Characterization of detergent-solubilized pseudorabies virus antigens

by

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A Thesis Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE

Department: Veterinary Microbiology and Preventive Medicine

Major: Veterinary Microbiology

Iowa State University
Ames, Iowa
1981

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EXPLANATION OF THESIS FORMAT

This thesis consists of an introduction (includes literature review and objectives), two separate manuscripts, a general conclusion, references and acknowledgements. The masters candidate, Melissa Anne Lum, is the senior author and principal investigator for each of the manuscripts.

LIST OF ABBREVIATIONS

Ag	Antigen
B	Bound fraction (from concanavalin A separation of crude antigen)
BL	Bovine lung
CA	Crude antigen extract
CAM	Chorioallantoic membrane
CBB	Coomassie brilliant blue R250 stain
CEF	Chick embryo fibroblast
CIE	Crossed immunoelectrophoresis
con A	Concanavalin A
DEAE	Diethylaminoethane dextran
DNA	Deoxyribonucleic acid
EHV-1	Equine herpes virus type-1
FBS	Fetal bovine serum
HSV-1	Herpes simplex virus type-1
IBRV	Infectious bovine rhinotracheitis virus
LD_{50}	Lethal dose - 50%
MDV	Marek's disease virus
MEM	Minimum essential medium
NP-40	Nonidet P-40
PB	Phosphate buffer
PFU	Plaque forming unit
PI	Post-inoculation
PRV	Pseudorabies virus

SDS-PAGE	Sodium dodecylsulphate - polyacrylamide gel electrophoresis
SN	Serum neutralization
TTT	Tris-Tricine Triton
UB	Unbound fraction (from concanavalin A separation of crude antigen)
VP	Virion protein

INTRODUCTION

Pseudorabies is an acute, infectious and often fatal disease occurring in many domestic animals of economic importance. The disease, caused by a herpesvirus, is prevalent in many countries throughout the world, including North, South and Central Americas, Africa, Europe, Russia and China. Literature reports deal primarily with the disease incidence in Europe and North America, where swine, cattle, sheep and goats, among others, may be affected (4). Primary economic losses occur in both swine and cattle industries, but the severest losses are met in swine breeding. The disease in swine causes a host of reproductive problems, including abortion, production of mummified fetuses, stillbirth and death in neonatal pigs.[1] Pseudorabies is generally milder in older pigs, but recent trends toward increased mortality in adult animals suggest that more virulent strains of virus are present in the hog population (17,37). Swine serve as the only known reservoir for the virus, and carrier animals contribute to disease spread to penmates as well as to other domestic animals (14). Pseudorabies in cattle is almost invariably fatal, causing intense pruritis and neurologic damage before death (14). The endemic nature of the disease in Europe[2] and its increased incidence and severity in the United States (17,37) has heightened concern for economic

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[1]Economic Analysis of Pseudorabies Program Options report made available by USDA-APHIS at the National Pseudorabies Hearing, St. Louis, May, 1981.

[2]Control of Aujeszky's Disease in Europe by Vaccination, presented by Norden Laboratories (Lincoln, Nebraska) at the National State Veterinary Meeting, Lincoln, May 1978.

losses and encouraged the development of vaccines to control losses.

Available Vaccines

Many pseudorabies vaccines are presently available for commercial use. In European countries, vaccines of the modified live type have been in use since the 1960s. The Bucharest virus strain, that was adapted to grow in the chorioallantoic membrane (CAM) of eggs, has been the source of several vaccines. Bran et al. (5) developed a vaccine by emulsifying Bucharest strain infected CAMs with aluminum hydroxide. This vaccine has been widely used in Rumania. In Czechoslovakia, Žuffa and Polak (51) developed the BUK vaccine by passing the Bucharest strain in chick embryo fibroblast (CEF) cell cultures, until it had lost virulence for calves and pigs. Škoda et al. (34) attenuated the Bucharest strain by extensive passage in CAMs and in CEF cell cultures, developing the TK200 vaccine. In Hungary, Bartha and Kojnok (3) developed the K strain vaccine, using a virus strain isolated from a clinical pseudorabies outbreak in pigs. The K strain, which was nonvirulent for pigs, was chosen for its atypical cytopathic effect on pig kidney monolayers at 32°C (2). Toneva (39) developed an attenuated vaccine by blind passage of pseudorabies virus in pigeon brains.

