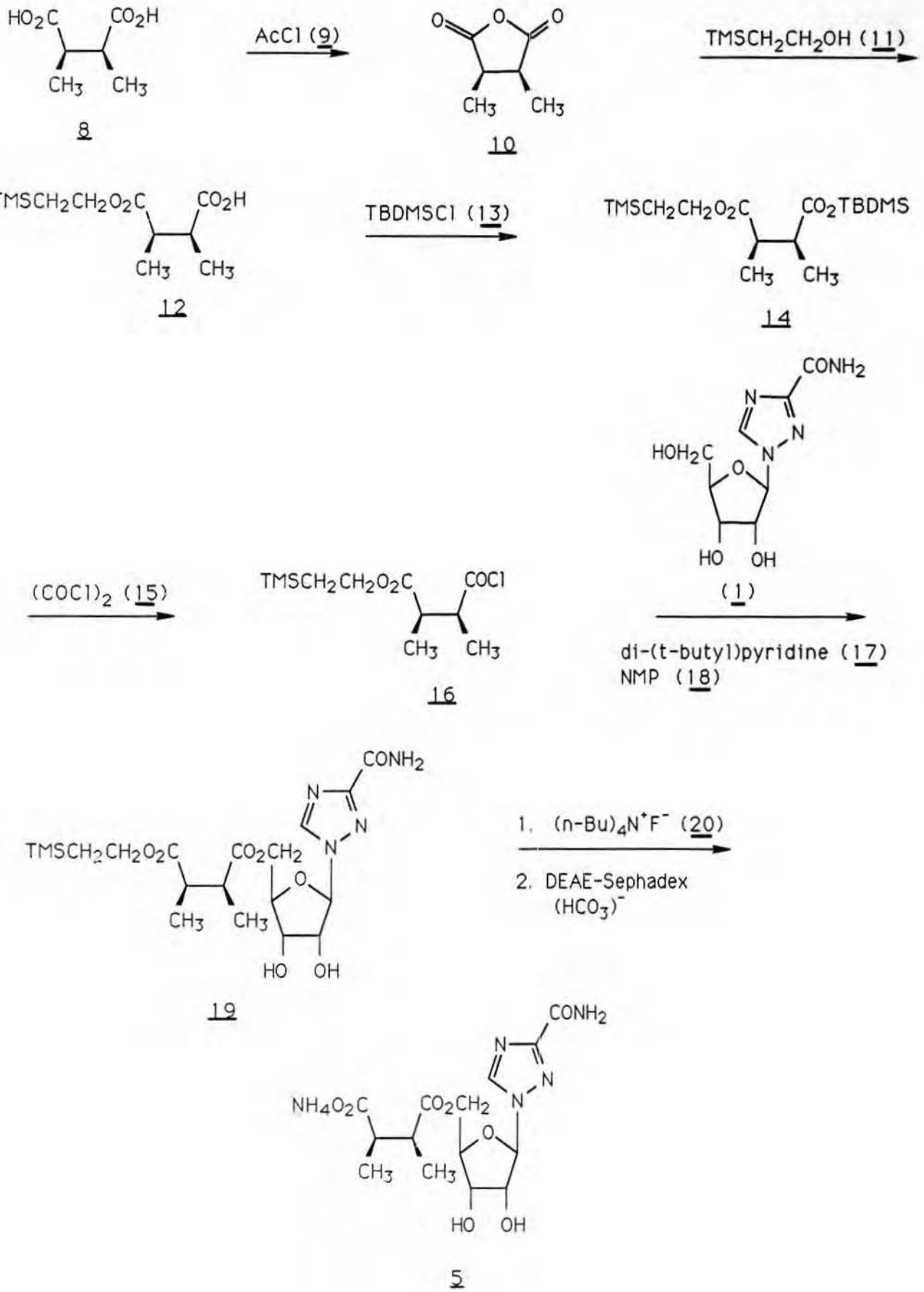
In this section, the syntheses of the ribavirin ester derivatives will be described first, followed by the syntheses of the MAb-ribavirin ester conjugates and or ribavirin phosphate derivatives and the methodological studies undertaken to determine conjugate loading.

Our studies had indicated that ribavirin-5'-hemisuccinate (3) slowly hydrolyzed under physiological conditions, giving ribavirin. Because this hydrolytic instability may render the hemisuccinate group unsuitable for use as a tether in an immunodrug conjugate, we undertook the synthesis of the more highly substituted

and therefore more sterically hindered 2,3-dimethylhemisuccinate 5. For steric reasons, the ester bond of 5 should be more hydrolytically stable than that of 3.

meso-2,3-Dimethylsuccinic acid (8) was heated at reflux with acetyl chloride (9) to afford 2,3-dimethylsuccinic anhydride (10) in 89% yield (Scheme I-1). Treatment of anhydride 10 with 2-trimethylsilylethanol (11) gave the hemiester 12 in 82% yield. Reaction of 12 with t-butyldimethylsilyl chloride (13) in dimethylformamide afforded diester 14 in 76% yield after vacuum distillation. Reaction of 14 with oxalyl chloride (15) produced, in 80% yield, the unstable and extremely moisture-sensitive acid chloride 16, which was not purified but was immediately allowed to react with ribivarin (1) in N-methylpyrrolidinone (NMP, 18) in the presence of 0.1 equivalent of 2,6-di(t-butyl)pyridine (17). Workup and chromatography on silica gel gave the trimethylsilyl succinate ester of ribavirin (19) in 36% yield. Treatment of 19 with tetra(n-butyl)-ammonium fluoride (20) in tetrahydrofuran afforded the free acid in 85% crude yield. This material was purified by ion-exchange chromatography on DEAE-Sephadex A-25 using a 0.0 to 0.25 M ammonium bicarbonate gradient to obtain the ammonium salt 5 of the 2,3-dimethylhemisuccinate of ribavirin in 60% yield.

Scheme I-1. Synthesis of Ribavirin-5'-(2,3-dimethyl)hemisuccinate



Hydrolysis studies indicated that 5 remained intact after 13 days at 37°C in phosphate-buffered saline (PBS) at pH 7.4, whereas 20% of the unsubstituted hemisuccinate 3 had undergone hydrolysis over the same period of time (Table I-2). These studies indicate that the 2,3-dimethylhemisuccinate of ribavirin should be a better candidate for tethering to monclonal antibodies than the hemisuccinate of ribavirin.

Ribavirin, ribavirin-5'-hemisuccinate, and ribavirin-5'-(2,3-dimethyl)hemisuccinate were screened for antiviral activity against PCV using an immunofluorescence assay (Table I-3). Ribavirin has an ED_{50} between 3.2 and 10 µg/ml and a maximum tolerated dose (MTD) of 320 µg/ml. Ribavirin-5'-hemisuccinate had an ED_{50} for antiviral activity that was one-log higher and a MTD that was three-fold higher. Therefore, this derivative had a more favorable therapeutic index than did ribavirin. In contrast, the 5'-(2,3-dimethyl)hemisuccinate has an ED_{50} that was two-logs higher than that of ribavirin and a MTD that was one-tenth that of ribavirin, indicating a far less favorable therapeutic index than that of either ribavirin or its 5'-hemisuccinate. Although the therapeutic index of the derivatives may change after conjugation to a MAb, for preliminary experiments we decided to use the MAb-ribavirin-5'-hemisuccinate conjugate because of its greater activity.

Table I-2. Hydrolysis of 2,3-Dimethylhemisuccinate and Hemisuccinate of Ribavirin.

Tether	Ribavirin Released (%)[a]				
	0 h	48 h	144 h	240 h	312 h
Hemisuccinate 3	0	7	11	15	20
2,3-Dimethylhemisuccinate 5	0	0	0	0	0

[a]Estimated using high-performance chromatography on a reverse-phase Waters Radialpak A column using 0.1 M ammonium acetate buffer/methanol (98/2) at a flow rate of 2.0 ml/min, detection at 260 nm and benzoic acid as the internal standard. The retention times were: ribavirin, 3.6 min; benzoic acid, 3.9 min; 5'-hemisuccinate of ribavirin, 4.6 min; and 5'-(2,3-dimethyl)hemisuccinate of ribavirin, 7.8 min (SRI Experiment 7794-57).

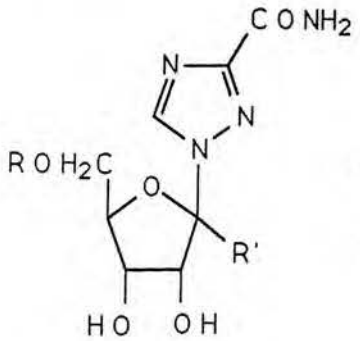
Table I-3. Immunofluorescence Assay for Antiviral Activity against Pichinde Virus.

Drug Conc. (μg/ml)	Percent Inhibition			
	Ribavirin	Ribavirin-5'-hemi-succinate (3)	Ribavirin-5'-(2,3-dimethyl)-hemisuccinate (5)	Ribavirin-5'-phosphate (26)
1000	100		94	100
320	100		65	100
100	100		20	100
32	100		5	66
10	54		0	23
3.2	15		0	5
1.0	0		0	5
ED_{50} (μg/ml)	3.2-10	10-32	100-320	10-32
MTD (μg/ml)	320	1000	32	320

Because ribavirin has a weak UV chromophore, the absorption maximum of which occurs at 206 nm (ε 1.2 x 10^4 in H_2O), which is coincident with that of protein, precluding spectroscopic drug-loading estimations, we initially decided to prepare a radiolabeled conjugate from radiolabeled ribavirin to determine drug loading onto MAbs. Process-development work was initiated using unlabeled ribavirin to prepare ribavirin-5'-hemisuccinate (3) on a small scale in order to develop methodology for preparing [1-^{3}H]ribavirin-5'-succinate from the tritiated drug. After the methodology had been developed, radiolabeled 4 (specific activity, 0.778 mCi/mmol) was prepared in two steps in 28% overall yield on a 0.032-mmol scale (Scheme I-2). This labeled material was used for the initial conjugation with MAb PC 4.9A6. Protein recovery after conjugation and chromatographic purification was 27%. Loading on conjugate 24 was determined to be 6.65 mol of drug/mol MAb from the molar concentrations determined by scintillation counting for the drug and by absorbance at 280 nm for the protein.

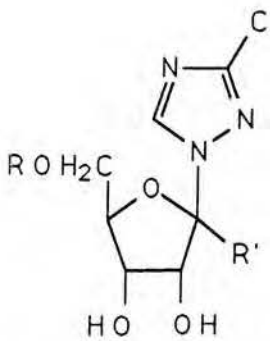
A 2-mg sample of the conjugate of ribavirin-5'-hemisuccinate with MAb PC 4.9A6 was required for initial *in vitro* antiviral screening. Because the quantity of radiolabeled [1'-^{3}H]ribavirin was limited, we decided to explore alternative methods of preparing the conjugate that would permit us to determine drug loading. The synthetic method selected was to use an activated ester derivative of ribavirin-5'-hemisuccinate that would release a chromophoric group during the conjugation step. The 4-nitrophenyl ester 6 of 3

Scheme I-2. Synthesis of Radiolabeled MAb PC 4.9A6-Ribavirin-5'-hemisuccinate Conjugate



1. $Me_3Si(CH_2)_2O_2C(CH_2)_2COCl$ (21), NMP (18)

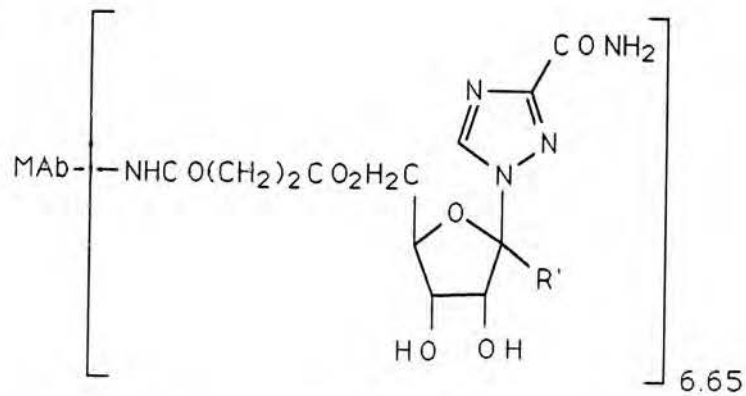
2 R = H, R' = ^3H



1. $(n\text{-}Bu)_4NF$ (20), THF
2. EDIC (23), MAb PC 4.9A6 (24), 0.01 M K Phosphate, 0.5 M NaCl, pH 7.6

22 R = $Me_3Si(CH_2)_2O_2C(CH_2)_2CO$, R' = ^3H

4 R = $NH_4O_2C(CH_2)_2CO$, R' = ^3H

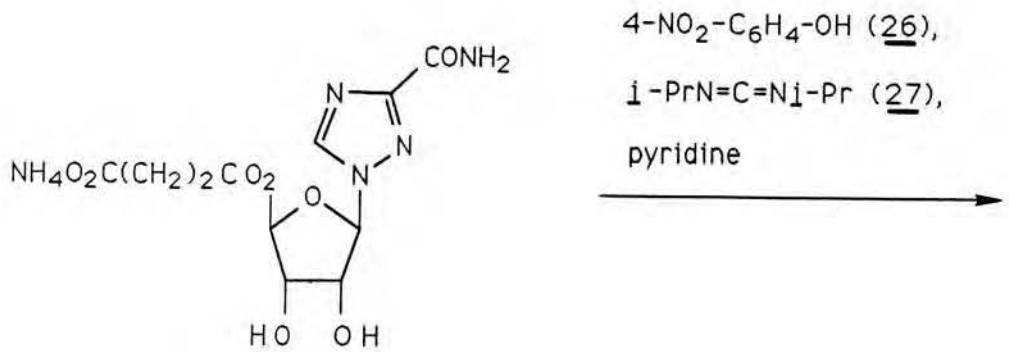


25 R' = ^3H

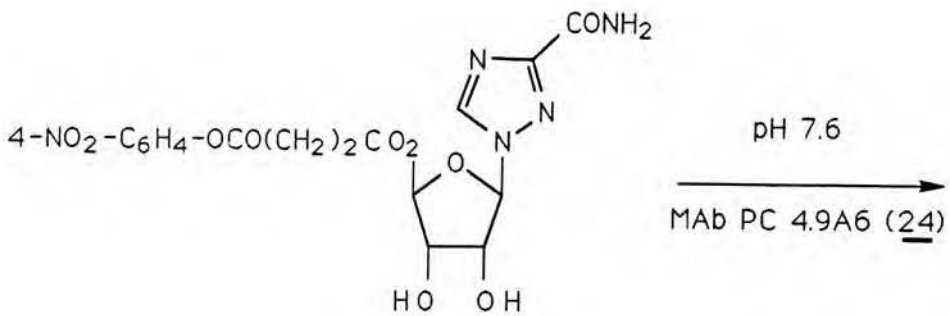
was selected as the active ester species (Scheme I-3). Release of the 4-nitrophenoxide anion during alkylation of the Mab would afford an indirect method of determining loading of the drug onto the MAb by monitoring the absorbance at 404 nm, which is the absorbance maximum of 4-nitrophenoxide at pH 7.6. Protein recovery after conjugation and loading was 66%. Loading of ribavirin onto the conjugate 25 was determined to be 4.77 mol of drug/mol of MAb from the 4-nitrophenoxide released during the conjugation after that released by hydrolysis was subtracted. This conjugate was supplied to Dr. Bill Barnett at Utah State University for *in vitro* screening. A synthesis of this conjugate on a 30-mg scale is in progress.

We had previously prepared ribavirin-5'-phosphate (26) as a potential precursor for the diphosphate esters 7 and 29 (Dawson et al., 1986). These diphosphate esters would be far more hydrolytically stable than the hemisuccinates and would not hydrolyze under physiological conditions but would be cleaved by lysozomal enzymes. Esterification of this phosphate with a tether having a carboxylic acid function would provide a means of tethering the derivative to MAbs. Esterification of the 5'-phosphate ester group requires protection of the 3'-hydroxyl group of the sugar to avoid formation of the cyclic phosphate. Therefore, the hydroxyl groups of 26 were protected by acetylation with acetic anhydride in pyridine (Scheme I-4). Unfortunately, a complex mixture resulted that only afforded a low yield of the desired diacetate phosphate 27

Scheme I-3. Synthesis of MAb PC 4.9A6-Ribavirin-5'-hemisuccinate Conjugate



3



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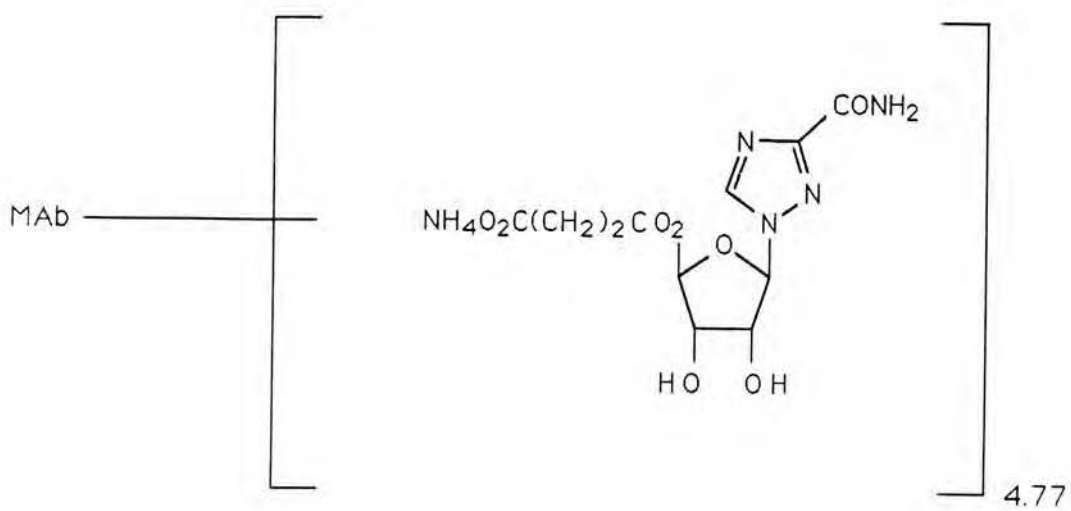
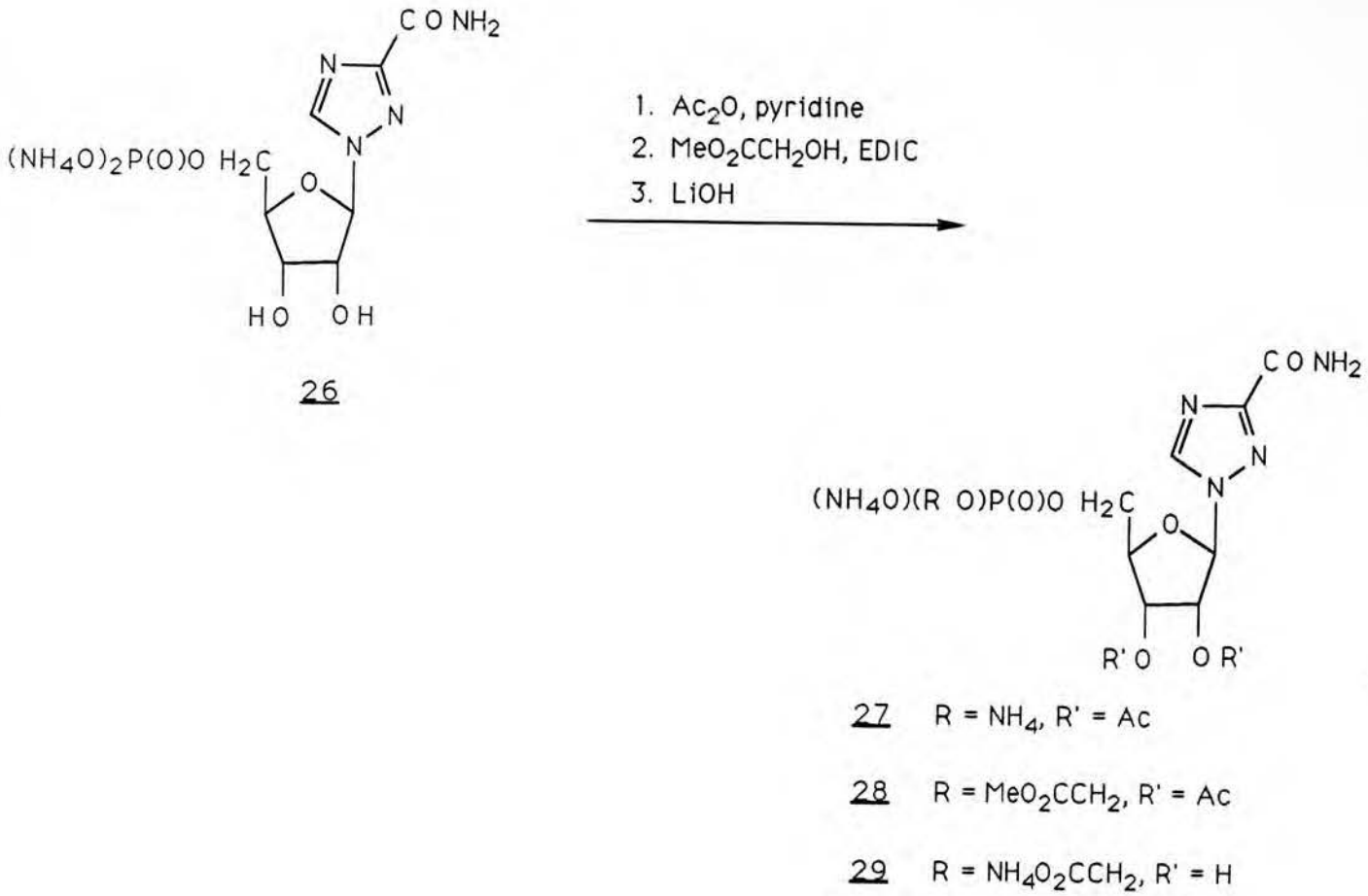


28

Scheme I-4. Studies on the Preparation of an Alkyl Ester of Ribavirin-5'-phosphate



26

27 R = NH_4, R' = Ac

28 R = MeO_2CCH_2, R' = Ac

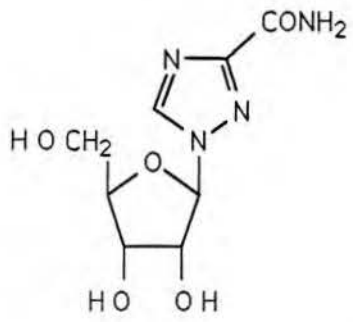
29 R = $NH_4O_2CCH_2$, R' = H

after DEAE-Sephadex chromatography using an ammonium sulfate gradient.

Alternative, more direct methods of preparing the phosphate diesters were investigated. The ammonium salt 7 of the 5-carboxypentyl ester of ribavirin-5'-phosphate was prepared from ribavirin (1) (Scheme I-5). The 2' and 3' hydroxyl groups of ribavirin were first protected by ketalization with acetone using perchloric acid as the catalyst in the presence of triethyl orthoformate (31) to remove the water formed in the reaction. The acetonide 32, which was prepared in 92% yield, was then allowed to react with (5-carbomethoxypentyl)(2-cyanoethyl)diisopropylphosphoramidite (33) in the presence of tetrazole (34) in acetonitrile. The phosphite that formed was oxidized to the phosphate 36 with iodine in the presence of 2,6-lutidine (35) in water/tetrahydrofuran. The cyanoethyl and methyl ester protecting groups were removed from 36 by treatment with ammonium hydroxide at room temperature, and the acetonide protecting group was removed from the product 37 by treatment with aqueous acetone, giving 7. This derivative will be conjugated to MAb 4.9A6 using methodology developed for the hemisuccinate derivatives.

The UV chromophore of ribavirin absorbs at 206 nm in water. Therefore, drug-loading determinations on immunoconjugates cannot be made using UV spectrophotometry because the ribavirin absorption maximum occurs at the end-absorption region of the protein. We had

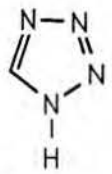
Scheme I-5. Synthesis of Ribavirin-5'-(5-carboxypentyl)phosphate Bisammonium Salt



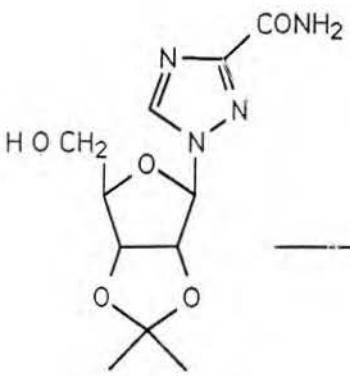
1

CH_3COCH_3 (30),
$(EtO)_3CH$ (31),
$HClO_4$

$(i\text{-}Pr)_2NP(OCH_2CH_2CN)[O(CH_2)_5CO_2Me]$ (33),

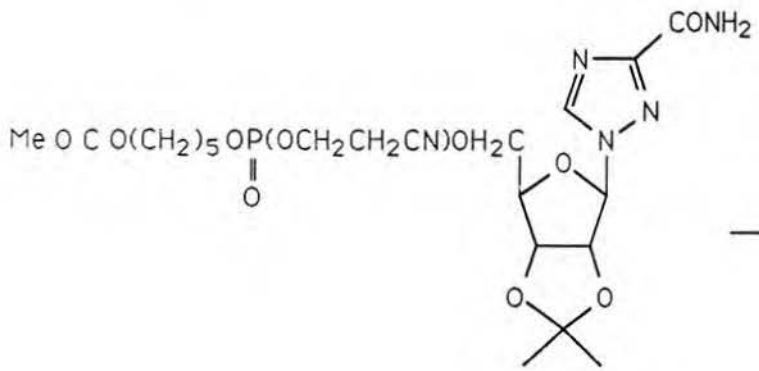


34



32

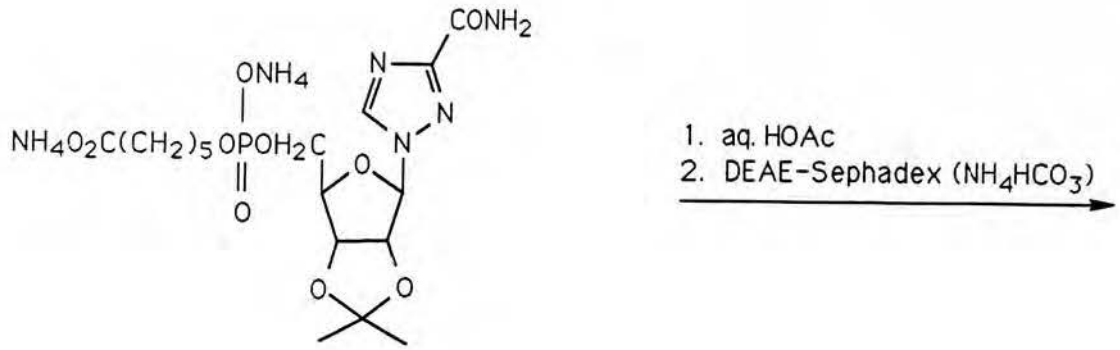
I_2, 2,6-lutidine (35)



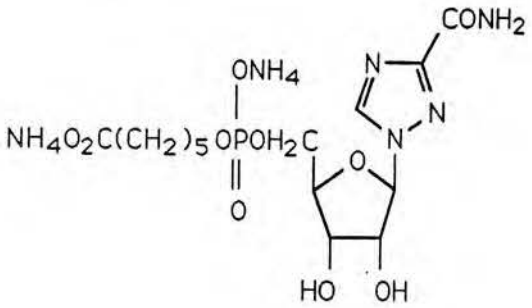
36

NH_4OH

Scheme I-5. Synthesis of Ribavirin-5'-(5-carboxypentyl)phosphate Bisammonium Salt (concluded)



37

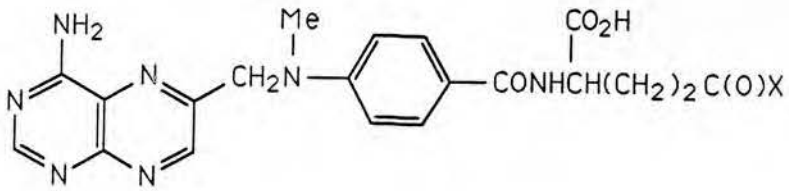


7

proposed to determine loading by using radiolabeled drug. Unfortunately, although this method is very accurate, it also necessitates the use of radiolabeled drug for every conjugation experiment. In an attempt to circumvent this problem, we investigated an alternative drug-loading determination method that employed the spectrophotometric measurement of the number of free amino groups on the protein. In the immunoconjugate preparation method that we currently use, the drug is linked to the amino groups of the antibody by an amide bond. Therefore, these reacted amino groups are no longer able to react with trinitrophenylsulfonate (Habeeb primary amino group assay for proteins) (Habeeb, 1966) to give a yellow color at 335 nm. The difference in the number of free amino groups on the protein before [there being approximately 90 amino groups per mouse antibody (Habeeb, 1966)] and after the conjugation step would give the number of amino groups reacting with drug and, hence, the number of drugs bound per antibody.

To ascertain the accuracy of the Habeeb method, studies on methotrexate-immunoglobulin conjugates were performed concurrently with the studies on the ribavirin conjugates. Because methotrexate (MTX, 38, Chart I-2) has a UV chromophore at 370 nm (ε 6.64 x 10^3) in PBS, pH 7.6, loadings can be readily determined spectrophotometrically as a check of the Habeeb method. Protein concentrations were determined by the absorbance at 280 nm (ε 2.5 x 10^5), with MTX and ribavirin controls being run. MTX had an appreciable absorbance at 280 nm (0.4 of the 370-nm adsorption) that had to be subtracted

Chart I-2. Methotrexate and Its Immunoconjugate



	X
38	-OH
39	-NH-MAb

to accurately assess the protein concentration of immunoconjugate solutions. At 280 nm, the absorbance of ribavirin was negligible. This method was verified by the Lowry procedure (Lowry et al., 1951). Neither MTX nor ribavirin provided any absorbance at 660 nm under Lowry conditions and therefore did not interfere with the assay. There was excellent agreement between the immunoglobulin concentrations determined by the UV spectrophotometric and Lowry methods (Table I-4). Unfortunately, this agreement could not be obtained between the UV spectrophotometric and Habeeb methods. UV absorption indicated that there was an average of 4.3 MTX molecules bound per molecule of mouse antibody, whereas the Habeeb method indicated 3.9. UV absorption indicated 3.4 MTX molecules bound per human antibody, whereas the Habeeb method indicated 5.7. The different loading results of the Habeeb method may reflect the inaccuracy of this modification of the Habeeb procedure in which two large numbers are subtracted from each other to give a small number. Greater accuracy would be achieved with much higher drug-loading levels. These higher loadings could theoretically be obtained using the water-soluble, modified ribavirins but not using MTX, which has low solubility in the conjugation reaction medium. However, preliminary experiments in progress indicate a maximum loading of 11 ribavirins per MAb by the 4-nitrophenyl ester method. This level of loading may not be sufficient for measurement by the Habeeb method.

Table I-4. Immunoglobulin-Drug Conjugates.

Immunoconjugate	Protein Conc. (μmol/ml) UV (280 nm)	Protein Conc. (μmol/ml) Lowry	Drug Conc. (μmol/ml) UV (370 nm)	Drug Conc. (μmol/ml) Habeeb	Loading (Drug/IgG) UV (370 nm)	Loading (Drug/IgG) Habeeb
mouse IgG	3.5×10^{-6}	3.5×10^{-6}	-	-	-	-
mouse IgG-MTX	4.5×10^{-4}	4.7×10^{-4}	19×10^{-4}	17×10^{-4}	4.3	3.9
mouse IgG-ribavirin-5'-hemisuccinate	1.39×10^{-6}	1.36×10^{-6}	-	5.8×10^{-6}	-	4.2
human IgG	4.6×10^{-6}	4.6×10^{-6}	-	-	-	-
human IgG-MTX	6.4×10^{-6}	6.7×10^{-6}	22×10^{-6}	37×10^{-6}	3.4	5.7

Some studies were performed to develop a RIA to quantitate drug-loading. A RIA for ribavirin in serum has been developed using rabbit polyclonal serum (Austin et al., 1983). The polyclonal antibodies in this serum should recognize the ribavirin tethered to MAb 4.9A6 because they are directed against ribavirin-5'-hemisuccinate covalently linked to ovalbumin--a conjugate prepared by the same method of attachment as that used to prepare the MAb conjugate. Rabbit anti-ribavirin serum was obtained from J. D. Connor, M.D., University of California Medical School, San Diego, CA, and [^{14}C]ribavirin of high specific activity (33 mCi/mmol) was obtained from Viratek, Irvine, CA. Unfortunately, we were not able to reproduce the reported assay procedure using unbound ribavirin. For this reason, Dr. Connor graciously offered to screen the MAb-ribavirin conjugate to determine the amount of ribavirin covalently linked to the MAb-ribavirin conjugate 25. A loading of 2.00 mol of ribavirin/mol of MAb was found by the RIA assay. Therefore, only 42% of the available covalently linked ribavirin was accessible to the polyclonal antibodies. Further, development work using other immunoconjugates will be required to determine whether this type of assay is reproducible and quantitative. In this regard, Dr. Michael Ussery of USAMRIID has kindly offered to determine ribavirin loading on our conjugates using the polyclonal serum that he developed.

In summary, it appears that the radiolabeled and active 4-nitrophenyl ester methods are the best methods to quantitate drug-loading developed thus far.

II. In Vitro Antiviral Evaluations. I. Development and Application of an Immunofluorescence Assay Suitable for in Vitro Antiviral Drug Screening against Pichinde Virus

A. Introduction

Pichinde virus (PCV) produces only marginal cytopathogenic effect (CPE) in vitro. Pichinde virus-induced CPE develops 10 to 14 days postinoculation (p.i.) in Vero 76 cells. Antiviral assays against Pichinde virus often utilize plaque reduction (PR), which requires relatively large amounts of drug and is slow to reach an acceptable endpoint. We have developed an immunofluorescence assay (IFA) that can be used in antiviral drug assays against Pichinde virus. Antiviral drug activity is determined by measuring the inhibition of PCV infectivity in Vero 76 cells. The assay is performed using an immunofluorescent cell-count assay to detect Pichinde virus-infected cells in 96-well cell culture plates. The presence or absence of fluorescing cells is easy to score, the assay is read after only 24 h, the amount of drug required to perform an assay is reduced by over 80%, and the sensitivity and reproducibility are comparable with that achieved with assays based on plaque reduction. This section describes the immunofluorescence antiviral assay and demonstrates the performance of the assay in the evaluation of the antiviral drugs selenazofurin, ribavirin, 3-deazauridine, and (S)-DHPA against PCV. The PCV immunofluorescence assay was then

used to determine the in vitro antiviral activity of several ribavirin derivatives.

B. Materials and Methods

Cells. The Vero 76 line of African green monkey kidney cells obtained from the American Type Culture Collection (ATCC, Rockville, MD) was used as the host cell line for all PCV assays. Cells were passaged in polystyrene, disposable cell-culture flasks using MEM (GIBCO Labs, Grand Island, NY) containing 9% fetal bovine serum (FBS) (HyClone Labs, Logan, UT). For antiviral assays, cells were seeded at 6×10^4 cells/well in 96-well, polystyrene cell culture plates.

Virus. The An 4763 strain of PCV (obtained from Dr. Joseph D. Gangemi, University of South Carolina School of Medicine, Columbia, SC) was used in these studies.

Immunofluorescence Assay for Pichinde Virus-Infected Cells. The immunofluorescence assay for PCV used fluorescein-conjugated murine MAb toward PCV prepared as described in Section XI of this annual report. At the end of the 20- to 24-h incubation the media was aspirated and the infected cell cultures were allowed to dry thoroughly at room temperature. If the cell sheets were not allowed to dry thoroughly before 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 deionized water (50 µl/well) followed by the addition of 200 µl of cold (-15°C) acetone 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 min. The fixative was poured from the plate and the cell sheets were allowed to dry. Cells were either immunostained immediately or stored at -15°C. Fluorescein-labeled MAb toward PCV was used at a dilution of 1:500 to provide intense specific staining, yet 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 h before the immunostain was poured from the plate. The plate was inverted on absorbent paper and allowed to drain. Immunostained cells were viewed through the bottom of the plate using a 16x objective and 10x eyepieces on a Zeiss epifluorescence microscope.

Test Compounds. All of the compounds were soluble in the MEM cell culture medium used as compound diluent. Compounds were stored in sealed vials, in the presence of desiccant, at room temperature. Once placed into solution, all compounds were stored at 4°C.

IFA Antiviral Assay. 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. The virus

inoculum had approximately 10 fluorescing cells per microscope field in the absence of drug. The virus was allowed to adsorb for 30 min, at which time 100 µl of test compound diluted in MEM with 2% FBS was added. 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 and incubated for 20 to 24 h at 37°C. The cells were fixed and immunostained to detect Pichinde virus-infected cells. The number of fluorescing cells was determined and compared to the number of fluorescing cells in wells without drug. Each assay included toxicity controls, as well as the virus and normal cell controls similar to those of the plaque reduction assay described previously (Huffman et al., 1973). Three virus-containing wells were used for each compound dosage level, with one additional well being used for toxicity controls (cells + sterile virus diluent + compound). Six wells in each panel were used for normal cell controls (cells + sterile virus diluent + drug diluent). The antiviral activity was expressed as the concentration of drug required to reduce the number of infected cells by 50% (ED_{50}). The ED_{50} was determined by plotting the percent inhibition versus compound concentration on semilogarithmic paper, with the ED_{50} level being that concentration causing a 50% reduction in the number of fluorescing cells.

C. Results

The immunofluorescence assay was used to measure the antiviral activity of several compounds against PCV; data from such an experiment using ribavirin and ribavirin 5'-phosphate are shown in Table II-1. In the process of verifying the utility and reliability of the antiviral assay using immunofluorescence as the parameter for scoring infection, several other compounds were also evaluated against PCV and a summary of these results is presented in Table II-2. The *in vitro* antiviral assay based on immunofluorescence was then used to evaluate the antiviral activity of several ribavirin derivatives synthesized by Dr. Dawson at SRI. The results of these assays are listed in Table II-3.

D. Discussion

The ED_{50} of ribavirin using inhibition of immunofluorescence as the parameter for scoring antiviral activity was 4.5 µg/ml (Table II-2), which is in the same range as the antiviral activity measured using inhibition of CPE, reading a marginal endpoint, or using PR. The antiviral assay based on inhibition of immunofluorescence was also shown to be applicable to antiviral compounds other than ribavirin. Thus, the antiviral drug assay based on inhibition of immunofluorescence is as sensitive as antiviral assays based on inhibition of CPE or PR and offers the advantages of economy of reagents, rapidity, and a less-ambiguous endpoint.

Table II-1. Immunofluorescence Assay for Antiviral Activity against Pichinde Virus.

Ribavirin 5'-phosphate		Ribavirin	
Conc. (µg/ml)	% Inhibition	Conc. (µg/ml)	% Inhibition
1000	100	1000	100
320	100	320	100
100	100	100	100
32	100	32	65
10	74	10	39
3.2	30	3.2	0
1.0	4	1.0	0

Ribavirin-5'-phosphate ED_{50} = 3.2-10 µg/ml.

Ribavirin ED_{50} = 10-32 µg/ml.

Table II-2. Activity of Antiviral Drugs against Pichinde Virus as Measured by Immunofluorescence Assay.

Drug Tested	ED_{50} (μg/ml)
Selenazofurin	1.0
Ribavirin	4.5
3-Deazauridine	7.5
(S)-DHPA	505.5

Table II-3. Immunofluorescence Assay for Antiviral Activity of Ribavirin Derivatives against Pichinde Virus.

	Percent Inhibition		
Conc. (μg/ml)	Ribavirin	Ribavirin-5'-phosphate	Ribavirin-5'-(2,3-dimethyl)-hemisuccinate
1000	100	100	94
320	100	100	65
100	100	100	20
32	100	66	5
10	54	23	0
3.2	15	5	0
1.0	0	5	0

Ribavirin, ED_{50} = 3.2-10 μg/ml, MTD = 320 μg/ml.

Ribavirin-5'-phosphate, ED_{50} = 10-32 μg/ml, MTD = 320 μg/ml.

Ribavirin-5'-hemisuccinate (data not shown), ED_{50} = 10-32 μg/ml, MTD = 1,000 μg/ml.

Ribavirin 5'-(2,3-dimethyl)hemisuccinate, ED_{50} = 100-320 μg/ml, MTD = 32 μg/ml.

Ribavirin-5'-hemisuccinate was found to have an ED_{50} of 10 to 32 µg/ml and an MTD of 1,000 µg/ml. These values suggest that ribavirin-5'-hemisuccinate may be a suitable derivative for tethering to antibodies. However, because of concerns over the potential lability of ribavirin-5'-hemisuccinate, the more-hindered ester ribavirin 5'-(2,3-dimethyl)hemisuccinate was synthesized by SRI chemists. Although ribavirin-5'-(2,3-dimethyl)hemisuccinate was much more stable than ribavirin-5'-hemisuccinate (for stability data, see First Year Annual Report, Dawson et al., 1986a), the addition of the two methyl groups had a deleterious effect on both the antiviral activity and the cytotoxicity of ribavirin-5'-(2,3-dimethyl)hemisuccinate (ED_{50} of 100 to 320 µg/ml and MTD of 32 µg/ml) compared with ribavirin-5'-hemisuccinate (ED_{50} of 10 to 32 µg/ml and MTD of 1,000 µg/ml). However, these antiviral assays were conducted in vitro using the ribavirin adducts without conjugation to a MAb. It is quite likely that the 5'-ester linkage must be cleaved before the dimethylhemisuccinate or hemisuccinate derivatives can express antiviral activity. When an immunoconjugate enters a target cell by endocytosis, the ester linkage between ribavirin and the tether to the MAb will be exposed to hydrolytic enzymes in the lysosomes, an environment that may be much different from the conditions experienced by the nonconjugated ribavirin adducts in our in vitro antiviral assays. Thus, although the ribavirin-5'-(2,3-dimethyl)hemisuccinate was less active in the in vitro antiviral assay, the compound may be active when conjugated to a MAb.

III. Immunofluorescence Assay Suitable for Evaluating Antiviral Immunoconjugates. Development and Application for Antiviral Evaluation of Ribavirin-Immunoconjugates

A. Introduction

In a traditional *in vitro* antiviral assay, the test compound is added within an hour of the virus inoculum. The test compound then has the opportunity to exert an antiviral effect prior to the first round of viral replication. Although this type of antiviral assay is effective for screening traditional antiviral drugs, it is not suitable for evaluating antiviral immunoconjugates. An antibody-targeted antiviral drug is produced by conjugating an antiviral drug to an antibody that targets a marker antigen defining the population of target cells. The targeted antivirals being produced in this project utilize MAbs specific for viral antigens expressed on the surface of virus-infected cells. Thus, the targeting potential of these immunoconjugates cannot be realized until virus-infected cells have begun to express viral antigens at their cell surface. The initial multiplicity of infection (MOI), the time of the addition of the test compound, and the time at which the test is read take on more critical roles in an assay with antiviral immunoconjugates. In this report we describe an antiviral assay utilizing inhibition of immunofluorescent foci formation for the evaluation of antibody-targeted immunodrugs. This is a modification of an immunofluorescence assay that we developed for evaluating antiviral drugs against

Pichinde virus. The primary modifications were to use a much lower MOI and to add the drug after the Pichinde virus infection was established. A small amount of an immunoconjugate consisting of ribavirin attached to MAb PC 4.9A6 was evaluated using this modified anitiviral assay.

B. Materials and Methods

Cells. The Vero 76 (African green monkey kidney) cell line obtained from the American Type Culture Collection was used as the host cell line for all PCV assays. Cells were passaged in polystyrene, disposable cell-culture flasks using MEM containing 9% FBS. For antiviral assays, cells were seeded at 6×10^4 cells/well in 96-well, polystyrene cell-culture plates.

Virus. The An 4763 strain of PCV was used in these studies.