Only one modified live vaccine has been federally licensed in the United States. In 1977, Norden Laboratories (Lincoln, Nebraska) (37) developed this vaccine from the BUK vaccine strain, which they adapted to grow on a porcine kidney cell line.

Many inactivated pseudorabies vaccines were developed in Europe during the 1960s. The most effective of these vaccines for swine were inactivated

with formaldehyde or glutaraldehyde, and mixed with oil adjuvant (8,38).

The availability of pseudorabies inactivated vaccines in the United States has been more recent. In 1978, Salsbury Laboratories (Charles City, Iowa) (17) was the first to develop a killed vaccine that was prepared from virus propagated in primary porcine tissue culture. The virus was chemically inactivated and mixed with adjuvant. Norden Laboratories developed an adjuvant-emulsified, inactivated vaccine[1] that was federally licensed in 1980.

Pseudorabies vaccines in the United States have been only recently available for commercial use, allowing insufficient time for a fair evaluation of field performance. Most of the European vaccines, however, have been in use for many years, permitting some conclusions to be drawn concerning their safety, efficacy and role in disease eradication.

Modified live vaccines are generally preferred because small doses of virus will induce a higher level and longer lasting immunity than killed vaccines (12). Multiplication of the vaccine strain creates high levels of antigen without producing a typical disease.[2] The greatest problem with attenuated vaccines is one of safety. Incomplete attenuation of the vaccine strain has, in some cases, resulted in vaccine-induced temperature responses, clinical signs, abortions, disease in neonatal pigs and shedding of vaccine virus (4,37). Excretion of vaccine virus causes inadvertent immunization

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[1]Information from vaccine product insert, PR-Vac, Norden Laboratories, Lincoln Nebraska.

[2]Control of Aujeszky's Disease by Vaccination, presented by Norden Laboratories (Lincoln, Nebraska) at the National State Veterinary Meeting, Lincoln, May 1978.

of in-contact swine. This would permit possible reversion to virulence by repeated passage through hosts (4). While vaccine virus may be quite safe in pigs, it may be insufficiently attenuated to prevent infection and disease in other species (37). Live herpesvirus vaccines also are potential vehicles for transfection (16) and establishing oncogenic and persistent infections (12,48).

Safety is the primary reason for using inactivated vaccines. In general, however, they have not been very successful in the field. They are expensive and difficult to prepare with sufficient antigen and lack effectiveness in stimulating local immunity (4,37). Because oncogenic potential persists in killed herpesvirus preparations (30), vaccine-induced transformation of normal host cells is possible.

Both types of pseudorabies vaccines have reduced economic loss and clinical symptoms. However, they do not necessarily prevent superinfection with virulent virus, permitting its subsequent excretion and persistence on an infected farm (4,28). Hogs that have been vaccinated with attenuated or killed products are indistinguishable serologically from infected animals.[1] Presently available vaccines are thus not compatible with eradication programs.[2]

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[1] Economic Analysis of Pseudorabies Program Options, report made available by USDA-APHIS at the National Pseudorabies Hearing, St. Louis, May 1981.

[2] Control of Aujeszky's Disease in Europe by Vaccination, presented by Norden Laboratories (Lincoln, Nebraska) at the National State Veterinary Meeting, Lincoln, May 1978.

Subunit Vaccines

Subunit vaccines, containing only those viral structural components required for evoking a protective immune response, have been reviewed by Rubin and Tint (33) and Reed (31). Preparatory procedures may contribute to the safety of the vaccine by eliminating whole virus, viral nucleic acid, immunosuppressive fractions, pyrogens and allergenic components (31,33). The removal of nucleic acids precludes the establishment of latent infections and oncogenesis in the vaccinate (31,33). Large doses of immunogen from fully virulent virus also may be administered without adverse effects (31). The efficacy of subunit vaccines has been confirmed by recent work with infectious bovine rhinotracheitis virus (21) and pseudorabies virus (32) infected cell extracts. Because the vaccine contains only a portion of the virus, serologic tests easily may be designed to distinguish vaccinates from infected animals, thus making their use compatible with eradication programs (31).

Viral subunit components may require the addition of adjuvant for immunogenicity. If they are of low molecular weight, aggregation or carrier coupling may also be necessary (31). Present subunit extraction procedures are expensive, making the vaccines costly to produce (31). Mass production of these vaccines may become more economical when additional progress is made in the areas of synthetic vaccine technology, involving chemical synthesis of immunologically active viral antigenic determinants (1), and recombinant DNA (deoxyribonucleic acid) technology, involving the development of microbial factories to produce the viral immunogen in sufficient quantities (9).