Immunofluorescence Assay for Antibody-Targeted Antiviral Drugs. In the antiviral assay described in this report, the test compound was added 20 h p.i. rather than concurrently with the inoculation. In the development of this modified antiviral assay, ribavirin was added 20 h p.i., and the length of time the infection was allowed to proceed was varied. Infections were terminated at 48 h p.i., at 72 h p.i., and at 96 h p.i. Growth medium was removed 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. The

virus inoculum had 5 to 10 immunofluorescent cell-forming units of PCV per well, resulting in an MOI of approximately 1:10,000. The inoculated cultures were incubated for 20 h at 37°C, at which time 100 µl of test compound diluted in MEM was added. The plates were sealed with plastic wrap and incubated at 37°C until the incubation was terminated by pouring the medium from the cultures and letting the cell sheet air-dry prior to fixing with acetone. In these experiments the duration of the infection was varied; assays were terminated at 48 h, at 72 h, and at 96 h p.i. Optimal conditions were determined to be addition of compound at 20 h p.i. followed by an incubation period of 96 h. The fixed cells were immunostained to detect Pichinde virus-infected cells, and the number and size of foci of fluorescing cells were determined. Each assay included toxicity controls, as well as virus and normal cell controls similar to the PR assay described previously (Huffman et al., 1973). Four wells were used for each compound dose, one for toxicity assessment (cells + sterile virus diluent + compound), and three wells for antiviral assessment (cells + virus + compound). Six wells in each panel were used for normal cell controls (cells + sterile virus diluent + drug diluent).

The PCV infection as detected by immunofluorescence was graded from 0 (normal cells) to 4 (virtually all cells fluorescing). The antiviral activity of a compound was determined in a manner analogous to measuring activity as a reduction in CPE except that reduction in immunofluorescent foci (IF) was the scoring parameter. The

antiviral activity was expressed as the concentration of drug required to reduce the IF score by 50% (ED_{50}).

Immunofluorescence Assay for Pichinde Virus-Infected Cells. The immunofluorescence assay for PCV used fluorescein-conjugated murine MAb toward PCV prepared as described in Section XI of this annual report. At the end of the infection, the medium was aspirated, and the infected cell cultures 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 deionized water (50 µl/well), followed by the addition of 200 µl of cold (-15°C) acetone 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 min. Then the fixative was poured from the plate and the cell sheets were allowed to dry. Cells were either immunostained immediately or stored at -15°C. Fluorescein-labeled MAb toward PCV was used at a dilution of 1:500 in PBS to provide intense specific staining, yet 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 h before the immunostain was poured from the plate. The plate was inverted on absorbent paper and allowed to drain. A drop of Elvanol

mounting medium was added to each well to increase the clarity of the microscopic image, and immunostained cells were viewed through the bottom of the plate using a 16x objective and 10x eyepieces on a Zeiss epifluorescence microscope.

Ribavirin. Ribavirin (ICN Pharmaceuticals, Costa Mesa, CA) was dissolved in MEM. Prior to use, ribavirin was stored in sealed vials, in the presence of desiccant, at room temperature. Once placed into solution, ribavirin was stored at 4°C.

Ribavirin-Immunoconjugate. The ribavirin-MAb PC 4.9A6 conjugate had approximately 4.8 molecules of a 5'-hemisuccinate derivative of ribavirin covalently bound to each molecule of immunoglobulin through an amide linkage at the 5'-hemisuccinate group. The MAb used to produce this conjugate was PC 4.9A6, an IgG_{2a} immunoglobulin specific for an antigenic determinant expressed on the surface of Pichinde virus-infected cells. The characterization of MAb PC 4.9A6 is described in Section XII of this annual report. The ribavirin content of the immunoconjugate was 0.65% ribavirin by weight and the concentration of protein in the conjugate was 0.6 mg/ml in PBS. Thus, the concentration of ribavirin in the conjugate was 3.9 µg/ml.

C. Results

Development of an Immunofluorescence Assay for Antibody-Targeted Antiviral Drugs. When the infections were allowed to proceed for 48 h, there were mainly discrete foci comprising 20 to 60 fluorescing cells (mean of 33, standard deviation of ±12) and occassional solitary fluorescing cells. When the infections were terminated at 72 h p.i., the foci of infected cells rarely contained fewer than 40 fluorescing cells, most were made up of over 100 fluorescing cells, and there were areas of overlapping foci. When the infections were terminated 96 h p.i., most cells were infected, as indicated by immunofluorescence in over 90% of the cells, and individual foci of fluorescing cells were no longer distinguishable because most foci were overlapping.

When ribavirin was added to the infected cells at 20 h p.i., there was a dose-dependent inhibition of immunofluorescent foci formation. At the highest noncytotoxic dose of ribavirin (320 µg/ml) there were no fluorescent foci formed at 48 to 96 h p.i. and only occasional solitary fluorescing cells; this result was scored as 0-1. As the concentration of ribavirin was reduced, the size and number of fluorescent foci increased (see Table III-1). The 50% effective dose (ED_{50}) for ribavirin was 32 µg/ml. The assay could be read at either 72 h or 96 h p.i. with the same results, but the extent of immunofluorescence had not reached 4+ by 72 h p.i., so the

Table III-1. Immunofluorescent Foci Assay for Antiviral Activity of Ribavirin[a] against Pichinde Virus.

Ribavirin Conc. (μg/ml)	Extent of Pichinde virus infection as measured by immunofluorescence[b] at 48 h p.i.	at 72 h p.i.	at 96 h p.i.
1000	CT[c]	CT[c]	CT[c]
320	0-1	0-1	0-1
100	0-1	0-1	1
32	1-2	2	2
10	2	2-3	4
3.2	2	3	4
1.0	2	3	4
0	2	3	4

[a]Ribavirin at the indicated concentrations was added 20 h after inoculation of Vero cells with PCV at an MOI of 1:10,000.

[b]Immunofluorescence was graded on a scale of 0 (normal cells) to 4 (confluent fluorescing foci).

[c]CT: Cytotoxicity was observed at a ribavirin concentration of 1,000 μg/ml.

difference between no antiviral activity and strong antiviral activity was more apparent when the assay was read at 96 h p.i.

Ribavirin-Immunoconjugate. The ribavirin-5'-hemisuccinate-immunoconjugate was examined using the modified antiviral assay, which gave an ED_{50} for ribavirin of 32 µg/ml and the MTD of 320 µg/ml. The ribavirin-immunoconjugate was evaluated over a concentration range of 0.001 to 1 µg/ml (a ribavirin content of 0.15 to 150 µg/ml based on protein content). There was no observable antiviral effect nor was there any apparent cytotoxicity; the results of the assays are shown in Table III-2. Unconjugated MAb PC 4.9A6 over the concentration range of 0.15 to 150 µg/ml exhibited no antiviral or cytotoxic effects.

D. Discussion

The antiviral activity (ED_{50}) of ribavirin when added 20 h p.i. as measured in the assay described in this report was 32 µg/ml compared with an ED_{50} of 3.2 to 10 µg/ml when ribavirin was added concurrently with PCV and measured by inhibition of immunofluorescent cell formation (see data in Section II of this annual report). It has been our experience that the in vitro antiviral activity of ribavirin diminishes as the time between virus inoculation and the addition of ribavirin increases.

Table III-2. Immunofluorescent Foci Assay for Antiviral Activity[a] against Pichinde Virus.

Ribavirin		PC 4.9A6[c]		Ribavirin-5'-hemisuccinate-PC 4.9A6[d]	
(μg/ml)	IF Score[b]	(μg/ml)	IF Score	(μg/ml)[e]	IF Score
1000	CT[f]	150	4	1	3.5
320	0.5	47.5	4	0.32	4
100	1	15	4	0.1	4
32	2.5	4.75	4	0.032	4
10	4	1.5	4	0.01	4
3.2	4	0.475	4	0.0032	4
1.0	4	0.15	4	0.001	4
0	4	0	4	0	4

[a]Drug at the indicated concentrations was added 20 h after inoculation of Vero cells with PCV at an MOI of 1:10,000.

[b]Extent of PCV infection as measured by immunofluorescence, following treatment with the indicated samples (concentrations in μg/ml). Immunofluorescence was graded on a scale of 0 (normal cells) to 4 (confluent fluorescing foci) at 96 h p.i. with PCV.

[c]PC 4.9A6 is a MAb specific for PCV.

[d]The ribavirin-5'-hemisuccinate-immunoconjugate had 4.77 molecules ribavirin attached to each MAb PC 4.9A6. In 300 μg of conjugate, there was approximately 2 μg of ribavirin, thus the selection of PC 4.9A6 concentrations.

[e]Ribavirin-5-'hemisuccinate-MAb PC 4.9A6 conjugate concentration based on ribavirin content.

[f]CT: cytotoxicity.

The selection of an MOI that would yield five to ten infected cells per well in a 96-well plate (6×10^4 cells per well) was crucial to the success of the assay. If the MOI was higher, the virus infected too many cells before the ribavirin had an opportunity to inhibit replication and, if the MOI was too low, the IF readings fluctuated independently of the drug concentration (data not shown).

There was a very limited amount (2 mg) of the ribavirin-5'-hemisuccinate-immunoconjugate available. The maximum concentration of bound ribavirin in the immunoconjugate solution was 1 μg/ml. Because the ED_{50} for ribavirin was 32 μg/ml, the lack of antiviral activity with the immunoconjugate at 1 μg/ml was not surprising. The obvious problem was that the concentration of ribavirin in the immunoconjugate was 32-fold less than the ED_{50} of nonconjugated ribavirin. There was a slight indication of reduction in the number of cells infected following treatment at the 1-μg/ml dose of the ribavirin-5'-hemisuccinate-immunoconjugate that did not occur at the corresponding level of antibody alone. The immunofluorescence scores at the highest concentration of immunoconjugate were reduced to 3.5 from the 4.0 observed in the controls. However, this small reduction in immunofluorescence scores could not be considered significant. In a future assay, the ribavirin-5'-hemisuccinate-immunoconjugate will be concentrated fourfold and then reassayed in this system.

IV. Derivation of Hybridoma Cell Lines that Secrete Monoclonal Antibodies toward Pichinde Virus Antigens Expressed on the Surface of Infected Cells. I. Fusion, Selection, and Expansion

A. Introduction

The hybridization technique described in this report was used to derive hybridoma cell lines that secrete MAbs directed against PCV antigens expressed on the surface of Pichinde virus-infected cells. This hybridization technique includes several modifications of previous procedures that failed to yield sufficient numbers of hybridoma cell lines. The limited number of hybridoma cell lines produced by the previous hybridization technique did not include any that secreted MAbs of the desired specificity. Therefore, the hybridization procedure was modified to include peritoneal macrophages in the fusion wells. These macrophages serve as feeder cells, they condition the media, and they phagocytize dead cells and debris, thereby reducing the toxicity of the fusion culture media. Other modifications included an antigen boost administered to the spleen donor mouse five days prior to the fusion, the avoidance of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer when dimethyl sulfoxide (DMSO) was present, more gentle manipulation of the fusion cell mixture during the critical fusion steps, and, finally, omitting aminopterin from the fusion cell cultures until 24 h postfusion.

B. Materials and Methods

The complete modified hybridization procedure that was used was as follows:

1. The female RBF/DnJ mouse that had been immunized against PCV six months, eight weeks, and six weeks prior to fusion (each injection was intramuscular, with a 50/50 mixture of PCV-infected suckling mouse brain in Freund's adjuvant) was boosted five days prior to the fusion by direct injection of 0.2 ml of PCV antigen in saline into the tail vein.

2. The spleen donor mouse was anesthesized with ether and exsanguinated. Blood was collected from the subclavean artery. The serum was used as a positive control in antibody assays.

3. The animal was saturated with 70% ethanol. The skin was peeled from the entire peritoneal and chest area and the skin was pinned back out of the way. The animal was again saturated with 70% ethanol.

4. The spleen was removed aseptically and placed in serum-free RPMI-1640 medium (RPMI-1640-SF) in a petri dish. Excess fat and connective tissue were removed from the spleen. The spleen

was transferred to a 60-mesh, hat-shaped, stainless-steel screen inverted over a 15-ml plastic centrifuge tube in an ice bath. The spleen was kept moist by the constant dropwise addition of Tris-NH_4Cl (recipe given at end of procedure). The Tris-NH_4Cl also served to lyse any erythrocytes present.

5. The spleen was minced with sterile scissors. The spleen was dissociated into a cell suspension by forcing it through the stainless-steel strainer, using the sterile plunger from a 3.0-ml disposable syringe and rinsing with Tris-NH_4Cl. This process was continued until only connective tissue remained. The total volume of cell suspension was 10 ml.

6. The cells were spun down at 400 x g for 5 min.

7. The supernatant fluids were decanted and the cells were resuspended in 12 ml of RPMI-1640-SF, with 0.01 M HEPES. Large chunks were allowed to settle (2 min). The cell suspension was transferred to a duplicate tube.

8. The tube was topped off with medium and the cells were spun down at 400 x g for 5 min.

9. These spleen cells and the myeloma cells were washed three times in RPMI-1640-SF, with 0.01 M HEPES.

10. Spleen cells and myeloma cells were resuspended in RPMI-1640-SF containing no HEPES (HEPES was avoided during all steps while cells were permeablized with DMSO). The cells were counted [spleen cells (10 ml at 6.3 x 10^6/ml, with 82% viable); FOX-NY cells (10 ml at 1.3 x 10^6/ml, with 93% viable)].

11. The spleen cells and myeloma cells were combined in a 15-ml conical polyprcpylene tube in a ratio of five spleen cells to one myeloma cell. The cells were spun down at 400 x g for 5 min.

12. The supernatant fluids were carefully decanted, the tube was drained, and the last drops were removed with a sterile swab. The tube was gently tapped to spread cells up the wall of the tube.

13. To the cell slurry was added 1 ml of fusion mixture (50% PEG and 5% DMSO; see recipe at end of procedure) warmed to 37°C (warming is necessary to get efficient fusion) over 60 sec with gentle swizzling between fingers. The slurry was not vortexed or pipetted to disrupt the cells. The slurry was incubated at 37°C for 90 sec. Clumps were visible at this stage. The clumps were allowed to remain intact for the cells were fusing at this time.

14. The fusion mixture was diluted by the dropwise addition of RPMI-1640-SF containing no HEPES, and again was mixed by gently swirling the tube between fingers, with no rough agitation.

The diluent buffer was added as follows: 1.0 ml over 30 sec, 3.0 ml over 30 sec, and finally 10.0 ml over 60 sec.

15. The suspension was allowed to stand at room temperature for 5 min. The cells were spun down at 400 x g for 5 min. The supernate was decanted and the cells were resuspended in 15 ml of RPMI-1640-SF containing no HEPES. The cells were counted (15 ml at 4.4 x 10^6/ml, with 70% viable).

16. The cells were spun down at 400 x g for 5 min and resuspended in 240 ml of RPM1-1640 with 15% FBS, adenine, and thymidine, and no aminopterin buffer, so there were approximately 2 x 10^5 viable cells/ml. The FBS was used without heat inactivation.

17. The fusion mixture of cells was distributed at 1 ml/well to 24-well plates that had been seeded with macrophages six days earlier. The isolation of macrophages is described in Section V of this annual report. The following controls were included: FOX-NY cells only, spleen cells only, and a nonfused mixture of FOX-NY cells and spleen cells. The wells of all three controls were maintained in RPMI-1640 with and without aminopterin. The media are referred to as AAT media (RPMI-1640 with aminopterin, adenine, and thymidine) and AT media (the same as AAT except without aminopterin). The recipes are given at end of the procedure description. The FOX-NY cells quickly died out in the wells with aminopterin; in wells without aminopterin they overgrew within seven

days to the point where most appeared dead. The spleen cell and nonfused mixture control supernatants were very useful as controls in the IFA screening procedures as an indication of the level of antibody contributed by residual spleen cells.

18. The fusion cell suspension was incubated 24 h, then 1 ml of AAT medium containing aminopterin (2X) was added to each well. From this point on the cells were protected from light and handling of the plates was minimized. On Days 7, 9, 11, 14, 17, 21, and 24 the cells were fed by removing 1 ml of medium and replacing it with 1 ml of AAT medium.

19. Supernatants from the fusion plates were assayed several times, beginning when colonies of a few hundred cells became visible.

20. Those hybridomas that secreted antibody toward PCV were cloned. For cloning, one well of a 24-well plate was cloned into all 96 wells of a 96-well cell culture plate.

21. AT RPMI-1640 (containing adenine and thymidine but no aminopterin) was used for the first cloning. One-half of the plate was seeded with a cell suspension diluted sufficiently to yield 1 cell/well (in 0.3 ml). The remaining wells were seeded with an average of 0.5 cells/well (in 0.3 ml).

22. On Day 4 or 5, all wells were examined using low power (40x). The lids of those wells with 1 clone were circled, and the lids of those wells with more than a single clone were marked with a "X". Only those wells with a single clone were expanded.

23. When colonies covered 20 to 50% of the floor of the well, 0.1 ml of the supernatant medium was removed and assayed for antibody activity. The removed medium was replaced with fresh RPMI containing AT.

24. The selected clones were expanded by using RPMI containing AT. One well from the 96-well plate was expanded to one well of a 24-well plate (2 ml/well).

25. When colonies covered half of the floor of the well, they were expanded to three wells of a 24-well plate using RPMI-1640 containing AT as above. They were reassayed for antibody to confirm positives.

26. When clones were 50% confluent, they were expanded by placing all three wells into one 25-cm^2 flask containing 10 ml of RPMI containing AT. Again, when cell densities reached 0.5 to 1.0 x 10^6 cells/ml, clones were transferred to a 75-cm^2 flask with 20 ml of RPMI.

27. When cell densities reached 0.5 to 1.0 x 10^6 cells/ml, the cultures were expanded as needed, using RPMI-1640 without AT.

Media recipes:

RPMI 1640: RPMI 1640 containing 15% FBS
10 mM HEPES (Research Organics)
100 U/ml penicillin
100 µg/ml streptomycin
2 mM L-glutamine (additional)

RPMI 1640-SF: RPMI 1640 as above, except without FBS

AAT: 4×10^{-7} M aminopterin
7.5×10^{-5} M adenine
1.6×10^{-5} M thymidine

AT: AAT minus aminopterin

Tris-NH_4Cl: 17 mM Tris base
140 mM NH_4Cl

Fusion Mixture: 50% PEG 4000 (Merck) and 5% DMSO (Sigma) in water (sterilized at 121°C on liquid setting for 30 min) and aliquoted into polystyrene tubes (2.0 ml/tube),

kept sterile and sealed with parafilm, and stored at room temperature in the dark.

C. Results and Discussion

When this modified fusion procedure was used, the yield of hybridoma cell lines was greatly increased. More than 50 antibody-producing hybridoma cell lines were cultured beyond the 200-cell stage. Four of these produced antibody to PCV antigens expressed on the surface of infected cells. These four were each cloned twice. The resulting series of hybridoma cell lines all produced MAb specific for PCV.

V. Derivation of Hybridoma Cell Lines that Secrete Monoclonal Antibodies toward Pichinde Virus Antigens Expressed on the Surface of Infected Cells. II. The Use of Macrophage Feeder Cells to Increase Hybridoma Yields

A. Introduction

Many of the hybridoma cells derived by the procedure described in the fourth quarterly report (Dawson et al., 1986) died after only a few divisions. This same problem was noted using the hybridization procedure described in Section IV unless macrophages were included in the fusion cultures. The inclusion of macrophages was

shown to significantly increase the yield of viable hybridoma cell lines. To determine the effect of using macrophages in the fusion cultures, the fusion mixture resulting from the hybridization procedure described in Section IV was plated onto two sets of 24-well plates, five plates with macrophage feeder cells, and five plates without macrophages. The procedure used for macrophage isolation was patterned after one described to B. Barnett by Clay Osterling of USAMRIID.

B. Materials and Methods

The following procedure was used for the isolation of peritoneal exudate cells (macrophages):

1. BALB/c mice (8 to 30 weeks old) were sacrificed by cervical dislocation. One mouse at a time was processed through Step 5, pooling macrophages at that step.

2. The mouse was disinfected by dropping it into a beaker of 70% aqueous ethanol (w/v). The mouse was pinned to a clean dissection board.

3. The abdominal skin was opened using sterile forceps and scissors and pulled back to completely expose the peritoneal wall. The skin flaps were secured with pins.

4. The abdominal muscle layer was lifted, using a second pair of sterile forceps. A 5-ml aliquot of sterile cold RPMI-1640 (without serum) was injected using a 5-cc syringe and a 20-gauge needle. The abdomen was gently massaged for 10 sec and the solution was withdrawn by keeping the bevel of the needle down and lifting the peritoneal membrane to form a "tent", taking precautions not to puncture the intestine. The withdrawn suspension should be clear. If it was noticeably cloudy, the solution and the mouse were discarded.

5. The fluids containing the macrophages were placed into a sterile centrifuge tube in an ice bath.

6. Step 4 was repeated, and the macrophage suspensions were pooled and centrifuged at 500 x g for 5 min. Note: The pellet was not readily visible.

7. The supernatant fluids were decanted, and the cell pellet was resuspended in a small amount (approximately 2 ml/mouse) of serum-free medium. Using trypan blue, the macrophages were counted using the trypan blue method and diluted to the desired cell number. Red blood cells or lymphocytes were not counted.

8. One mouse typically yielded about 1×10^6 macrophages. Aliquots of 2.5×10^4 macrophages/1.0 ml were used in 24-well plates or 5×10^3 macrophages/0.2 ml in 96-well plates.

9. The macrophages were resuspended in HAT or other selective medium and placed into the culture plates before the fusion products (hybrids). This procedure was done 24 h before the addition of fusion products so that macrophage wells could be checked for contamination.

10. Plates of macrophages could be used on Days 1 to 6. Plates were usable after longer periods if the medium were changed.

11. The macrophages were attached. The culture was cleared of debris in 6 to 10 days.

C. Results and Discussion

At seven days postfusion there was a difference in size of the colonies on the two sets of plates. The colonies in wells with macrophages had an average population of 98 ± 90 cells, whereas those in wells without macrophages averaged 24 ± 28 cells/colony. The percentage of wells yielding hybridoma colonies large enough to warrant antibody screening (at least 200 cells) was 9% in plates without macrophages and 22% in plates with macrophages. All four of the hybridoma cell lines that produced MAb toward PCV antigens expressed on the surface of infected cells were obtained from the fusion mixtures that had been cultured with macrophages.

VI. Derivation of Hybridoma Cell Lines that Secrete Monoclonal Antibodies toward Pichinde Virus Antigens Expressed on the Surface of Infected Cells. III. Cloning and Storage of Hybridoma Cell Lines that Produce Monoclonal Antibodies toward Pichinde Virus

A. Introduction

Representative doubly cloned hybridoma cell lines from each of the four anti-PCV MAb secretor hybridoma lines were expanded to cell densities of 1 to 2 x 10^6 cells/ml in T75 cell culture flasks and then 1.0-ml aliquots were frozen. It was determined that cloning in RPMI medium without feeder cells was generally satisfactory. For cloning hybridoma cells, most procedures call for conditioned medium. However, we found that cloning in fresh RPMI medium prepared with FBS that had not been heat-inactivated resulted in a higher yield of mature clones than did cloning in conditioned medium.

Previous cloning experiments had shown that FOX-NY cells died in RPMI-1640 supplemented with AAT but were viable in the presence of adenine and thymidine. It was also shown that FOX-NY cells could be cloned in RPMI-1640 without using feeder cells. However, in those experiments the clones died out before reaching a size that covered 25% of the well. The results with conditioned medium were

more encouraging, but the clones appeared to deteriorate as they exceeded about 10% confluency. On the one hand, efficient cloning may require media with growth factors that are contributed by vigorously growing cells but, on the other hand, perhaps the process of conditioning the media may deplete essential ingredients such as glutamine. Heat-inactivation of the FBS may also inactivate essential growth factors. This experiment compared cloning in several different media. In the previous experiments, the media were prepared using heat-inactivated FBS, whereas in this experiment nonheat-inactivated FBS was used and the conditioned medium was prepared from the freshly prepared RPMI-1640 medium, using the nonheat-inactivated FBS, and then supplemented with glutamine.

B. Materials and Methods

Cells. Because hybridoma cells were not available at the time, FOX-NY cells were used for these studies. Their use precluded the use of AAT in the cloning medium because the FOX cells are killed by aminopterin. FOX-NY cells (Passage 12) were used for this experiment. The cells had been recovered from liquid nitrogen storage in Passage 10 and then maintained in exponential growth phase until used for this cloning experiment eight days later.

Media. Prior to cloning, the cells were grown in RPMI-1640 with 15% FBS, 0.01 M HEPES, and Pen-Strep (100 U/ml penicillin and 100 g/ml streptomycin sulfate). The FBS was not heat-inactivated as

this might be detrimental to the nutrients and growth factors. The conditioned medium was prepared by culturing FOX-NY cells in logarithmic phase growth in RPMI-1640 for 48 h, and then filter-sterilizing the medium. Glutamine was added to conditioned medium from a 200 mM working solution that was stored frozen until the time of the cloning experiment.

Cloning. Cells were diluted and plated in each of the various media on seperate 96-well cell culture plates at a dilution calculated to yield 0.5 cell/well. The plates were then placed in a humidified incubator and left undisturbed until observed for clones. The plates were examined four days later, and a dot was placed over wells containing a single clone, and the number of cells in each clone was determined. At eight days postseeding, the condition of the cells and the size of the clones were again determined.

C. Results

The clones in the plates with conditioned medium reached a maximum size of 30 cells and did not appear healthy. In fact, these clones died out by eight days, at which time four to 20 dead cells were all that remained. The clones in the plate with RPM-1640 were the most vigorous of the lot; they contained over 200 cells at eight days. There appeared to be some dead cells at the center of the colonies, yet very healthy cells at the edges. It appeared that

crowding was a problem with these rapidly growing clones. Therefore, in a cloning experiment, the rapidly growing clones should be expanded to a 24-well plate by the time they reach several hundred cells in size. The clones cultured in a 50/50 mixture of RPMI-1640 and conditioned medium did much better than those in conditioned medium but did worse than those in RPMI-1640 alone. As is shown in Table VI-1, many of these colonies were dying out by the time they reached a size of 200 cells.

D. Discussion

There was no advantage to cloning in conditioned medium. In fact, the data indicated that conditioned medium was less desirable than fresh medium prepared with FBS that had not been heat-inactivated. The conditioned medium may be toxic to the cells either as a result of toxic products introduced from the conditioning process or through depletion of essential nutrients. This experiment utilized FOX-NY cells and not hybridoma cells. The FOX-NY cells did very well in regular RPMI-1640 medium, whereas hybridoma cells, which may be more fastidious, required conditioned medium or feeder cells. Subsequent experience with hybridoma cells indicated that most do very well in regular RPMI-1640 medium prepared with FBS that had not been heat-inactivated.

Table VI-1. Effects of Different Media on Cloning of FOX-NY Cells.

Medium	No. wells with one or more clones at four days postseeding	No. wells with mature clones eight days postseeding
RPMI-1640	22	15
Conditioned medium	7	0
Conditioned medium with 2mM glutamine	13	0
50/50 mix of RPMI-1640/-conditioned medium (with 2mM glutamine)	20	10

VII. Derivation of Hybridoma Cell Lines that Secrete Monoclonal Antibodies toward Pichinde Virus Antigens Expressed on the Surface of Infected Cells. IV. Freezing and Storage of the Hybridoma Lines

A. Introduction

Based on the results described in the previous section, hybridoma cell lines that secreted antibody towards PCV were cloned in RPMI-1640 with 15% FBS (not heat-inactivated). Vigorous clones were obtained from four hybridoma lines of interest, and after a second cloning these were expanded as previously outlined in the description of the hybridization procedure. These doubly cloned, monoclonal antibody-secreting hybridomas were then frozen and stored in liquid nitrogen. The cell-freezing procedure used is described in this section.

B. Materials and Methods

Cells. Cells were vigorous and in the logarithmic phase of growth at the time of freezing.

Freezing Media. RPMI-1640 with 40% FBS and RPMI-1640 with 20% DMSO (sterile-filtered and used at 4°C).

Freezing Procedure. Cells were pelleted by centrifugation. The cell pellet was resuspended in RPMI-1640 without serum. The total number of cells was determined. The cells again were pelleted and resuspended in cold RPMI-1640 with 40% FBS to yield between 2 x 10^6 to 2 x 10^7 cells per ml. At this point the volume was one-half the final volume. From this step on, the cells and medium were held at 0°C to 4°C. An equal volume of cold RPMI-1640 with 20% DMSO was added dropwise to the cell suspension. Aliquots (1 ml/tube) were dispensed into 2-cc polypropylene cryotubes having a silicone gasket (Nunc, Denmark) on ice. The cryotubes were placed in styrofoam test-tube holders (50-tube package of 15-ml disposable centrifuge tubes, Item 25319, Corning Glass Works, Corning, NY) and placed in a -90°C freezer. After 12 to 24 h, cryotubes were transferred to boxes or canes and stored in liquid nitrogen or an ultracold freezer.

Recovery of Frozen Cells. A cryotube of cells was allowed to partially thaw at 37°C. As soon as the pellet of ice was free from the walls of the tube, the contents were poured into a centrifuge tube containing 10 ml of RPMI-1640 with 40% FBS. The cells were pelleted and the medium was removed. The cells were resuspended very gently in 10 ml of RPMI-1640 with 15% FBS, seeded into a T25 flask, and placed in a 37°C incubator.

Cell Counting and Viability Determinations. Cell counting was performed with a hemacytometer. For viability determinations 0.04%

trypan blue (Sigma Chemical, St. Louis, MO) was included in the cell suspensions.

C. Results

FOX-NY cells, frozen according to the procedure described in this report, were thawed 48 h after freezing. The cell counts and viability of this thawed culture were determined at the times indicated in Table VII-1.

D. Discussion

By 96 h after thawing, the FOX-NY cell culture had increased in cell number by a factor of ten (just over three doublings) and had returned to >98% viability. The cell-freezing procedure described in this report did not adversely affect the viability of the FOX-NY myeloma cell line.

Table VII-1. Viabilty of FOX-NY Cells Recovered from Frozen Cultures.

Time post-thawing	Cell count	Viability
4 h	1.8×10^5/ml	82%
48 h	3.0×10^5/ml	97%
then diluted 1:5		
96 h	3.4×10^5/ml	98%

VIII. Ascites Tumor Formation Using Hybridomas Derived from the Fusion of FOX-NY Myeloma Cells with Spleen Cells from RBF/Dn Mice

A. Introduction

Hybridoma cell lines derived from the fusion of FOX-NY myeloma cells with spleen cells from RBF/Dn mice are more stable in cell cuture than are conventionally derived hybridoma cell lines (Taggart and Samloff, 1983). Desiring to take advantage of this increased genetic stability, we derived a series of FOX-NY x RBF/Dn hybridoma cell lines that secreted MAbs toward PCV antigens. Four of these MAbs recognized PCV antigens expressed on the surface of Pichinde virus-infected cells *in vitro* and *in vivo*. Because large quantities of these MAbs were required for *in vivo* antiviral studies, the corresponding hybridoma cell lines were injected into mice for ascites production. However, we found that the usual protocols for the production of ascites tumors when applied to these hybridoma cell lines resulted in erratic, inefficient ascites tumor production. Attempts to propagate these FOX-NY x RBF/Dn hybridoma cell lines by generally accepted protocols in recipient mice of the RBF/Dn or BALB/c strain or the (RBF/Dn x BALB/c)F_1 hybrid of the two resulted in limited ascites production. More aggressive immunosuppression of the recipient mice did increase the success rate for ascites tumor production. By increasing the pristane dose or by including total-body gamma irradiation in the immunosuppression protocols, ascites

production was greatly enhanced. This section describes a series of experiments directed at defining conditions for efficient ascites fluid tumor production from FOX-NY x RBF/Dn hybridoma cell lines.

B. Methods and Procedures

Hybridoma Cells. The PC 4.7C2, PC 4.8D2, PC 4.9D3, and PC 4.9A6 hybridoma cell lines were derived through the fusion of FOX-NY myeloma cells with the spleen cells from RBF/Dn mice that had been hyperimmunized with PCV. The hybridoma cell lines had been cloned twice and the cells were in the exponential growth phase when injected into recipient mice. The viability of the cells was determined by trypan-blue exclusion. At the time of inoculation, at least 90% of the cells were viable in all of the cultures. The hybridoma cell lines used in these studies all secreted anti-Pichinde virus MAb. All four MAbs are of the IgG_{2a} isotype.

Mice. Female BALB/c mice were obtained from Simonsen Laboratories (Gilroy, CA). RBF/Dn mice were obtained from Jackson Laboratories (Bar Harbor, ME). At the time of injection of hybridoma cells, mice varied from 8 to 13 weeks of age. F_1 hybrid mice were bred in the Utah State University Laboratory Animal Research Center using RBF/Dn males from Jackson laboratories and BALB/c females from Simonsen Laboratories. The resulting (RBF/Dn x BALB/c)F_1 mice after weaning were housed one sex to a cage.

Pristane Priming and Hybridoma Cell Inoculation. The general protocol was a single intraperitoneal (i.p.) injection (26-gauge needle) of pristane (2,6,10,14-tetramethylpentadecane). Six to seven days later, mice received 5 $x10^6$ to 1 x 10^7 hybridoma cells in 1.0 ml of RPMI-1640 cell culture medium without FBS, injected (20-gauge needle) into the peritoneal cavity.

Collection of Ascites Fluids. The date of tumor appearance was noted for each mouse and ascites fluids were collected every one to three days after the tumors were readily apparent, usually within four days of the initial appearance of the tumor. The rate of collection of ascites fluids depended on how rapidly the individual tumors grew. Ascites fluids were collected by inserting a 20-gauge needle into the peritoneal cavity and allowing the fluids to drain into a centrifuge tube. The fluids were clarified by centrifugation at 600 x g for 5 min. The clear ascites fluids were removed from the pelleted red blood and hybridoma cells and the small amount of residual pristane overlaying the fluids in the tube.

Characterization of Ascites Fluids. The volume of ascites fluids per mouse was recorded. Specific antibody titers were determined by limiting dilution in an indirect immunofluorescent antibody (IFA) assay for antibody to PCV as described in Section II.

The Effect of Pristane on Ascites Tumor Formation. To determine the effect of pristane dosage upon ascites tumor yields,

mice (5 to 6 mice per group) received 0, 0.1, 0.5, 1.0, or 2.0 ml of pristane in a single i.p. injection seven days prior to injection of 5×10^5 PC 4.9A6 hybridoma cells.

FOX-NY x RBF/Dn Hybridoma Cell Ascites Formation in BALB/c mice Primed with High Pristane Dosage. The FOX-NY x RBF/Dn hybridoma cell line PC 4.9A6 produced ascites tumors in BALB/c mice if the mice were treated with relatively large doses of pristane. To determine whether high pristane dosage would lead to similar results with other FOX-NY x RBF/Dn hybridoma cell lines, three cell lines--PC 4.9D3, PC 4.7C2 and PC 4.8D2--were inoculated into mice. Female BALB/c mice (11 to 12 weeks old) were injected (i.p.) with 2.0 ml of pristane. Seven days later each mouse received (i.p.) 5×10^6 hybridoma cells. The production of ascites tumors and the resulting ascites fluids were monitored.

Effect of Using (RBF/Dn x BALB/c)F_1 Mice. Hybrid (RBF/Dn x BALB/c)F_1 mice (6 to 7 weeks old) were primed with 1.0 ml of pristane (i.p.) and seven days later were inoculated with 5×10^6 to 1×10^7 PC 4.7C2, PC 4.8D2, PC 4.9D3, or PC 4.9A6 hybridoma cells. Mice were examined daily (through 88 days p.i.) for ascites tumor development. As an addendum to this experiment, nonproducing (RBF/Dn x BALB/c)F_1 mice in the PC 4.7C2 group received an additional 0.5 ml of pristane 88 days after the original inoculation with PC 4.7C2 cells and were irradiated four days later with a ^{137}Cs source, receiving a whole-body radiation dose of 350 rad. One of

the seven mice died about 22 h after the radiation exposure. One day after radiation treatment, the mice received hybridoma cells isolated from the ascites fluids of the lone mouse that had produced a PC 4.7C2 ascites tumor. The cells had been cultured in vitro for nine days, the cell viability was 98%, and 3.1×10^6 cells were injected into each mouse.

Effect of Total-Body Irradiation on Ascites Production. Hybrid (RBF/DnJ x BALB/c)F_1 mice, (11 to 12 weeks old) received 1.5 ml of pristane (i.p.) followed six days later with 350 rads of total-body irradiation delivered by a ^{137}Cs gamma-source. Hybridoma cells were injected the following day. PC 4.8D2 hybridoma cells (8×10^6/mouse) were injected (i.p.) into 16 mice (12 irradiated and four not irradiated). PC 4.7C2 cells (7×10^6/mouse) were injected (i.p.) into 16 mice (10 irradiated and six not irradiated). All mice had been treated with pristane.

C. Results

The Effect of Pristane on Ascites Tumor Formation. When the FOX-NY x RBF/Dn hybridoma cell line PC 4.9A6 was injected (i.p.) into BALB/c mice, increasing the dosage of pristane resulted in increased efficiency of ascites tumor formation. Without pristane priming, none of the mice developed ascites tumors, and priming with either 0.1 ml or 0.5 ml of pristane led to a very low percentage of responders, with one out of five mice responding at either pristane

dose (Table VIII-1). In contrast, all of the mice pretreated with either 1.0 or 2.0 ml of pristane produced ascites tumors. The response in the 2.0-ml group was very uniform, with an average time to first ascites fluid collection of 8 ± 1 days. The yields of ascites fluids were similar from the groups primed with 2.0 ml of pristane (6.3 ± 3.5 ml/mouse) and 1.0 ml of pristane (5.1 ± 1.9 ml/mouse). The anti-Pichinde virus antibody titers of the ascites fluids from all the mice ranged from 10^5 to $10^{5.5}$, as measured by limiting dilution in an indirect immunofluorescent antibody assay.

FOX-NY x RBF/Dn Hybridoma Cell Ascites Formation in BALB/c Mice Primed by High Pristane Dosage. In the previous experiments designed to determine the effect of pristane dosage, hybridoma cell line PC4.9A6 produced ascites tumors in BALB/c mice if the mice were treated with relatively large doses of pristane. To determine if high pristane dosage would lead to similar results with the other FOX-NY x RBF/Dn hybridoma cell lines, PC 4.9D3, PC 4.7C2, and PC 4.8D2 hybridoma cells were inoculated into mice. All of the mice that were inoculated with the PC 4.9D3 cell line developed ascites tumors that were visible by seven days p.i. Fluids were drained from all mice eight days p.i. The ascites fluids volumes were low, only 3.1 ml/mouse, and the tumors were very virulent. All the mice died within four days of developing visible tumors. Two mice that received the PC 4.7C2 cell line developed ascites tumors 45 days after receiving the cells (data shown in Table VIII-2). Pools of both the PC 4.7C2 and PC 4.9D3 ascites had similar titers by

Table VIII-1. Effect of Pristane Dose on Ascites Production Following Inoculation of BALB/c Mice with FOX-NY x RBF/Dn Hybridoma Cell Line PC 4.9A6.

Pristane dose (ml)	% Mice developing tumors (number/total)	Total vol. of ascites fluids collected (ml)	Time from inoculation with hybridoma to first collection (days)		
			Earliest	Latest	Ave ± SD
2.0	100 (6/6)	37.6 (6.3 ± 3.5/mouse)	7	9	8.0 ± 1.1
1.0	100 (5/5)	25.6 (5.1 ± 1.9/mouse)	9	16	12.6 ± 3.2
0.5	20 (1/5)	1.0	15	-	-
0.1	20 (1/5)	2.8	18	-	-
0.0	0	0	-	-	-

Table VIII-2. Effect of Hybridoma Line on Ascites Production with Time.

Hybridoma cell line	% Mice developing tumors (number/total)	Total vol. of ascites fluids collected (ml)	Time from inoculation to first collection (days)		Anti-PCV titer[a]
			Earliest	Latest	
PC 4.9D3	100 (10/10)	31.2 (3.1 ± 2.1/mouse)	8	8	$10^{-5.7}$
PC 4.7C2	20 (2/10)	8.4 (4.2 ± 0.6/mouse)	45	46	$10^{-5.5}$
PC 4.8D2	0	0	-	-	

[a]The anti-PCV antibody titers of the ascites fluids were determined by limiting dilution in an indirect immunofluorescent antibody assay for antibody to PCV.

indirect IFA. The MAb PC 4.9D3 titer was $10^{-5.7}$ and that for MAb PC 4.7C2 was $10^{-5.5}$ (dilution assay). None of the mice that had received cell line PC 4.8D2 developed ascites tumors.

Anti-Pichinde Virus Ascites Production. Effect of Using (RBF/Dn x BALB/c)F_1 Mice. Of the (RBF/Dn x BALB/c)F1 mice that received the PC 4.9A6 hybridoma cells, four of five developed ascites tumors. The fifth mouse quickly developed a large solid tumor on a hind leg and was sacrificed 22 days after the hybridoma cells were injected without producing ascites fluids (Table VIII-3). Six of the seven mice that received PC 4.9D3 hybridoma cells developed ascites tumors. The only mouse that did not produce ascites fluids was killed 22 days p.i. and had a large black tumor on the abdomen. One of the eight mice that received PC 4.7C2 hybridoma cells developed ascites fluids. The ascites developed in this sole responder long after all of the mice in the PC 4.9A6 and PC 4.9D3 groups had died. The ascites fluids were collected 78 days after the PC 4.7C2 hybridoma cells were injected into the mice. The ascites fluids were assayed for anti-Pichinde virus activity because this late-developing ascites tumor could have been a spontaneous tumor rather than a tumor arising from the PC 4.7C2 hybridoma cell line. The antibody titer directed against PCV was over 10^5 as measured by indirect IFA (Table VIII-4). In an attempt to get ascites from the seven surviving, nonproducing mice in the PC 4.7C2 group, the mice were once again primed with pristane 88 days after the original inoculation with PC 4.7C2 hybridoma cells. Four days

Table VIII-3. Development of Ascites Tumors in (RBF/Dn x BALB/c)F_1 Mice.

Hybridoma cell line	% Mice developing tumors (number/total)	Total vol. of ascites fluids collected/mouse (ml)	Time from inoculation with hybridoma to first collection (days)		
			Earliest	Latest	Ave ± SD
PC 4.9A6	80 (4/5)	9.4 ± 5.2	26	32	29.0 ± 3.5
PC 4.9D3	86 (6/7)	5.8 ± 3.0	26	36	33.5 ± 4.2
PC 4.7C2	13 (1/8)	24.5	78	-	78
PC 4.8D2	0 (0/8)	-	-	-	-

Table VIII-4. Titration of PC 4.7C2 Ascites Fluids.

Sample	Dilution	IFA Result
PC 4.7C2 (AF) (02/03/88) GBG	10^{-1}	4 (no nonspecific Rx)
PC 4.7C2 (AF) (02/03/88) GBG	10^{-2}	4
PC 4.7C2 (AF) (02/03/88) GBG	10^{-3}	4
PC 4.7C2 (AF) (02/03/88) GBG	10^{-4}	4
PC 4.7C2 (AF) (02/03/88) GBG	10^{-5}	2-3
PC 4.7C2 (AF) (02/03/88) GBG	10^{-6}	0-1

later these mice were irradiated (350 rad) and PC 4.7C2 hybridoma cells were injected the next day. One of the irradiated PC 4.7C2 mice died 20 to 22 h after irradiation, apparently as a result of the irradiation. Of the remaining six mice, four produced ascites tumors, which were tapped 16 days after the cells had been injected into the irradiated mice. At the time of preparing this report (03/15/88), it had been 18 days since the PC 4.7C2 cells were injected and the experiment is still in progress. The first collection of ascites fluids from these irradiated mice produced 7.8 ± 1.8 ml/mouse.