Herpes Split Vaccines

A variety of viral subunit vaccines has been used experimentally with some success. Herpes subunit vaccines include those for herpes simplex virus type 1 (HSV-1), (6,29,35), pseudorabies virus (PRV) (32), infectious bovine rhinotracheitis virus (IBRV) (21), equine herpesvirus type 1 (EHV-1) (26) and Marek's disease virus (MDV) (18,49). Most of these herpesvirus vaccines are not true subunit vaccines, but are crude extracts of virus-specific host cell proteins rather than purified subunits. These, therefore, will be referred to as "split" vaccines.

Cappel et al. (6) solubilized HSV-1 envelope proteins with the nonionic detergent, Nonidet P-40 (NP40). The solubilized material was mixed with adjuvant and used to immunize rabbits. Twice-vaccinated rabbits developed both humoral and cell-mediated immunity and demonstrated an 80% protection rate against lethal challenge. Rajčani et al. (29) prepared a vaccine by NP-40 solubilization of HSV-1 infected cells. The solubilized extract was mixed with Freund's incomplete adjuvant and was administered in 3 doses to rabbits. Immunized rabbits were challenged by HSV-1 infection of scarified corneas and were later sacrificed to examine trigeminal ganglia. Vaccinates were found to have a reduced number of virus-carrier ganglion cells when compared to controls. Šlichtová et al. (35) also used NP-40 to solubilize HSV-1 infected cells. Mice were vaccinated subcutaneously with a single dose of this adjuvanted vaccine. After intradermal challenge, vaccinates and controls were observed for skin lesion development and neurological symptoms. Vaccinates were fully protected from viral encephalitis and the severity of their lesions were limited. Immunization also appeared to result in the accelerated clearance

of virus.

Rock and Reed (32) prepared an experimental split vaccine from nonionic detergent extracts of PRV infected cells. The solubilized antigens, free of infectious virus, were mixed with Freund's incomplete adjuvant and used to vaccinate mice. Vaccinates produced both a cellular immune response and neutralizing antibody. Twice-vaccinated mice were protected from challenge and demonstrated neutralizing antibody titers of approximately 1:4,000.

An experimental, detergent-split IBRV vaccine was prepared with NP-40 (21). The adjuvanted vaccine, administered to calves in two doses, produced high serum neutralizing titers and prevented clinical disease. In addition, calves were thought not to be infected by challenge virus and did not shed virus after challenge.

An EHV-1 vaccine was prepared by treating purified virus preparations with the detergent Rexol 25J and mixing the envelope extracts with Freund's adjuvant (26). Hamsters receiving two doses of the vaccine were protected against challenge.

Two adjuvant-emulsified, detergent split vaccines against MDV were investigated. Separate vaccines were prepared from MDV (18) and turkey herpesvirus infected cells (49). The MDV vaccine protected the majority of vaccinated chickens from disease. Turkey herpesvirus detergent extracts were passed through a concanavalin A (con A) Sepharose column to separate out glycoproteins. The eluted glycoprotein fraction was purified further by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and found to contain 3 viral glycopeptides. These glycopeptides were cut

from the gel, pooled, mixed with Freund's incomplete adjuvant, and administered to chickens. Vaccinates formed neutralizing antibodies but only partial protection resulted from challenge with virulent Marek's disease virus. Incomplete protection may have been due to SDS denaturation of immunizing proteins (49).

Protective Antigens

Herpesviruses basically are composed of a nucleocapsid and an envelope. The immunogenic properties of nucleocapsid and envelope antigens have been investigated and compared (13,22,26,27). The envelope proteins, specifically the glycoproteins, are the major targets of neutralizing antibodies and therefore play an important protective role in the infected host (6,13,19,22,26,27,50). Rabbits inoculated with HSV-1 envelope preparations developed high titers of neutralizing antibodies (19) and a cellular immune response (6). Immunization with HSV-1 envelope preparations induced higher neutralizing antibody titers in humans and rabbits than did nucleocapsid (13,22), and protected rabbits from lethal challenge (50). Similar differences in titers were found with equine herpesvirus type-1 (EHV-1) envelope and nucleocapsid preparations in hamsters (27); lethal challenge of hamsters vaccinated with nucleocapsid or envelope antigens resulted in protection of envelope vaccinates only (26).