Effect of Total Body Irradiation on Ascites Production. This experiment is still in progress; several mice are currently producing ascites, some have already died, and others have yet to begin producing ascites. The cells were inoculated into the mice 33 days ago, and this is a report of the results to date (03/15/88). All 10 of the irradiated mice that received the PC 4.7C2 cell line developed ascites tumors, which appeared 18 to 31 days after the cells were injected. Four of these 10 also developed solid tumors, and in one case the solid tumor grew so rapidly that the mouse had to be killed prior to developing a sufficient volume of ascites fluids for harvest. Eight of the nine ascites fluid-producing mice have now died. The yields from those eight were 6.3 ± 4 ml/mouse. If the mice that developed solid tumors were excluded, the yields were 7.3 ± 4 ml/mouse. None of the six nonirradiated mice had developed ascites tumors or solid tumors by 33 days p.i. Twelve

irradiated mice received cell line PC 4.8D2. At 27 days p.i., ascites fluids were collected from three of these mice. By 33 days p.i., five of the 12 had developed ascites tumors. One of these mice also developed a large solid tumor in the abdomen. Because ascites tumors were still appearing as of 33 days p.i., the efficiency of production cannot be determined at this time. However, the volumes of ascites fluids per collection have been quite high. Only two of the mice have died so far. Total volumes of ascites fluids collected from the two mice were 8.5 ml and 11.0 ml. None of the four nonirradiated mice developed ascites tumors or solid tumors.

D. Discussion

The FOX-NY cell line is a murine myeloma cell line that does not produce immunoglobulin heavy or light chains and thus is desirable as a fusion partner for hybridoma production. The FOX-NY cell line is deficient in the enzyme adenosine phosphoribosyltransferase (APRT), which forms the basis for the selection procedure described in the original reference on the use of FOX-NY cells for hybridoma production (Taggart and Samloff, 1983). RBF/DnJ mice are characterized by a mutation resulting in a Robertsonian 8.12 translocation that places portions of chromosomes 8 and 12 on a single 8.12 translocation chromosome. Therefore, the gene coding for APRT is on chromosome 8 and the heavy chain immunoglobulin locus is on chromosome 12. Thus in the RBF/DnJ mouse the enzyme marker sensitive to the selection procedure is genetically linked to the

ability to produce immunoglobulin. Therefore, hybridoma cell lines derived from the fusion of FOX-NY myeloma cells to spleen cells from RBF/DnJ mice are stable, antibody-producing hybridoma cell lines. However, in spite of the advantages of genetic stability, unless these hybridoma cell lines can be readily propagated as ascites tumors, their utility is seriously limited. When we attempted to produce ascites fluids from several RBF/DnJ x FOX-NY hybridoma cell lines, ascites-production procedures that were suitable for conventional BALB/c-based hybridoma cell lines failed. In this section a series of experiments directed at efficient ascites production have been described. Because some of the experiments are still in progress and only four RBF/DnJ x FOX-NY hybridoma cell lines have been studied, the overall conclusions could be modified in the future.

1) By increasing the pristane dosage to 1.0 to 2.0 ml/mouse, the efficiency of ascites tumor formation was dramatically increased. However, this procedure was not universally satisfactory for all RBF/DnJ x FOX-NY hybridoma cell lines. In these studies two out of four cell lines failed to produce ascites even in recipient mice that had received 2.0 ml of pristane.

2) Using hybrid (RBF/Dn x BALB/c)F_1 mice as the RBF/DnJ x FOX-NY hybridoma cell line recipients, the increased pristane dosage levels did not dramatically increase the ascites tumor yields over those obtained using BALB/c mice.

3) An aggressive immunosuppression regimen of pristane priming followed by total-body irradiation (gamma, 350 rad) in hybrid (RBF/Dn x BALB/c)F1 mice produced recipient mice suitable for the propagation of RBF/DnJ x FOX-NY hybridoma cell lines as ascites tumors.

The Effect of Pristane on Ascites Tumor Formation. Mice primed by i.p. injection with pristane demonstrate an increased susceptibility to the development of spontaneous plasmacytomas. This phenomenon, apparently based upon suppression of normal immune surveillance, has been exploited for the propagation of hybridoma cells as ascites tumors. Freund and Blair (1982) demonstrated that pristane markedly depressed natural killer-cell activity in BALB/c mice. It has been suggested that the BALB/c strain of mouse is particularly susceptible to plasmacytoma formation (Potter and Wax, 1981). Because most murine hybridomas are derived from mice and cells of the BALB/c strain, the in vivo propagation of MAbs has usually been straightforward. When BALB/c-derived hybridomas were seeded into BALB/c mice (Hoogenraad and Wraight, 1986), 0.1 ml of pristane was shown to be as effective as 0.5 ml of pristane. In contrast, in the case of ascites tumor formation in BALB/c mice using hybridomas derived from RBF/Dn mice (FOX-NY x RBF/Dn hybridoma cell lines), either 0.1 ml or 0.5 ml of pristane yielded a very low percentage of responders. Using the FOX-NY x RBF/Dn hybridoma cell line PC 4.9A6, we found that an increased dosage of pristane resulted in an increased efficiency of ascites tumor formation in

BALB/c mice. When 0.5 ml of pristane was used to prime BALB/c mice for ascites tumor formation with the PC 4.9A6 hybridoma cell line, only one of five mice responded and the time between inoculation with the hybridoma cells and the formation of the tumor was long in comparison with mice receiving higher pristane dosages. All of the mice pretreated with either 1.0 or 2.0 ml of pristane produced ascites tumors. The response in the 2.0-ml group was very uniform, with an average time to first ascites fluid collection of 8 ± 1 days. The yield of ascites fluids was similar in the 1.0-ml (5.1 ± 1.9/mouse) and 2.0-ml (6.3 ± 3.5/mouse) groups of mice, both being much higher than the volumes obtained from the sporadic responders in the lower pristane-dose groups.

FOX-NY x RBF/Dn Hybridoma Cell Ascites Formation in BALB/c Mice Primed by High Pristane Dosage. The results of pristane dose experiments using the PC 4.9A6 hybridoma cell line in BALB/c mice indicated that this FOX-NY x RBF/Dn hybridoma cell could be grown as an ascites tumor in BALB/c mice if the mice were primed with 1 to 2 ml of pristane. However, we found that even with a high pristane dosage, ascites formation in this system was very dependent on the cell line. Of the three other FOX-NY x RBF/Dn hybridoma cell lines examined, only PC 4.9D3 produced efficient ascites formation in the BALB/c mice, whereas 20% of the mice receiving the PC 4.7C2 cell line developed ascites and none of the mice inoculated with PC 4.8D2 developed ascites tumors. The ascites fluids produced in this experiment were of high titer, but the volumes were low. The mice

were 12 weeks old when injected with pristane. Perhaps younger mice would have been more suitable for ascites production. However, subsequent experiments with (RBF/Dn x BALB/c)F_1 mice demonstrated that mice of this age would produce ascites, and older mice produced larger volumes of ascites than did younger mice. The conclusion of this set of experiments was that even with large pristane doses, BALB/c mice did not develop FOX-NY x RBF/Dn hybridoma cell ascites tumors efficiently with some FOX-NY x RBF/Dn hybridoma cell lines.

Effect of Using (RBF/Dn x BALB/c)F_1 Mice. Although initial experiments utilizing increased dosages of pristane resulted in satisfactory production of MAb PC 4.9A6 and PC 4.9D3 and a small amount of MAb PC 4.7C2, there was no success with PC 4.8D2. These four hybridoma cell lines carry cell-surface antigens from both the RBF/Dn and BALB/c strains of mice. This may have accounted for the failure of some of these hybridoma lines to grow as ascites tumors in either RBF/Dn or BALB/c strains of mice. In an experiment not described in this report, PC 4.9A6 hybridoma cells failed to grow as ascites in RBF/Dn mice. We next used (RBF/Dn x BALB/c)F_1 mice as recipients. We determined that the use of (RBF/Dn x BALB/c)F_1 mice in place of BALB/c mice resulted in efficient ascites production with PC 4.9A6 and PC 4.9D3 cell lines, the same as was observed with BALB/c mice. Although the time between the injection of the PC 4.9A6 and PC 4.9D3 cells into the mice and the time of ascites appearance was longer (29 to 34 days compared with 8 to 13 days; see Tables VIII-1, VIII-3, and VIII-4), the volumes of ascites fluids

were greater from the (RBF/Dn x BALB/c)F_1 mice (7.6 ml) compared with the BALB/c mice (4.8 ml). Ascites tumor formation with the PC 4.7C2 cell line in both the (RBF/Dn x BALB/c)F_1 and BALB/c mice was delayed and sporatic. In BALB/c mice, two of ten mice developed ascites tumors 45 and 46 days after inoculation with PC 4.7C2 cells. In (RBF/Dn x BALB/c)F_1 mice, one of eight mice developed an ascites tumor; in this case the tumor appeared 78 days after inoculation with PC 4.7C2 cells. Neither the BALB/c nor the (RBF/Dn x BALB/c)F_1 mice developed ascites tumors following inoculation with the PC 4.8D2 cells.

In an attempt to get ascites from the surviving, nonproducing mice in the PC 4.7C2 group, the mice were aggressively immunosuppressed with pristane and gamma irradiation. These mice were then inoculated with the corresponding hybridoma cell line that had been taken from the (RBF/Dn x BALB/c)F_1 mouse that had produced ascites fluids. This attempt was initiated after the observation that irradiated mice in an ongoing experiment were producing ascites. Of the six mice in this group, four produced ascites tumors, which were drained 16 days after the cells had been injected into the irradiated mice. The experiment is still in progress (03/15/88). The first collection of ascites fluids from these irradiated mice produced 7.8 ± 1.8 ml/mouse; it appears that these mice will produce 15 to 20 ml of ascites fluids/mouse. The results from this uncontrolled experiment indicate that even relatively old mice (21 to 23 weeks old) can be induced to form ascites tumors,

that previous inoculation with a hybridoma cell line does not preclude the growth of that cell line as an ascites (as might be expected if the mice mounted an immune response to the surface antigens of the hybridoma cell line), and that aggressive immunosuppression appeared to be a key in growing the RBF/Dn x BALB/c hybridoma cell lines as ascites. Another possibility that needs to be examined in a controlled experiment is that by using RBF/Dn x BALB/c hybridoma cells that had been passaged as ascites tumors, the efficiency of ascites induction might be increased.

Effect of Total-Body Irradiation on Ascites Production.

Previous experiments utilizing either (RBF/Dn x BALB/c)F_1 mice or BALB/c mice with increased dosages of pristane resulted in production of moderate amounts of MAbs PC 4.9A6 and PC 4.9D3 and a small amount of MAb PC 4.7C2, but no ascites from the PC 4.8D2 hybridoma cell line. That these four hybridoma cell lines carry different arrays of surface antigens from both the RBF/Dn and BALB/c strains of mice may account for the failure of some of these hybridoma lines to grow as ascites tumors in either RBF/Dn or BALB/c strains of mice. However, the failure of two out of four of these cell lines to grow as ascites in hybrid (RBF/Dn x BALB/c)F_1 mice that should be tolerant to surface antigens from the RBF/Dn and BALB/c strains was somewhat surprising. Because the efficiency of ascites production with the PC 4.9A6 cell line in BALB/c mice was directly related to pristane dosage, we thought that perhaps an even more aggressive immunosuppression might facilitate ascites production from the

hitherto recalcitrant PC 4.7C2 and PC 4.8D2 hybridoma cell lines. The next step was to add total-body irradiation to the immunosuppression protocol. These experiments, still in progress, utilizing a combination of pristane priming and gamma irradiation indicate that aggressively immunosuppressed (RBF/Dn x BALB/c)F_1 mice do develop ascites tumors at a high frequency even with the very refractory PC 4.7C2 and PC 4.8D2 hybridoma cell lines. Because only 33 days have elapsed since the hybridoma cells were injected into the mice, some of the nonresponding mice may yet develop ascites tumors. However, at this time 10 out of 10 mice that were treated with pristane and then irradiated (350 rad) developed ascites tumors after receiving PC 4.7C2 hybridoma cells. The ascites tumor development with cell line PC 4.8D2 has been slower than that with PC 4.7C2, but five of 12 pristane-primed, irradiated mice have developed ascites tumors after receiving PC 4.8D2 hybridoma cells. None of the nonirradiated mice developed ascites tumors with either cell line; however, in the case of PC 4.7C2 hybridoma cells, ascites tumors have typically not appeared until 40 or more days p.i. The combination of pristane priming and gamma irradiation produced the best ascites yields to date. Pristane-primed, irradiated (RBF/DnJ x BALB/c)F_1 mice produced ascites from both the PC 4.7C2 and PC 4.8D2 hybridoma cell lines. This was the first time that the PC 4.8D2 hybridoma cell line produced ascites.

IX. Purification of Anti-Pichinde Virus Monoclonal Antibodies

A. Introduction

MAbs PC 4.9A6 and PC 4.9D3 were purified from murine ascites fluids using ammonium sulphate precipitation followed by affinity chromotography on protein A. The purification procedure and yields at each step are described in this section. The overall yields for the purification of MAbs PC 4.9A6 and PC 4.9D3 were 34% and 9%, respectively. The purified monoclonal antibodies retained complete antigen-binding activity.

B. Materials and Methods

Ascites Fluids. Ascites fluids from individual mice bearing ascites tumors originating from either PC 4.9A6 or PC 4.9D3 hybridoma cells were pooled. Residual hybridoma cells and debris were removed by centrifugation at 500 x g. The MAbs secreted by these hybridoma cell lines are of the IgG_{2a} isotype and bind to PCV antigens expressed on the surface of cells infected with PCV. The two MAbs recognize distinct epitopes, as evidenced by their failure to compete with each other in competitive binding assays.

Precipitation with Ammonium Sulphate. Immunoglobulins were precipitated from the ascites fluids with 50% ammonium sulphate. While the ascites fluids in an Erlenmeyer flask were gently swirled,

an equal volume of saturated ammonium sulphate in distilled water was added dropwise. The suspension was allowed to stand overnight at 4°C and then the precipitate was pelleted at 500 x g for 15 min. The precipitated immunoglobulins were dissolved in Dulbecco's PBS (0.5 mM $MgCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, and 140 mM NaCl, pH 7.2) to return to the original sample volume and then reprecipitated by the addition of an equal volume of saturated ammonium sulphate. The precipitated immunoglobulins were collected as before by centrifugation. The precipitation was performed one more time. The ascites PC 4.9A6 was precipitated a fourth time because there was still some visible hemoglobin in the pellet after the third precipitation. The immunoglobulins recovered from the ammonium sulphate precipitations were dissolved in PBS and then dialyzed against at least 100 volumes of PBS, i.e., 20 ml of immunoglobulin dialyzed against 2,000 ml of PBS. The dialysis fluids were changed at least four times at intervals of a few hours or more.

Determination of IgG_{2a} Concentrations. The concentration of IgG_{2a} in various sample fractions was determined by radial immunodiffusion (RID) with antisera specific for IgG_{2a}. The reagents were purchased as a IgG_{2a} quantitation kit from ICN Biomedicals, Inc. (Costa Mesa, CA). The procedures used were described in the product enclosures of the supplier.

Affinity Chromatography Using Protein A-Sepharose. The ammonium sulphate-purified fraction from the ascites fluids was

further purified by affinity chromatography on protein A-Sepharose using the BioRad Affi-Gel Protein A MAPS II Kit (BIO-RAD Laboratories, Richmond, CA), following the instructions supplied with the kit. The protein concentration in the samples was estimated by OD_{280} measurements. The affinity-chromatography column utilized the entire 5 ml of gel suspension supplied with the kit, which should be sufficient to bind 30 to 40 mg of IgG. The samples were diluted one to one with binding buffer, and 4 ml of the diluted sample (20 to 30 mg of protein) was applied to the column for each run. The sample-application rate was 0.5 ml/min. The column was regenerated between runs as described in the instructions. The acidic solution (the composition of the binding buffer and elution buffer were not disclosed by the supplier) containing the eluted IgG was neutralized immediately by adding 1 ml of 1 M Tris, pH 9.0, to each 13 ml of eluted sample. After the completion of antibody purification, the protein A sepharose was regenerated and stored in PBS containing 0.05% NaN_3 at 4°C.

Storage of Purified Monoclonal Antibodies. The purified MAb pools were dialyzed extensively against PBS without azide. They were then filter-sterilized by filtration through sterile membrane filters with a pore size of 0.22-μ. The IgG_{2a} concentration was measured by RID. The purified MAbs were then aliquoted at 5 ml per vial, lyophilized, and stored at -15°C.

Antigen-Binding Activity of Monoclonal Antibodies. The samples were assayed by an indirect immunofluorescence assay as described in Section XI of this report. The antigen-binding titers were expressed as the reciprocal of the highest dilution of sample that produced readily recognizable immunostaining of Pichinde virus-infected Vero cells.

C. Results

The yields based on IgG_{2a} recovery at each step of the purification procedure are given in Table IX-1. Because the IgG_{2a} content did not necessarily indicate active antibody, the antigen-binding activity of the purified MAbs was measured by indirect immunofluorescence (see Table IX-2). The binding activity was $10^{4.5}$ for both MAbs PC 4.9A6 and PC 4.9D3 preparations having IgG_{2a} contents of 1.1 mg/ml and 0.34 mg/ml, respectively. The antigen-binding activity of the original ascites fluids were $10^{5.5}$ and $10^{5.7}$, respectively, and the corresponding IgG_{2a} concentrations were 14 mg/ml and 17 mg/ml for MAbs PC 4.9A6 and PC 4.9D3, respectively. The residual binding activity normalized to the IgG_{2a} content was 126% and 300% of the original for MAbs PC 4.9A6 and PC 4.9D3, respectively.

Table IX-1. Purification of Monoclonal Antibodies: Yields and Recoveries.

	Ascites		After $(NH_4)_2SO_4$		After Protein A		
Hybridoma	Volume (ml)	$[IgG_{2a}]^a$ (mg/ml)	Volume (ml)	[Protein]^b (mg/ml)	Volume (ml)	$[IgG_{2a}]^a$ (mg/ml)	Yield (%)
PC 4.9A6	23	14	25	15	100	1.1	34
PC 4.9D3	8	17	10	11	35	0.34	9

[a] $[IgG_{2a}]$ in mg/ml as determined by radial immunodiffusion with antisera specific for IgG_{2a}.

[b] [Protein] in mg/ml determined by optical density measured at 280 nm.

Table IX-2. Antigen-Binding Activity of Monoclonal Antibodies Before and After Purification from Murine Ascites Fluids.

	Ascites		After Purification	
Hybridoma	IgG_{2a} (mg/ml)	Binding activity	IgG_{2a} (mg/ml)	Binding activity
PC 4.9A6	14	$10^{5.5}$	1.1	$10^{4.5}$
PC 4.9D3	17	$10^{5.7}$	0.34	$10^{4.5}$

D. Discussion

The immunoglobulin samples after ammonium sulfate precipitation appeared to be approximately 75% pure IgG based on examination by SDS-PAGE; the purity after the affinity-chromatography step appeared to be in excess of 95%. The yield of IgG after the ammonium sulfate precipitation was about 85% for MAb PC 4.9A6 and 60% for MAb PC 4.9D3. Recovery for the affinity-chromatography step was 15% to 40%. MAb PC 4.9D3 was purified after MAb PC 4.9A6 using the regenerated affinity column. Thus, the lower efficiency for recovery of MAb PC 4.9D3 may have been a result of the degradation of the protein A-Sepharose rather than an intrinsic difference between MAbs PC 4.9D3 and PC 4.9A6. The final products contained 110 mg of MAb PC 4.9A6 and 12 mg of MAb PC 4.9D3.

X. The Effect of Lyophilization on the Binding Activity of Anti-Pichinde Virus Monoclonal Antibodies PC 4.9A6 and PC 4.9D3

A. Introduction

Lyophilization usually does not grossly affect the activity of polyclonal antisera but some MAbs lose binding activity upon lyophilization. In this experiment the effect of lyophilization on the anti-Pichinde virus MAbs PC 4.9A6 and PC 4.9D3 was examined.

B. Materials and Methods

Infected Target Cells. Vero 76 cells growing in 24-well cell culture plates were inoculated with the An 4763 strain of PCV at a MOI of 0.01. The infection was allowed to proceed at 37°C for 20 h. The medium was then removed from the monolayers and the cells were allowed to dry thoroughly. The dry cells were fixed for 5 min with 80% acetone by adding 0.2 ml of water followed by 0.8 ml of cold acetone to each well.

Lyophilization. Ascites fluids and purified MAbs were aliquoted at 5 ml per 25-ml serum vial, frozen in a -80°C freezer, and then lyophilized on a model FDX-1-84-VP lyopohilizer (FTS Systems, Inc., Stone Ridge, NY). Lyophilization to dryness was accomplished in 6 to 12 h. After lyophilization, the vials were sealed and the samples were stored at -20°C.

Purification of Monoclonal Antibodies. The purification of the MAbs was as described in Section IX. Briefly, the immunoglobulins were isolated from ascites fluids by ammonium sulphate precipitation and then further purified by affinity chromatography on protein A-Sepharose. The purified MAb pools were dialyzed extensively against Dulbecco's PBS. They were each filter-sterilized by filtration through 0.22-μ pore-size membrane filters (Gelman Sciences, Inc, Ann Arbor, MI) to yield the sterile purified MAbs utilized in this study.

Titration of Monoclonal Antibodies. A half-log dilution series of each antibody sample in PBS was titered by limiting dilution using an indirect fluorescent antibody assay on acetone-fixed Pichinde virus-infected Vero 76 cells in 24-well cell culture plates. For the assay, 200 µl of diluted sample was placed into each well as the first antibody and incubated 2 h at 37°C. The first antibody was then removed and the cell sheets were rinsed twice with PBS. The second antibody, 100 µl of fluorescein-labeled anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 1:250 was added and allowed to react at 37°C for 1 h. The excess second antibody was removed, and the cell sheets were rinsed once with distilled water and examined for fluorescing cells. The endpoint was taken as the highest dilution of antibody sample that produced readily recognizable immunofluorescent staining of infected cells. Samples were run in triplicate.

C. Results

There was no significant increase or decrease in MAb activity associated with lyophilization (see Table X-1).

Table X-1. Effect of Lyophilization on Binding Activity of Monoclonal Antibodies.

Sample	Titer[a] Before lyophilization	Titer[a] After lyophilization
PC 4.9A6 purified on protein A	$10^{-4.5}$	$10^{-4.5}$
PC 4.9D3 purified on protein A	$10^{-4.5}$	$10^{-4.2}$
PC 4.9A6 ascites fluids	$10^{-5.5}$	$10^{-5.7}$
PC 4.9D3 ascites fluids	$10^{-5.7}$	$10^{-5.5}$
PC 4.7C2 ascites fluids	$10^{-5.5}$	$10^{-5.5}$

[a]Assayed by limiting dilution in an indirect fluorescent antibody assay on Pichinde virus-infected Vero 76 cells.

D. Discussion

MAbs PC 4.9A6 and PC 4.9D3, either as crude ascites preparations or as highly purified immunoglobulins, were not adversely affected by lyophilization. Because the MAbs are produced at Utah State University, whereas the conjugation of drugs to these MAbs are performed at SRI in California, the MAbs may be lyophilized and shipped in this form very conveniently. Lyophilization may also prove useful as a method for further concentrating the MAbs, which are diluted during the purification and conjugation procedures.

XI. Preparation of Fluorescein-Labeled Monoclonal Antibodies

A. Introduction

MAbs PC 4.9A6 and PC 4.9D3 specific for PCV antigens expressed on the surface of Pichinde virus-infected cells were conjugated with fluorescein isothiocyanate (FITC). These fluorescein-labeled MAbs may be used as reagents in PCV assays, for antiviral assays, as *in vivo* tracers for MAb distribution studies, and in competitive binding experiments to determine the epitope specificity of anti-Pichinde virus MAbs.

B. Materials and Methods

Preparation of Fluorescein-Labeled Monoclonal Antibodies. Immunoglobulins, predominantly MAbs, were isolated from ascites fluids by precipitation with ammonium sulphate and further purified by affinity chromatography on protein A-Sepharose using the Bio-Rad Affi-Gel Protein A MAPS II Kit as previously described (Section IX). Immunoglobulin samples were dialyzed against 0.1 M Na_2HPO_4, pH 9.0. Following dialysis, precipitates were removed by centrifugation for 15 min at 550 x g. Sufficient FITC (Sigma Chemical Co., St. Louis, MO) was added to give a protein to FITC ratio (w/w) 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 Na_2HPO_4, 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 the dropwise addition of 0.2 M Na_3PO_4 and allowing the reaction to proceed at 25°C for 2.5 h. The conjugation conditions (see Table XI-1), i.e., pH and FITC-to-immunoglobulin ratio, were selected to yield a fluorescein-to-protein ratio in the final conjugate of approximately 10 fluorescein molecules per molecule of IgG (Hebert et al.,1972). The unconjugated FITC was removed by dialysis against Dulbecco's PBS containing 0.1% sodium azide. The fluorescein-labeled MAb preparations were then further purified by gel exclusion chromotography on Sephadex G-75 (Pharmacia Fine

Chemicals, Piscataway, NJ). The resulting FITC anti-Pichinde reagents were aliquoted at 0.25 ml/vial and lyophilized.

Evaluation of FITC-MAb Conjugates by Direct Immunofluorescence. The three fluorescein-labeled MAb preparations were titered by limiting dilution in a direct immunofluorescent cell assay using Pichinde virus-infected Vero 76 cells on coverslips as the targets. The fluorescein-labeled MAbs were diluted in PBS. Staining was at 37°C for 90 min, after which the coverslip cell cultures were rinsed once in distilled water and then mounted in Elvanol. The quality and intensity of immunofluorescent staining was determined by epifluorescence microscopy. The specific staining intensity was scored on a scale of 0 to 4+: 0 (no visible immunofluorescent staining); 1+ (weak immunofluorescent staining, but definitely positive;); 2+ (easy to read, definitely positive, suitable for competitive binding assays); 3+ (very strong staining but not quite maximum); 4+ (extremely intense immunofluorescent staining).

C. Results

All three conjugations resulted in fluorescein-labeled antibody preparations with excellent immunostaining characteristics. The volumes and amounts of immunoglobulins and FITC used in the conjugations are shown in Table XI-1. The three fluorescein-labeled MAb preparations were titered in a direct immunofluorescent cell assay using Pichinde virus-infected Vero cells on coverslips

Table XI-1. Conjugation Conditions for Producing Fluorescein-Labeled Monoclonal Antibodies.

IgG Source	IgG conc.[a] (mg/ml)	Vol. (ml)	Total IgG (by 280 nm) (mg)	Total IgG (by FITC) (mg)
Ammonium sulphate-precipitated ascites PC 4.9A6	15	5.5	82	1.6
Ammonium sulphate-precipitated ascites PC 4.9D3	11	4.5	50	1.0
Protein A-affinity-purified PC 4.9A6	2.5	6.0	15	0.3

[a]IgG concentration determined assuming protein at 1 mg/ml gives $OD_{280} = 1.0$.

(Table XI-2). There was only the slightest nonspecific staining at dilutions of 1:100 or greater, whereas the specific staining was very clear and intense out to dilutions of at least 1:1,600.

D. Discussion

The immunofluorescent conjugates described in this section have proved to be very useful tools. Several of the applications for these conjugates are described in other sections of this annual report. The fluorescein-labeled MAbs were subsequently used in direct immunofluorescent cell count assays for PCV, where they elimated the need for a second antibody as is required with indirect immunostaining assays and reduced the assay time by about 40%. The fluorescein-labeled MAbs were also used to determine the in vivo distribution of MAbs PC 4.9A6 and PC 4.9D3 in infected and noninfected hamsters (see Section XIII). The fluorescein-labeled MAbs were used in place of radiolabeled antibodies in competitive binding assays to determine the epitope-binding specificity of several anti-Pichinde virus MAbs (see Section XII).

Table XI-2. Immunofluorescent Staining of Pichinde Virus-Infected Vero 76 Cells with Fluorescein-Labeled Monoclonal Antibodies.

FITC-MAb[a]	Dilution FITC-MAb	Specific Staining Intensity[b]
Ammonium sulphate-		
precipitated ascites PC 4.9A6	1:100	4+
precipitated ascites PC 4.9A6	1:400	4+
precipitated ascites PC 4.9A6	1:1,600	2+
precipitated ascites PC 4.9A6	1:6,400	1+
precipitated ascites PC 4.9A6	1:25,600	-
Ammonium sulphate-		
precipitated ascites PC 4.9D3	1:100	4+
precipitated ascites PC 4.9D3	1:400	4+
precipitated ascites PC 4.9D3	1:1,600	4+
precipitated ascites PC 4.9D3	1:6,400	2+
precipitated ascites PC 4.9D3	1:25,600	-
Protein A-affinity-		
purified PC 4.9A6	1:100	4+
purified PC 4.9A6	1:400	3+
purified PC 4.9A6	1:1,600	2+
purified PC 4.9A6	1:6,400	1+
purified PC 4.9A6	1:25,600	-

[a]The FITC-MAb conjugates are those listed in Table XI-1.

[b]The specific staining intensity was scored on the 0 to 4+ scale: 0 (no visible immunofluorescent staining); 1+ (difficult to read, but definitely positive); 2+ (easy to read, definitely positive, suitable for competitive binding assays); 3+ (very strong staining but not quite maximum); 4+ (extremely intense immunofluorescent staining). Note that none of these FITC-MAbs at 1:100 or greater dilutions gave significant nonspecific staining.

XII. Anti-Pichinde Virus Monoclonal Antibodies. Characterization: Binding to Pichinde Virus Antigens Expressed on the Surface of Living Cells, Isotype Analysis, Epitope Specificity, and Viral Protein Specificity

A. Introduction

This section describes the characterization of several MAbs derived against PCV. It was determined that MAbs PC 4.7C2, PC 4.8D2, PC 4.9D3, and PC 4.9A6 bind to antigens expressed on the surface of Pichinde virus-infected cells. These four MAbs were selected for further characterization. The epitope- and polypeptide-binding specificities of the four MAbs were examined using competitive binding assays and radioimmunoprecipitation (RIP). By performing competitive binding assays, it is possible to determine if two MAbs recognize the same or different epitopes. There may be several epitopes on a single polypeptide. For the purpose of targeted delivery with a MAb, some epitopes will be more suitable than others. Therefore, a knowledge of the epitope specificity of the candidate MAbs will aid in the assembly of a representative panel of MAbs for studies of antibody-mediated, targeted drug delivery. A knowledge of the polypeptide specificity of a MAb is also necessary for predicting the suitablity of that MAb as a drug-delivery vector. Some polypeptides contain epitopes that are displayed on the surface of infected cells, whereas others do not. The polypeptide-binding specificities of the four candidate

anti-Pichinde virus MAbs had been examined previously by Western-blot analysis, with no apparent reactivity with any of the PCV protein bands on the electrophoretic blots. However, fluorescein conjugates of these MAbs had repeatedly been shown to react with PCV epitopes expressed on the cytoplasmic membrane of Pichinde virus-infected cells. The most likely candidate viral proteins would be GP1 and GP2--glycoproteins that span the cytoplasmic membrane of Pichinde virus-infected cells. The three-dimensional structure of the hydrophilic and hydrophobic domains of membrane-spanning glycoproteins is very dependent on the nature of their immediate environment. Thus, the conformation of these glycoproteins following extraction from the virus envelope or cell membrane and SDS-gel electrophoresis may be quite different from the conformation recognized by the MAbs. Binding of MAbs to such glycoproteins may be conformationally dependent, and sometimes such MAbs are not detected by Western-blot analysis. We therefore examined the protein-binding specificity of the candidate MAbs by RIP using ^{35}S-methionine-labeled PCV proteins as targets. The cytosols were generated from the Pichinde virus-infected cells without the use of ionic detergents.

B. Materials and Methods

Monoclonal Antibodies. The derivation of the hybridoma cell lines that secrete anti-Pichinde virus MAbs was described in Section IV. Most of the work described in this section utilized MAbs PC

4.7C2, PC 4.8D2, PC 4.9D3, and PC 4.9A6, which are of the IgG_{2a} subclass and bind to PCV antigens expressed on the surface of Pichinde virus-infected cells. MAb WE 33.6, also of the IgG_{2a} subclass, was obtained from Dr. M. J. Buchmeir, Scripp's Clinic, La Jolla, CA. MAb WE 33.6 is directed against the GP2 glycoprotein of another arenavirus, lymphocytic choriomeningitis virus (LCMV).

Detection of Antibodies That React with Antigens Expressed on the Surface of Pichinde Virus-Infected Cells. Vero 76 cells were infected with PCV at an MOI of 0.01. Following an incubation period of 22 h to allow expression of PCV antigens on the surface of infected cells, the antibody samples were added to the medium that bathed the cells. After incubation for 1 h at 37°C, the medium was removed and cultures were rinsed twice with warm MEM, and then the cultures were dried and fixed with acetone. Cultures were then immunostained to detect murine antibody-binding to cells. Immunostaining was performed by adding 100 µl of fluorescein-labeled goat anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) to each well and incubating for 3 h. Cell sheets were rinsed twice with water and examined by epifluorescence microscopy for fluorescing cells.

Monoclonal Antibody Isotype Determinations. The isotypes of the MAbs were determined by enzyme-linked immunosorbent assay (ELISA) using anti-mouse sub-isotyping reagents (HyClone

Laboratories, Inc., Logan, UT), following the assay instructions of the supplier.

Cells. Confluent monolayer cultures of Vero 76 cells in 24-well cell culture plates were seeded 42 h prior to virus inoculation. Cells were grown in MEM with 5% FBS.

^{35}S-Labeled Pichinde Virus Proteins. Cell culture-grown PCV strain CoAN 4763 was used as the inoculum for producing ^{35}S-labeled PCV proteins. Vero 76 cells growing in 24-well cell culture plates were rinsed once with MEM and then inoculated at an MOI of 0.01 with PCV. Noninfected controls were processed in parallel. Following 1-h adsorption, the inocula were removed, and all wells received 2 ml of MEM. At 48 h p.i., the medium was removed, and the cells were rinsed twice with methionine-deficient MEM. Then 1 ml of methionine-deficient MEM containing ^{35}S-methionine (Trans^{35}S-label™ obtained from ICN Biomedicals, Inc. Irvine, CA) at 60 µCi/ml was added to each well. The cultures were incubated at 37°C for an additional 24 h to allow ^{35}S-labeling of proteins. At 72 h p.i. the supernatant fluids were removed and the monolayers were solubilized.

Solubilization Procedure. The cytosol solubilization buffer consisted of 0.05 M NaCl, 0.02 M Tris-HCl, 0.001 M EDTA, 2% Nonidet P-40 (NP-40), and 1 mM phenylmethyl sulfonyl fluoride (PMSF), pH 7.4. Solubilization buffer (0.5 ml/well) at 4°C was added to the cell monolayers in the 24-well plate and incubated at 4°C for 15

min. Wells were rinsed once with an additional 0.5 ml of solubilization buffer/well, and the rinse was added to the original lysates so that 1.0 ml of cytosol was derived from each well. The suspensions were vortexed for 15 sec. Nuclei and large debris were removed by centrifugation for 10 min at 15,000 x g. The solubilized cytosols and cell culture supernatant fluids were aliquoted at 1.0 ml/vial and held frozen at -80°C.

^{35}S Assays. The amount of radioactivity present in samples was determined by liquid scintillation counting using Beckman Ready Safe™ liquid scintillation cocktail. The extent of ^{35}S-labeled amino acid incorporation into protein was estimated by determining the percentage of ^{35}S activity that was precipitated by 10% trichloroacetic acid (TCA).

Immunoprecipitation. The immune complexes were formed by mixing 50 µl of ascites fluid or 200 µl of hybridoma supernatant fluid with 100 to 200 µl of ^{35}S-labeled cytosol containing approximately 10^6 cpm of TCA-precipitable material and incubating the mixture for 16 h at 4°C. To this mixture was added 200 µl of a 10% suspension of Staphylococcus aureus cells (Sigma Chemical Co., St. Louis, MO), which bind the Fc portion of immunoglobulins due to the presence of protein A on the surface of the bacteria cell membranes. This mixture was further incubated for 2 h on ice with occasional gentle swirling. The protein A-antibody-antigen complexes were pelleted by centrifugation. The pellets were washed

three times with TNE buffer (0.01 M Tris-HCl, 0.1 M NaCl, 0.001 M EDTA, pH 7.5) containing 0.5% NP-40. After the third wash, the complexes were resuspended in electrophoresis sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol) and boiled for 2 min. After boiling, the S. aureus cells present in the sample were removed by centrifugation and 50 to 60 µl of supernatant containing the MAb-reactive viral protein was loaded onto polyacrylamide gels for electrophoresis.

Polyacryamide Gel Electrophoresis (SDS-PAGE). All SDS-PAGE was performed under the following conditions using a Hoeffer Mighty Small II electrophoresis apparatus. The buffers used were according to Laemmli (1970). The resolving gels were 1.5-mm thick and were 10% total acrylamide (T) with 2.7% crosslinker (C), in 0.375 M Tris-HCl, pH 8.8, 0.1% SDS. The stacking gel was 4% T and 2.7% C. The tank buffer was 0.025 M Tris, pH 8.3, 0.192 M glycine, and 0.1% SDS. Electrophoresis sample buffer was 0.125 M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol. Gels were run at a constant current of 20 mA per gel for approximately 1.5 h. The gels were cooled by circulating cold tap water through the gel apparatus. Gels were then fixed in a solution of 30% methanol and 10% acetic acid for 15 min. The gels were stained for 1 h in a similar solution with 0.125% Coomassie Blue R-250 and then destained with fixing solution overnight. Molecular-weight markers (BioRad Chemical Division, Richmond, CA) were run with each gel and were used to determine the apparent molecular weights of the viral proteins.

Fluorography. Prior to drying, the gels were allowed to equilibrate in doubly distilled water and then impregnated with Fluoro-hance (Research Products International Corp., Mount Prospect, IL) for 30 min. After this treatment, the gels were dried onto a heavy filter-paper backing with a BioRad gel-drying apparatus for 1.75 h at 80°C. The dried gels were placed against Kodak X-OMat X-ray film and exposed for 3 h at -90°C. The films were developed according to the manufacturer's specifications.

Competitive Binding Assay. Anti-Pichinde virus MAbs PC 4.9A6 and PC 4.9D3 were labeled with FITC as described in Section XI. These FITC-labeled MAbs were used in competitive binding experiments to determine the epitope-binding specificity of the anti-Pichinde virus MAbs. The ability of the sample MAbs to block (competitive binding) the binding of the FITC-labeled MAbs to Pichinde virus-infected Vero cells in an immunofluorescent cell assay was measured. The fluorescein-labeled MAbs were used at a concentration that would yield strong staining; dilutions of the test MAb were mixed with the fluorescein-labeled MAb and then the mixture was added to an infected culture of Vero cells. Cell sheets were examined for evidence of competition between the two antibodies by comparing the intensity of immunostaining with test antibody added to the FITC-labeled MAb to the intensity of immunostaining when only sample diluent was added to the FITC-labeled MAb.

The nonlabeled MAbs being tested were diluted in Dulbecco's PBS and then 150 µl was added per well to Pichinde virus-infected Vero cells in a 24-well cell culture plate. These "competing" antibodies were incubated on the cell sheets for 30 min at 37°C prior to adding the FITC-labeled MAb. Sufficient FITC-labeled MAb was added to yield very intense immunofluorescent staining of infected cells in the absence of competing antibody. The antibody mixtures on the infected Vero 76 cells were incubated at 37°C for 2.5 h, after which the cell sheets were rinsed once in distilled water and then mounted with Elvanol. The relative intensity of the specific anti-PCV staining was then observed using an epifluorescence microscope. Absence of competition indicated that the antibodies were not directed toward the same epitope. Controls included: negative control (FITC-labeled MAb competing with diluent only to demonstrate fluorescent-staining intensity without competition), and positive control (nonlabeled form of the the FITC-labeled MAb to demonstrate competition).

Animals. Female hamsters, strain MHA, were obtained from Charles River Laboratories (Willmington, MA). The hamsters were 13 weeks old and weighed 150 g when immunized with PCV.

Hyperimmune Hamster Sera toward Pichinde Virus. A pool of liver homogenates from MHA hamsters that had been infected with PCV was the source of PCV antigens for immunization. The pool, a 10% suspension consisting of 1 part liver to 9 parts PBS, had been

homogenized and assayed for PCV titer by plaque assay in cell culture. The titer was 3×10^7 plaque-forming units/ml. Because the purpose was to produce antisera and not to cause fatal PCV infections in the hamsters, the PCV liver homogenate was heat-inactivated by placing the material in a 60°C water bath for 1 h. The liver homogenate containing PCV was mixed with an equal volume of Freund's complete adjuvant to generate a stable emulsion. The immunogen was administered by intramuscular injection, 0.25 ml in both hind flanks for a total volume of 0.5 ml per animal. Booster injections were administered at 21 and 32 days after the primary immunization. Serum was collected 10 weeks after the primary immunization.

Indirect Immunofluorescence Assay for Antibody toward Pichinde Virus. Antibody samples diluted in PBS were added to Pichinde virus-infected, acetone-fixed Vero 76 cell cultures in 24-well plates. The antibodies on the infected cell cultures were incubated for 2 h and then removed. The cell sheets were rinsed twice with PBS. The presence of anti-Pichinde virus antibody was then detected by adding 100 μl of fluorescein-labeled goat anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) and incubating for 3 h. Cell sheets were rinsed twice with water and examined for fluorescing cells, scoring on a -, +/-, 1+, 2+, 3+, 4+ scale for increasing relative immunofluorescence-staining intensity.

C. Results

Detection of Antibodies that React with Antigens Expressed on the Surface of Pichinde Virus-Infected Cells. To determine whether any of the anti-Pichinde virus MAbs react with antigens expressed on the surface of Pichinde virus-infected cells, the MAbs were added to the cell-culture medium bathing either infected or noninfected living cells. After a suitable reaction period, the free antibody was rinsed away and the cells were stained with fluorescein-labeled antibody toward mouse IgG. The results of the indirect immunofluorescence assays utilizing viable Vero cells and the MAbs generated toward PCV indicated that four of the MAbs recognized epitopes displayed on the surface of infected cells. MAbs PC 4.7C2, PC 4.8D2, PC 4.9D3, and PC 4.9A6 selectively bound to PCV antigens expressed on the surface of viable Vero 76 cells infected with PCV. Antibodies present in normal mouse serum were not selectively taken up by Pichinde virus-infected Vero cells; however, antibodies present in hyperimmune mouse serum did bind to the surface of infected Vero cells. None of the MAbs reacted with uninfected Vero cells. Neither the hyperimmune hamster serum nor MAb WE 33.6 was available for evaluation when these experiments were conducted.

Monoclonal-Antibody Isotype Determinations. Twenty-five anti-Pichinde virus MAbs resulting from the PC4 fusion experiment were examined by ELISA using isotype-specific reagents. The majority--22 of 25--were of the IgG_{2a} isotype, two were of the IgG_1 isotype, and

one was of the IgG_{2b} isotype. MAbs PC 4.7C2, PC 4.8D2, PC 4.9D3, and PC 4.9A6 reacted with PCV antigens expressed on the surface of infected cells and were of the IgG_{2a} isotype.

Anti-Pichinde Virus Activity in Hyperimmune Hamster Serum and Anti-LCMV Monoclonal Antibody WE 33.6 by Indirect IFA. The hyperimmune hamster serum exhibited a strong response (reciprocal titer of approximately 10^4) to Pichinde virus-infected Vero cells (Table XII-1). In the indirect immunofluorescence assays, FITC-labeled goat antimouse IgG was found to be suitable for detecting the hamster IgG. Anti-LCMV MAb WE 33.6, at dilutions of 10^{-3} and 10^{-4}, produced recognizable immunofluorescence staining of Pichinde virus-infected cells. However, there was such a high concentration of IgG in the WE 33.6 ascites that nonspecific staining overwhelmed any specific staining at all dilutions of 10^{-2} or less. This narrow window of staining may be interpreted to mean that MAb WE 33.6 has a relatively low specificity for PCV GP2. The endpoint dilution titer by IFA against Pichinde virus-infected target cells was 10^3 for MAb WE 33.6, compared with anti-Pichinde virus IFA titers of $10^{5.5}$ to $10^{6.5}$ for ascites fluids generated from hybridoma cell lines PC 4.9A6, PC 4.9D3, and PC 4.7C2.

Epitope Specifities. Competitive binding assays indicated that MAbs PC 4.9A6 and PC 4.7C2 recognized the same antigenic determinant, whereas MAb PC 4.9D3 recognized a distinct, nonoverlapping epitope (Table XII-2). Homologous antisera out to dilutions of

Table XII-1. Antibody Titers toward Pichinde Virus.[a]

Sample	Dilution	IFA Result
Hyperimmune hamster serum	neat	2+, extensive nonspecific staining
Hyperimmune hamster serum	10^{-1}	3+, extensive nonspecific staining
Hyperimmune hamster serum	10^{-2}	4+, minimal nonspecific staining
Hyperimmune hamster serum	10^{-3}	3+
Hyperimmune hamster serum	10^{-4}	2+
Hyperimmune hamster serum	10^{-5}	-
WE 33.6 (ascites fluids)	neat	No discernable specific fluorescing cells, extensive nonspecific staining
WE 33.6 (ascites fluids)	10^{-1}	No discernable specific fluorescing cells, extensive nonspecific staining
WE 33.6 (ascites fluids)	10^{-2}	No discernable specific fluorescing cells, extensive nonspecific staining
WE 33.6 (ascites fluids)	10^{-3}	2+
WE 33.6 (ascites fluids)	10^{-4}	1+
PC 4.9A6 (ascites fluids)	10^{-4}	4+
PC 4.9A6 (ascites fluids)	10^{-5}	4+

[a]The antibody titers toward PCV were measured by an indirect immunofluorescence assay utilizing fluorescein-labeled antibody toward mouse IgG as the second antibody and Pichinde virus-infected Vero cells as targets. Immunofluorescence was graded on a scale of 0 (normal cells with no fluorescence) to 4 (intense specific immunofluorescence in infected cells), and the endpoint titer was the reciprocal of the highest dilution yielding immunostaining of at least 2+ intensity. The resulting titers were: 10^4 for the hyperimmune hamster serum, 10^3 for the anti-LCMV MAb WE 33.6 (ascites fluids), and 10^5 for MAb PC 4.9A6, an anti-Pichinde virus MAb (ascites fluids).

Table XII-2. Data from Competetive Binding Assays for Determining Epitope Specificities.[a]

Labeled antibody	Competing antibody	Dilution	Staining Intensity
PC 4.9A6	Diluent (Neg. control)	-	4+
PC 4.9A6	Hyperimmune hamster serum	neat	-
PC 4.9A6	Hyperimmune hamster serum	1:10	1+
PC 4.9A6	Hyperimmune hamster serum	1:100	2+
PC 4.9A6	PC 4.9A6 ascites	1:100	-
PC 4.9A6	PC 4.9A6 ascites	1:1,000	-
PC 4.9A6	PC 4.9D3 ascites	1:10	4+
PC 4.9A6	PC 4.9D3 ascites	1:100	4+
PC 4.9A6	PC 4.7C2 ascites	1:10	-
PC 4.9A6	PC 4.7C2 ascites	1:100	+/-
PC 4.9A6	WE 33.6 ascites	1:10	2+
PC 4.9A6	WE 33.6 ascites	1:100	3+
PC 4.9A6	PC 4.9A6 supernate	neat	4+
PC 4.9A6	PC 4.7C2 supernate	neat	4+
PC 4.9A6	PC 4.8D2 supernate	neat	4+
PC 4.9A6	PC 4.9D3 supernate	neat	4+
PC 4.9D3	Diluent (Neg. control)	-	3+
PC 4.9D3	Hyperimmune hamster serum	neat	-
PC 4.9D3	Hyperimmune hamster serum	1:10	-
PC 4.9D3	Hyperimmune hamster serum	1:100	-
PC 4.9D3	PC 4.9D3 ascites	1:100	-
PC 4.9D3	PC 4.9D3 ascites	1:1,000	-
PC 4.9D3	PC 4.9A6 ascites	1:10	3+
PC 4.9D3	PC 4.9A6 ascites	1:100	3+
PC 4.9D3	PC 4.7C2 ascites	1:10	3+
PC 4.9D3	PC 4.7C2 ascites	1:100	3+
PC 4.9D3	WE 33.6 ascites	1:10	+/-
PC 4.9D3	WE 33.6 ascites	1:100	2+
PC 4.9D3	PC 4.9A6 supernate	neat	3+
PC 4.9D3	PC 4.7C2 supernate	neat	3+
PC 4.9D3	PC 4.8D2 supernate	neat	3+
PC 4.9D3	PC 4.9D3 supernate	neat	+/-

[a]The epitope-binding specificity of the anti-Pichinde virus MAbs was examined by competitive binding assays. The ability of the sample antibodies to block (competitive binding) the binding of FITC-labeled anti-Pichinde virus MAbs PC 4.9A6 and PC 4.9D3 to Pichinde virus-infected Vero cells in an immunofluorescence cell assay was measured. The fluorescein-labeled MAbs were used at a concentration that would yield strong staining. Dilutions of the test MAb were mixed with the fluorescein-labeled MAb and then the mixture was added to an infected culture of Vero cells. The presence or absence of competition between antibodies was determined by comparing the intensity of immunostaining with that obtained with corresponding controls. Cell sheets were examined for fluorescing cells, scoring on a -, +/-, 1+, 2+, 3+, 4+ scale for increasing relative immunofluorescent staining intensity.

1:1,000 completely blocked binding of MAb PC 4.9A6 or PC 4.9D3. Hyperimmune MHA hamster serum out to dilutions of 1:100 (the greatest dilution tested) completely blocked the binding of MAb PC 4.9D3 and at 1:10, but not at 1:100, blocked the binding of MAb PC 4.9A6. MAb PC 4.9D3 did not compete with MAb PC 4.9A6, nor did MAb PC 4.9A6 compete with MAb PC 4.9D3 in the reciprocal assay. PC 4.7C2 ascites did block the binding of MAb PC 4.9A6 out to dilutions of 1:100 (the greatest dilution tested) but did not compete with MAb PC 4.9D3. Anti-LCMV MAb WE 33.6 partially blocked the binding of both MAb PC 4.9A6 and MAb PC 4.9D3. Reagents were not available to test for competition with MAb PC 4.7C2 and MAb PC 4.8D2. In contrast to ascites fluids, cell-culture supernates failed to demonstrate competition for binding sites, even in the case of homologous antibodies. Therefore, nothing can be claimed about the epitope specificity of MAb PC 4.8D2, for which cell culture supernates but no ascites fluids were available.

^{35}S-Activity in Fractions from Metabolic Labeling Experiment. The ^{35}S-labeled cell-culture products were divided among the six fractions described in Table XII-3, each with a total volume of 11 to 12 ml. The PCV-infected and noninfected cytosols were used for the RIP experiments.

Polypeptide Specificities. All four anti-Pichinde virus MAbs precipitated a single ^{35}S-labeled protein that migrated between the heavy and light chains of the MAbs. The apparent molecular weight

Table XII-3. ^{35}S-Activity in Fractions from Metabolic Labeling Experiment.[a]

Fraction	Total cpm/20 µl	TCA[b]-precipitable ^{35}S (%)
PCV[c]-infected cytosols	6.36×10^5	18.3
Noninfected cytosols	6.52×10^5	5.2
PCV-infected cell-culture supernatants	12.32×10^5	3.1
PCV-infected cell-culture supernatants (solubilized)	5.66×10^5	1.6
Noninfected control cell-culture supernatants	8.77×10^5	2.9
Noninfected control cell-culture supernatants (solubilized)	1.54×10^5	1.0

[a] ^{35}S-methionine was used to metabolically label polypeptides synthesized in Vero cells, noninfected controls, and cultures infected with PCV. Cultures were rinsed twice with methionine-deficient MEM. Then 1 ml of methionine-deficient MEM containing ^{35}S-methionine (Trans^{35}S-label™ obtained from ICN Biomedicals, Inc., Irvine, CA) at 60 µCi/ml was added to each well in a 24-well plate. The cultures were incubated at 37°C an additional 24 h to allow ^{35}S-labeling of proteins, the supernatant fluids were removed, and the monolayers were solubilized. The amount of radioactivity present in samples was determined by liquid scintillation counting.

[b] The extent of 35S-labeled amino acid incorporation into protein was estimated by determining the percentage of the ^{35}S activity that was precipitated by 10% trichloroacetic acid (TCA).

[c] PCV, Pichinde virus.

of the polypeptide was 38,000 daltons. This 38K viral polypeptide band was not visible on gels stained with Coomassie blue, but was the most intense viral band on autoradiograms obtained from gels on which total cell lysates from infected cells were run. When mixtures of the immunoprecipitates obtained with all of the MAbs were run in a single lane, the ^{35}S-labeled proteins comigrated, indicating that all four MAbs bind to the same viral protein. When films were overexposed, a minor band, corresponding to a 60K polypeptide, appeared in the lanes containing the MAb immunoprecipitates.

When total cell lysates from infected and noninfected cells were electrophoresed in adjacent lanes, autoradiograms demonstrated two intense bands of definite viral origin corresponding to polypeptides with apparent molecular weights of 27,000 and 38,000 Daltons. There were also several faint bands between 58,000 and 69,000 Daltons. The intensity of the bands indicated that the 38,000-Dalton polypeptide precipitated by the MAbs was the most abundant of the ^{35}S-labeled viral polypeptides. The anti-LCMV MAb WE 33.6 did not precipitate detectable levels of ^{35}S-labeled viral polypeptides. When the four anti-Pichinde virus MAbs were allowed to react with ^{35}S-labeled lysates from noninfected cells, they did not precipitate ^{35}S-labeled polypeptides. When an isotype matched MAb specific for a Friend leukemia virus antigen was allowed to react with the ^{35}S-labeled lysates from Pichinde virus-infected cells, no ^{35}S-labeled proteins were precipitated. This latter

observation supports the absence of an interaction between the 38,000-Dalton PCV polypeptide and murine IgG_{2a} in general or other trivial interactions between the components of the RIP reaction mixture. Polyclonal mouse serum, hyperimmune toward PCV, precipitated ^{35}S-labeled 27K, 38K, and 58 to 69K polypeptides. The polyclonal hamster serum was not available for evaluation at the time these experiments were conducted.

D. Discussion and Conclusions

MAbs PC 4.7C2, PC 4.8D2, PC 4.9D3, and PC 4.9A6 are specific for PCV antigens expressed on the surface of viable Vero cells infected with PCV. This observation indicates that these MAbs may be suitable candidates as drug-delivery vectors for the antibody-mediated targeted delivery of antiviral drugs. All four of these MAbs were found to be of the IgG_{2a} isotype.

Anti-Pichinde virus MAbs PC 4.9A6 and PC 4.7C2 recognize the same epitope, whereas MAb PC 4.9D3 recognizes a second, nonoverlapping epitope. Competitive binding assays with anti-LCMV MAb WE 33.6, which cross-reacts with the GP2 glycoprotein of PCV, resulted in partial competition with MAbs PC 4.9A6 and PC 4.9D3. This was one of the indications that anti-Pichinde virus MAbs PC 4.9A6 and PC 4.9D3 bind to epitopes on the GP2 glycoprotein of PCV. The anti-Pichinde virus MAbs PC 4.7C2, PC 4.8D2, PC 4.9D3, and PC 4.9A6 precipitated a 38,000-dalton polypeptide that was the major viral

polypeptide in the cytosols from Pichinde virus-infected Vero cells. Of the three major polypeptides of PCV, the 38K polypeptide most likely corresponds to the GP2 glycoprotein.

Vezza et al. (1977) described three polypeptides obtained from intact PCV (strain An 3739)---GP1 and GP2, 64,000-dalton and 38,000-Dalton glycoproteins, respectively, which are found in approximately equal ratios in the viral envelope, and NP, a 66,000-dalton nonglycosylated internal protein that constitutes about 70% of the protein in purified PCV. Harnish et al. (1981) examined the viral proteins obtained from purified PCV (strain An 3739) and cytosols of Pichinde virus-infected BHK-21 cells. They found the virus to contain GP1 (52K), GP2 (36K), NP (64K), and a large L protein (200,000 daltons). However, the cytosols contained many additional polypeptides that exhibited a time- and MOI-dependent appearance and abundance. One of these, GPC (79K), was a precursor to GP1 and GP2. They also found high levels of 48K, 38K, and 28K polypeptides, which were fragments of NP. Buchmeier et al. (1977) found that the major soluble complement-fixing (CF) antigen obtained from cell cultures infected with PCV was a 20,000- to 30,000-dalton fragment of NP. Auperin et al. (1984) determined the nucleotide sequence of the gene coding for NP. According to their analysis, the NP polypeptide from PCV (strain An 3739) would have a calculated molecular weight of 62,911 daltons. Buchmeier et al. (1981) used RIP to determine the polypeptide specificities of MAbs toward LCMV and PCV. They noted that cytosol preparations contained all of the previously reported

LCMV polypeptides but that the relative proportion of NP was reduced in comparison with intact virions. They also found that a few MAbs precipitated both GP1 and GP2, but pointed out that this could have been the result of coprecipitation of a common macromolecular structure. When MAbs that precipitated either GP1 or GP2 were allowed to react with cytosols, they often precipitated a small amount of GPC, the precursor of GP1 and GP2. We observed a faint band corresponding to a 60K polypeptide on overexposed fluorographs of the immunoprecipitates obtained with the anti-Pichinde virus MAbs PC 4.7C2, PC 4.8D2, PC 4.9D3, and PC4.9A6. This observation is in line with anti-GP2 specificity.

It must be noted that the PCV characterized by us was of the strain CoAn 4763, whereas the PCV that has been characterized by others (Auperin et al., 1984; Buchmeier et al., 1977; Buchmeier et al., 1981; Harnish et al., 1981; Vezza et al., 1977) was of strain An 3739. However, it does not seem likely that there would be major polypeptide differences between these two strains of PCV. We used the CoAn 4763 strain because we found it to be more lethal in the in vivo MHA hamster model than the strain An 3739.

LCMV is the prototype of the arenavirus group and shares some antigenic relationship to PCV. Parekh and Buchmeier (1986), using a panel of MAbs against the GP1 and GP2 glycoproteins of LCMV, demonstrated the presence of four distinct epitopes on GP1 and three epitopes on GP2 of LCMV. They found that none of the MAbs directed

at GP2 were neutralizing but that MAbs directed against two of the four epitopes on GP1 did neutralize LCMV. The three epitopes on LCMV GP2 were overlapping in that one designated GP2A defined by several MAbs, including MAb WE 33.6, partially overlapped the other two epitopes GP2B and GP2C. However, MAbs against epitopes GP2B and GP2C did not compete with each other. MAb WE 33.6, generated against LCMV, cross-reacted with PCV (M. J. Buchmeier, personal communication, and the results presented in this report). In RIP experiments, we found that MAb WE 33.6 would not precipitate a discernable polypeptide from ^{35}S-methionine-labeled PCV cytosols. However, we found that MAb WE 33.6 did give positive staining of Pichinde virus-infected Vero cells by indirect immunofluorescence. In competitive binding assays, MAb WE 33.6, at a 1:10 dilution, definitely competed for the binding site recognized by MAb PC 4.9D3, but this competition was fairly weak because at a dilution of 1:100, MAb WE 33.6 failed to significantly inhibit the binding of MAb PC 4.9D3. Homologous MAb completely blocked binding of the FITC-labeled antibody at ascites dilutions of 1:10,000 (data not shown). MAb WE 33.6 inhibited the binding of MAb PC 4.9A6 only slightly, indicating at most only a partial overlap of the recognized antigenic sites. However, the results of these competitive binding assays with MAb WE 33.6 indicated that anti-Pichinde virus MAbs PC 4.9A6 and PC 4.9D3 probably do bind to epitopes on the GP2 glycoprotein of PCV.

We also examined the ability of hyperimmune hamster serum to compete for the antigenic sites recognized by anti-Pichinde virus MAbs PC 4.9A6 and PC 4.9D3. The hamster polyclonal serum at dilutions of 1:10 and 1:100 (highest dilution tested) completely inhibited the binding of MAb PC 4.9D3, but only partially inhibited the binding of MAb PC 4.9A6. Apparently the epitope recognized by MAb PC 4.9D3 is strongly immunogenic in hamsters, while that recognized by MAb PC 4.9A6 is less immunogenic.

The SDS-PAGE patterns obtained with the Pichnde virus-infected cytosols described in this report differed in some respects from some of those reported by Harnish et al. (1981). The cell-lysis and protein-solubilization procedures were patterned after those of Buchmeier and Oldstone (1979) and Smith and Pifat (1982). However, there were some notable differences. Smith and Pifat (1982) included 0.5% sodium deoxycholate, which is an anionic detergent, whereas Buchmeier and Oldstone (1979) did not. We did not include this anionic detergent either, but used the nonionic detergent NP-40. This may account for the low recovery of NP, which may have required the ionic detergent to be extracted from nucleoprotein complexes and virions. The solubilization buffer used by Buchmeier and Oldstone (1979) did not include EDTA but contained both $CaCl_2$ and $MgCl_2$. Both of these divalent cations would be chelated by the EDTA that was included in our solubilization buffer. The solubilization buffer used by Smith and Pifat included neither divalent cations nor EDTA. Finally, the pHs of the buffers

differed. We used 7.4, Smith and Pifat used 7.5, and Buchmeier and Oldstone used 9.0. How the differences in solubilization buffers would account for differences in the proportions of the different proteins recovered is not clear, but it seems likely that there would be solubilization buffer-dependent differences. We did not include anionic detergents such as SDS and sodium deoxycholate in the cytosol solubilization buffer because our aim was to determine which proteins were precipitated by the panel of MAbs and we had already determined that the MAbs would not recognize their corresponding epitopes following denaturation by SDS-PAGE buffer. The solubilization buffer used by Harnish et al. (1981) included both SDS and sodium deoxycholate, but their immunoprecipitations were performed with polyclonal sera, which probably would not be as sensitive to epitope conformational changes as MAbs would be.

The diversity and relative amounts of viral polypeptide obtained by RIP will vary, depending on the labeling conditions, as a result of temporal control of the synthesis and the incomplete translation and degradation of polypeptides. Buchmeier and Oldstone (1979) used a 2-h pulse of ^{35}S-methionine at 44 h p.i. and found two major polypeptides, NP (63K) and GPC (75K), in the cytosols from LCMV-infected BHK-21 cells. Using the same labeling procedure but including high-speed centrifugation to clarify the cytosol supernatant, Buchmeier et al. (1981) observed a substantial reduction in the proportion of NP in the labeled polypeptides and an increase in GP1 and GP2. The polypeptide distributions in the

cytosols from Pichinde virus-infected BHK-21 cells, reported by Harnish et al. (1981), were obtained with either 10-min or 1-h pulses of labeled amino acids. In contrast, we included ^{35}S-methionine for 24 h. Another factor leading to variation in RIP distribution patterns is traceable to differences in the hyperimmune sera used to precipitate total viral polypeptides. Harnish et al. (1981) used sera from either convalescent or hyperimmunized hamsters, whereas we used hyperimmune mouse serum from mice that had been immunized with suckling mouse brain homogenates. It is possible that the mouse and hamster sera had different distributions of antigen specificities. We now have a hyperimmune hamster serum pool that is of much higher titer than the mouse serum. In future immunoprecipitation studies, the pattern of polypeptides precipitated by the hamster serum will be compared to the pattern obtained using the mouse serum.

Arenaviruses appear to differentially regulate the expression of NP and the surface glycoproteins. This regulation probably plays a significant role in the ability of arenaviruses to establish persistent infections (Buchmeier and Parekh, 1987). Thus, the relative concentrations of NP, GP1, and GP2 in infected cells is variable, depending on MOI, time after infection, and the host cell type. In addition, several NP-related polypeptide fragments and the precursor glycoprotein GPC exhibit a highly variable intensity of expression in cytosols of infected cells (Buchmeier et al., 1977; Harnish et al., 1981). Therefore, the apparent differences in the

level of expression of the various PCV polypeptides reported from different laboratories or from different experiments in the same laboratory will be difficult to replicate unless the conditions of infection, labeling, and cytosol preparation are carefully reproduced.

In this section we described the binding specificities of four anti-Pichinde virus MAbs as revealed by applying radioimmunoprecipitation techniques using ^{35}S-labeled lysates from Pichinde virus-infected cells. All four precipitated a 38,000-Dalton polypeptide that was the major viral polypeptide in the cytosols. Because this protein was not precipitated by isotype-matched irrelevant MAbs or from noninfected Vero cell lysates by any of the anti-Pichinde virus MAbs, this 38K protein appears to be the PCV protein recognized by the four anti-Pichinde virus MAbs. That all four MAbs recognized the same protein was not too surprising, because each was selected for its ability to bind to PCV antigens expressed on the surface of Vero cells infected with PCV. However, competitive binding assays demonstrated that MAbs PC 4.9A6 and PC 4.7C2 recognize the same epitope and that MAb PC 4.9D3 recognizes a second, nonoverlapping epitope. Therefore, it appears that the four anti-Pichinde virus MAbs react with at least two different epitopes displayed on the portion of the 38K PCV protein exposed at the surface of infected cells. At this time it would appear that the most probable candidate is GP2; however, we are currently performing RIP studies utilizing [^{3}H]glucosamine to determine if the precipitated

polypeptide is GP2 or perhaps one of the NP fragments. Recently, we have obtained MAb WE 33.6 from Michael Buchmeier. MAb WE 33.6, generated toward LCMV and specific for GP2, also recognizes the GP2 of PCV (Parekh and Buchmeier, 1986). In RIP experiments utilizing ^{35}S-labeled lysates from Pichinde virus-infected cells, we were unable to detect any glycoproteins precipitated by MAb WE 33.6. In the future we will compare the polypeptides precipitated by our MAbs with those precipitated by MAb WE 33.6 in RIP experiments utilizing [^{3}H]-glucosamine-labeled lysates.

XIII. In Vivo Targeting with Anti-Pichinde Virus MAbs PC 4.9A6 and PC 4.9D3

A. Introduction

This section describes biodistribution of anti-Pichinde virus MAbs in infected and noninfected MHA hamsters. Fluorescein conjugates of MAbs PC 4.9A6 and PC 4.9D3, which bind to antigens expressed on the surface of Pichinde virus-infected cells, were injected into hamsters, and the distribution of the labeled antibodies was examined by fluorescence microscopy of thin-sections prepared from spleen, liver, lung, heart, kidney, and brain of the hamsters.

B. Methods

Animals. Female MHA hamsters (Charles River Breeding Laboratories, Inc., Willmington, MA), 30 days old, were divided into infected and control groups. Those to be infected were inoculated subcutaneously (s.c.) in the groin with 10^4 LD_{50} units of the An 4763 strain of PCV.

Monoclonal Antibody Conjugates. The MAbs were conjugated to FITC by the procedure of Hebert et al. (1972) as described in Section XI. The FITC-labeled MAbs had titers of approximately 1:6,000 by direct immunofluorescence assay on Pichinde virus-infected Vero cells.

Treatment. Eight days after virus challenge, 0.5-ml doses of the FITC-labeled MAbs were injected i.p. Three dosages were examined to avoid problems associated with too much or too little FITC-labeled MAb. The dosages were 250, 25, and 2.5 µg/animal with MAb PC 4.9A6 and 150, 15, and 1.5 µg/animal with MAb PC 4.9D3. The animals were killed by cervical dislocation 4 h after the antibody was administered. Tissues were immediately removed and frozen at -80°C in preparation for cryosectioning.

Virus Levels in Organs. The levels of PCV in 10% homogenates of the different organs were measured by in vitro virus assay on Vero cell monolayers using the immunofluorescent cell count procedure as described in Section II.

Examination. Spleen, liver, lung, heart, kidney, and brain sections were mounted on slides using Elvanol and a coverslip. Sections were not fixed. The tissue sections were examined by epifluorescence microscopy. Infected tissues were always examined in comparison with those from noninfected animals. Because the livers from the animals that received FITC-MAb PC 4.9A6 at either 250 or 2.5 µg were the most intensely fluorescing of the sections, these were taken as a relative fluorescence intensity of 100%, and all other sections were scored by comparison with these sections.

C. Results

At the time of antibody injection, the virus-inoculated hamsters showed obvious signs of infection, including bloody secretions from the eyes and the anal and genital openings. One infected hamster died on the day of treatment prior to injection with FITC-MAb conjugate. Because we had started this experiment with an extra infected hamster, there were enough animals to include three in each group. Care was taken to compare all sections with livers from the animals that received FITC-MAb PC 4.9A6 at either 250 or 2.5 µg, which were taken as the base-line readings and

defined as 100%. The fluorescence in these sections was very striking compared with that in noninfected controls.

The readings for the middle concentration with either conjugate did not correlate with those observed with the highest or lowest concentrations. It was as if there were hardly any conjugate put into these animals. For that reason only the data from the animals receiving the highest and lowest levels of labeled MAbs were used to produce the distribution graphs. The sections were observed and scored without the reader knowing which treatment groups were being read. The data are presented in Tables XIII-1 to XIII-6. The relative fluorescence intensities for sections from the different organs are shown in Figures XIII-1 to XIII-4. The levels of PCV in the different organs from infected hamsters was measured by in vitro virus assay using immunofluorescing-cell counts. The results are shown in Table XIII-7.

D. Discussion

There was a marked distribution of the labeled MAbs to the liver, kidney, spleen, and lung tissues of the infected animals compared with those of the noninfected animals. The difference was particularly dramatic for the livers. In infected and noninfected animals some of the fluorescence was diffuse and mainly in the epithelium covering the organs. The fact that this epithelial distribution was quite pronounced on brains from infected animals

Table XIII-1. Distribution of Fluorescein-Labeled Anti-Pichinde Virus MAbs in the Livers of Infected and Noninfected Hamsters.

Challenge	Treatment	Observations
PCV-infected	PC 4.9A6 at 250 µg	Many FCs[a] throughout liver; very intense fluorescence in epithelial layer. Intensity ~ 4+ or 100%.
PCV-infected	PC 4.9A6 at 25 µg	Few FCs; most in epithelial layer. Intensity ~ 25%.
PCV-infected	PC 4.9A6 at 2.5 µg	Many FCs throughout liver; very intense fluorescence in epithelial layer. Intensity ~ 4+ or 100%.
Noninfected	PC 4.9A6 at 250 µg	No obvious FCs; only fluorescence is at margin in epithelial layer. Intensity ~ 7%.
Noninfected	PC 4.9A6 at 25 µg	No FCs; no fluorescence at margin. Intensity ~ 0%.
Noninfected	PC 4.9A6 at 2.5 µg	No FCs; slight fluorescence at margin in epithelial layer. Intensity ~ 3%.
PCV-infected	PC 4.9D3 at 150 µg	Many FCs; intensity = 4+ around walls of vessels and 2+ in cells throughout liver. Intensity ~ 80%.
PCV-infected	PC 4.9D3 at 15 µg	No FCs; no fluorescence at margin in epithelileal layer. Intensity ~ 0%.
PCV-infected	PC 4.9D3 at 1.5 µg	FCs throughout the liver but less intense than with PC 4.9A6. Intensity ~ 40%.
Noninfected	PC 4.9D3 at 150 µg	No FCs but slight fluorescence at margin in epithelial layer. Intensity ~ 6%.
Noninfected	PC 4.9D3 at 15 µg	No FCs; no fluorescence at margin. Intensity ~ 0%.
Noninfected	PC 4.9D3 at 1.5 µg	No FCs; no fluorescence at margin. Intensity ~ 0%.

[a]FCs = fluorescent cells.

Table XIII-2. Distribution of Fluorescein-labeled Anti-Pichinde Virus MAbs in the Hearts of Infected and Noninfected Hamsters.

Challenge	Treatment	Observations
PCV-infected	PC 4.9A6 at 250 µg	FCs[a] in rare areas of layer covering the heart and a few in the linings of ventricles. Intensity ~ 15%.
PCV-infected	PC 4.9A6 at 25 µg	No FCs or fluorescence.
PCV-infected	PC 4.9A6 at 2.5 µg	A few FCs; no fluorescence. Intensity ~ 2%.
Noninfected	PC 4.9A6 at 250 µg	No FCs; slight diffuse fluorescence at margins. Intensity ~ 2%.
Noninfected	PC 4.9A6 at 25 µg	No FCs or fluorescence.
Noninfected	PC 4.9A6 at 2.5 µg	No FCs or fluorescence.
PCV-infected	PC 4.9D3 at 150 µg	A very few FCs; most of the fluorescence in cells covering heart. Intensity ~ 10%.
PCV-infected	PC 4.9D3 at 15 µg	No FCs or fluorescence.
PCV-infected	PC 4.9D3 at 1.5 µg	No FCs or fluorescence.
Noninfected	PC 4.9D3 at 150 µg	No FCs or fluorescence.
Noninfected	PC 4.9D3 at 15 µg	No FCs or fluorescence.
Noninfected	PC 4.9D3 at 1.5 µg	No FCs or fluorescence.

[a]FCs = fluorescent cells.

Table XIII-3. Distribution of Fluorescein-Labeled Anti-Pichinde Virus MAbs in the Lungs of Infected and Noninfected Hamsters.

Challenge	Treatment	Observations
PCV-infected	PC 4.9A6 at 250 µg	Most FCs[a] among epithelial cells covering the lung; quite patchy; some areas with no FCs, others with many FCs. Solitary FCs in interior tissues of lungs. Intensity ~ 30%.
PCV-infected	PC 4.9A6 at 25 µg	No FCs or fluorescence.
PCV-infected	PC 4.9A6 at 2.5 µg	Many FCs throughout interior of lungs. Intensity ~ 20%.
Noninfected	PC 4.9A6 at 250 µg	No FCs; very slight diffuse fluorescence (not FCs) in isolated areas along margins. Intensity ~ 3%.
Noninfected	PC 4.9A6 at 25 µg	No FCs or fluorescence.
Noninfected	PC 4.9A6 at 2.5 µg	No FCs or fluorescence.
PCV-infected	PC 4.9D3 at 150 µg	Many FCs throughout the interior of lung. Some patches of FCs in epithelial layer. Intensity ~ 30%.
PCV-infected	PC 4.9D3 at 15 µg	A few FCs in interior tissues. Intensity ~ 4%.
PCV-infected	PC 4.9D3 at 1.5 µg	No FCs or fluorescence.
Noninfected	PC 4.9D3 at 150 µg	No FCs or fluorescence.
Noninfected	PC 4.9D3 at 15 µg	No FCs or fluorescence.
Noninfected	PC 4.9D3 at 1.5 µg	No FCs or fluorescence.

[a]FCs = fluorescent cells.

Table XIII-4. Distribution of Fluorescein-Labeled Anti-Pichinde Virus MAbs in the Brains of Infected and Noninfected Hamsters.

Challenge	Treatment	Observations
PCV-infected	PC 4.9A6 at 250 µg	FCs[a] and diffuse fluorescence in epithelial cells covering the brain and some on the linings of blood vessels; no FCs in the interior of the brain. Intensity ~ 5%.
PCV-infected	PC 4.9A6 at 25 µg	No FCs or fluorescence.
PCV-infected	PC 4.9A6 at 2.5 µg	FCs in rare area of epithelial covering of brain; no FCs in interior tissues. Intensity ~ 2%.
Noninfected	PC 4.9A6 at 250 µg	No FCs or fluorescence.
Noninfected	PC 4.9A6 at 25 µg	No FCs or fluorescence.
Noninfected	PC 4.9A6 at 2.5 µg	No FCs or fluorescence.
PCV-infected	PC 4.9D3 at 150 µg	A few FCs in epithelium covering brain. Intensity ~ 5%.
PCV-infected	PC 4.9D3 at 15 µg	No FCs or fluorescence.
PCV-infected	PC 4.9D3 at 1.5 µg	A few FCs in epithelium covering brain. Intensity ~ 2%.
Noninfected	PC 4.9D3 at 150 µg	No FCs or fluorescence.
Noninfected	PC 4.9D3 at 15 µg	No FCs or fluorescence.
Noninfected	PC 4.9D3 at 1.5 µg	No FCs or fluorescence.

[a]FCs = fluorescent cells.

Table XIII-5. Distribution of Fluorescein-Labeled Anti-Pichinde Virus MAbs in the Spleens of Infected and Noninfected Hamsters.

Challenge	Treatment	Observations
PCV-infected	PC 4.9A6 at 250 µg	Intense fluorescence in epithelium covering the spleen. Intensity ~ 75% in that layer. Faint FCs[a] in interior tissue of spleen. Overall intensity ~ 40%.
PCV-infected	PC 4.9A6 at 25 µg	No FCs or fluorescence.
PCV-infected	PC 4.9A6 at 2.5 µg	Many FCs; easiest to see in the epithelial cells covering the spleen, but many faint FCs in interior tissue of spleen. Intensity ~ 5% inside and 40% in epithelial layer. Overall intensity ~ 25%.
Noninfected	PC 4.9A6 at 250 µg	Diffuse, relatively strong fluorescence over the exterior covering of spleen. Some intensely fluorescent debris-like clusters. Intensity ~ 30%.
Noninfected	PC 4.9A6 at 25 µg	About 10 solitary FCs, but not at margins. No bright infected areas. Intensity ~ 5%.
Noninfected	PC 4.9A6 at 2.5 µg	Some diffuse, weak fluorescence over the exterior covering of spleen. Intensity ~ 3%.
PCV-infected	PC 4.9D3 at 150 µg	Clearly defined FCs all along the epithelial covering of spleen. None in interior. Intensity ~ 20%.
PCV-infected	PC 4.9D3 at 15 µg	No FCs or fluorescence.
PCV-infected	PC 4.9D3 at 1.5 µg	A few areas of epithelial covering of spleen with bright FCs. Intensity ~ 10%.
Noninfected	PC 4.9D3 at 150 µg	Some isolated FCs and fluorescence at margins. Intensity ~ 6%.
Noninfected	PC 4.9D3 at 15 µg	No FCs or fluorescence.
Noninfected	PC 4.9D3 at 1.5 µg	No FCs, but many fluorescent blobs near spleen sections. No fluorescence in spleen.

[a]FCs = fluorescent cells.

Table XIII-6. Distribution of Fluorescein-Labeled Anti-Pichinde Virus MAbs in the Kidneys of Infected and Noninfected Hamsters.

Challenge	Treatment	Observations
PCV-infected	PC 4.9A6 at 250 µg	Intense FCs[a] in epithelial covering of the kidney; some weak diffuse fluorescence in interior. Intensity ~ 50%.
PCV-infected	PC 4.9A6 at 25 µg	No FCs or fluorescence.
PCV-infected	PC 4.9A6 at 2.5 µg	Several areas of FCs in interior tissue of kidney; some FCs in epithelial covering of the kidney, but more inside. Intensity ~ 40%.
Noninfected	PC 4.9A6 at 250 µg	Diffuse, relatively strong fluorescence over epithelium of kidney but no FCs. Intensity ~ 20%.
Noninfected	PC 4.9A6 at 25 µg	No FCs or fluorescence.
Noninfected	PC 4.9A6 at 2.5 µg	No FCs or fluorescence.
PCV-infected	PC 4.9D3 at 150 µg	Some FCs in interior tissue of kidney and in epithelial covering. Intensity ~ 25%.
PCV-infected	PC 4.9D3 at 15 µg	No FCs or fluorescence.
PCV-infected	PC 4.9D3 at 1.5 µg	Strong FCs in interior of kidney; epithelium may have been lost in sectioning. Intensity ~ 20%.
Noninfected	PC 4.9D3 at 150 µg	Weak fluorescence at epithelium; no FCs. Intensity ~ 2%.
Noninfected	PC 4.9D3 at 15 µg	No FCs or fluorescence.
Noninfected	PC 4.9D3 at 1.5 µg	Essentially no fluorescence, but a few isolated FCs. Intensity ~ 2%.

[a]FCs = fluorescent cells.

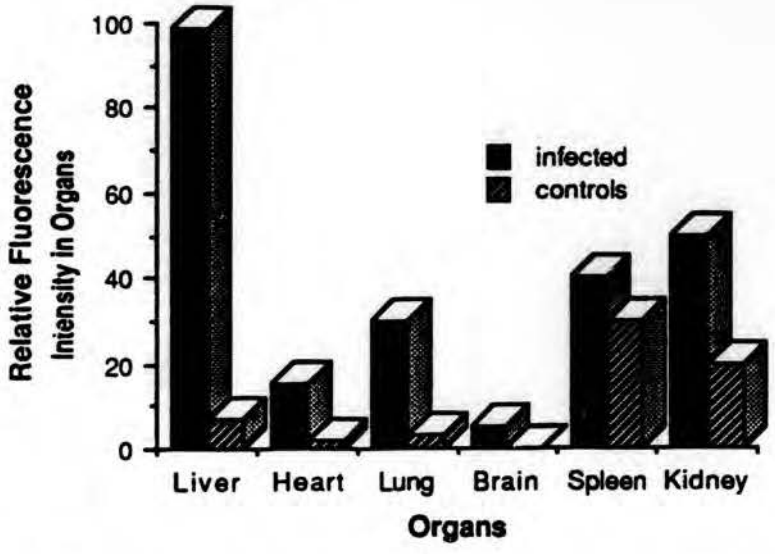


Figure XIII-1. In vivo Targeting with MAb PC 4.9A6 at 250 µg per Animal.

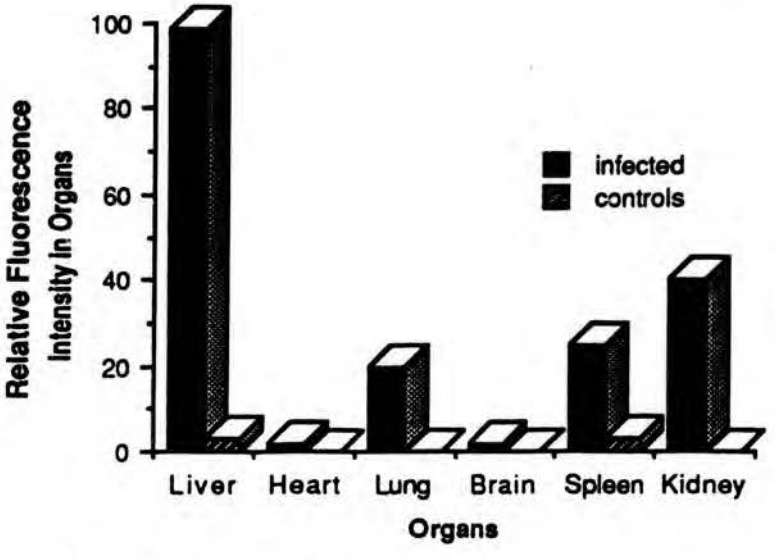


Figure XIII-2. In vivo Targeting with MAb PC 4.9A6 at 2.5 µg per Animal.

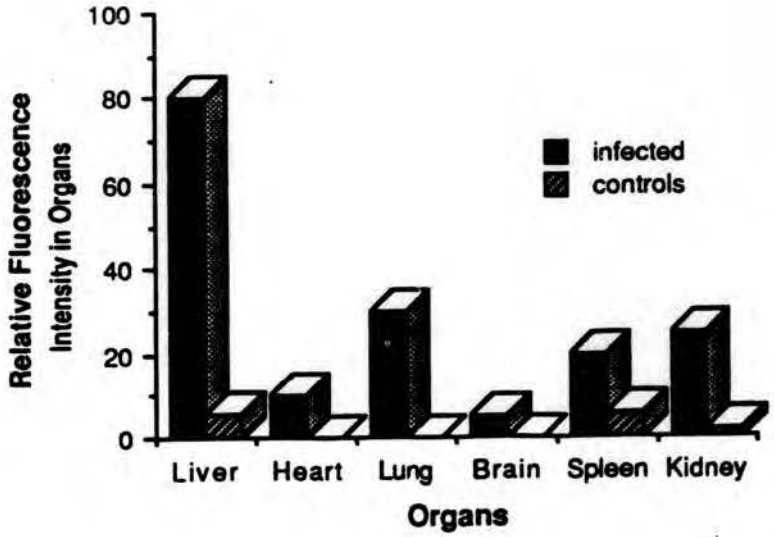


Figure XIII-3. In vivo Targeting with MAb PC 4.9D3 at 150 µg per Animal.

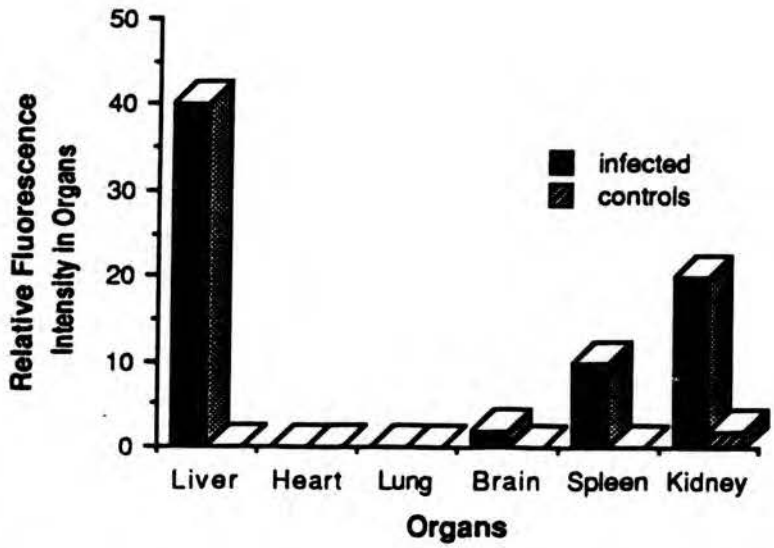


Figure XIII-4. In vivo Targeting with MAb PC 4.9D3 at 150 µg per Animal.

Table XIII-7. Pichinde Virus Distribution in Infected Hamsters at 8 Days Postinoculation.

Organ	Virus Titer (IFCFU/g[a])
liver	3.1×10^8
brain	4.4×10^5
spleen	7×10^4
kidney	3×10^4
lung	1×10^4
heart	1×10^4

[a]The levels of Pichinde virus in 10% organ suspensions were measured by an *in vitro* assay on Vero cells. The data are reported as immunofluorescent cell forming units (IFCFU) per gram of organ tissue.

was interesting because there was a significant virus titer in the brain and because the MAbs would not be expected to cross the blood-brain barrier. Some solitary isolated fluorescing cells were seen in the lungs and spleens in both infected and noninfected animals. These may have been macrophages that accumulated antibody as a result of being infected by PCV or by interacting with the Fc portions of the IgG_{2a} MAbs.

Those instances where the antibodies were found in relatively high concentrations in organs of noninfected animals were limited to animals that received the highest dosages of antibody. In those animals receiving 250 µg of MAb PC 4.9A6, the ratio of fluorescence intensity in the spleens and kidneys of the infected animals compared with the organs of noninfected animals was approximately 2:1, whereas the ratio in the livers was greater than 10:1. At the lower antibody dosages, the fluorescense intensity was always much greater (greater than 10:1) in organs from infected animals than in those from noninfected animals.

Stephen et al. (1980) studied the tissue tropism of PCV in MHA hamsters and reported virus titers of $10^{6.8}$ in both the plasma and spleen at 9 days p.i.; levels in other organs were not reported. Murphy et al. (1977) found that the major target organs for PCV infections in MHA hamsters were the liver, spleen, and kidney. They also observed viral antigen in brain sections from the infected animals. Jahrling et al. (1981) studied the tropism of PCV in

Strain 13 guinea pigs. They found infectious virus concentrations of 10^6 to 10^8 plaque-forming units (PFU)/ml in the liver, spleen, and lung; titers approaching 10^6 in kidney; and titers of 10^4 in brain. We, too, observed very high virus levels in the liver but found lower concentrations in the spleen, lung, and kidney. These lower virus levels (10^4-fold less) in the spleen and kidney correlate with the lower level of fluorescence in these organs after treatment with FITC-MAb conjugates. The virus levels that we observed in the brain were comparable with those reported by Jahrling et al. (1981).

E. Conclusions

The main target organ for PCV in the hamster model that we are using is the liver. The virus titer in the livers was a thousand-fold higher than titers observed in any other organs. The results of this in vivo targeting study indicate that MAbs PC 4.9A6 and PC 4.9D3 after i.p. administration to Pichinde virus-infected hamsters concentrate in the livers of the infected animals, reaching at least 10-fold greater levels in the livers of infected animals than in livers of noninfected animals. This is an indication that these MAbs have potential for evaluating the targeted delivery of antiviral drugs to virus target organs. The animals used in this study were killed by cervical dislocation. If a similar study is done again, it may be preferable to exsanguinate the animals and carefully rinse the organs before freezing them. These precautions

would reduce the amount of blood in the tissues and might reduce the background of labeled antibody.

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