Identification of Glycosylated Antigens

Studies by Kaplan (15) and Vestergaard (44) revealed that only a small fraction (total numbers and quantities) of herpesvirus envelope antigens becomes virion incorporated, while the majority remains associated with

the host cell membrane. Infected host cells have served as an excellent source of viral antigens for analytical work with SDS-PAGE and crossed immunoelectrophoresis (CIE) (25). Because herpes simplex antigens have been analyzed extensively with little information being available on other herpesviruses, discussion will be restricted to HSV-1.

Sodium dodecylsulphate-polyacrylamide gel electrophoretic analysis of ^{14}C-glucosamine or ^{14}C-amino acids labelled HSV-1 specific infected cell membrane antigens revealed 12 polypeptides, 9 of which were glycosylated. The membrane glycoproteins 7, 8 and 18 were found to be electrophoretically similar to the HSV-1 virion proteins (VPs) 7, 8 and 18, respectively (10).

The SDS-PAGE analysis denatures proteins and causes an alteration of their biological conformation and immunological activity (7). Nonionic detergents, such as Triton X-100, extract whole membrane proteins from infected cells by binding to the hydrophobic domain of intrinsic membrane proteins. Detergent replacement of the lipid environment results in the solubilization of protein in its native conformation (11).

Crossed immunoelectrophoresis of Triton X-100 solubilized infected cell extracts, followed by protein staining, consistently separated three distinct HSV-1 antigens - Ags 6, 8 and 11 (20,24,40,42,43,46,47). In separate experiments, crossed immunoaffinoelectrophoresis (con A Sepharose incorporated into an intermediate gel) and ^{14}C-glucosamine labelling of infected cell antigens identified all three antigens as glycoproteins (23,41,43). Antisera, individually prepared against Ags 6, 8 and 11, neutralized HSV-1 virus particles, indicating that these same or antigenically similar antigens were present on the virion envelope (44). Crossed immunoelectrophoresis of ^{14}C-protein hydrolysate

labelled infected cell extracts, followed by autoradiography, revealed four additional antigens, Ags 3, 3A, 6A and 7 (20,23,24,43,45,47), the glycosylation statuses of which have not been confirmed.

Biological Function of Glycosylated Antigens

The major HSV-1 glycoprotein antigens and their functional roles have been reviewed by Norrild (25) and Spear et al. (36). Glycoproteins gA, gB, gC, gD and gE (designated by the Workshop on Herpesviruses, Cambridge, England, 1978) are present in both the virion and plasma membrane of virus infected cells. The functions of each are as follows: gA--unknown, gB--required for infectivity, gC--suppression of polykaryocyte formation, gD--unknown and gE--Fc receptor. Crossed immunoelectrophoresis separated HSV-1 Ags 6, 8 and 11 correspond serologically to gC, gD and gA + gB, respectively. The SDS-PAGE separated virion proteins, VPs 7, 8, 8.5 and 18 correspond to gB, gC, gA and gD, respectively.

Objectives

The objectives in this study were to characterize pseudorabies virus specific antigens present in infected cell membrane extracts. Antigens were enumerated and characterized by glycosylation and protective activity.

PART I. CROSSED IMMUNOELECTROPHORETIC CHARACTERIZATION OF PSEUDORABIES (AUJESZKY'S DISEASE) VIRUS ANTIGENS

This manuscript has been submitted for publication to the Archives of Virology.

CROSSED IMMUNOELECTROPHORETIC CHARACTERIZATION OF PSEUDORABIES (AUJESZKY'S DISEASE) VIRUS ANTIGENS

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SUMMARY

Detergent solubilized antigens from pseudorabies virus (PRV) infected cells were characterized by crossed immunoelectrophoresis. Patterns revealed 16 virus-specific antigens, 13 of which were glycosylated.

INTRODUCTION

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is used frequently in the analysis of herpesvirus infected cell antigens (2,3,5,7,-8,13). The ionic detergent, SDS, denatures proteins causing a marked reduction in immunological function (22,27). Nonionic detergents, such as Triton X-100, are used to solubilize membrane bound proteins without altering their immunological activity (4,28). Herpesvirus-specific membrane antigens have been solubilized by Triton X-100 (7,10,11,13,14,18,19,20,21,22,23,24,25,26) and analyzed by crossed immunoelectrophoresis (CIE) (10,19,20,21,22,23,24,25,26). The purpose of the present study was to characterize Triton X-100 solubilized pseudorabies (Aujeszky's disease) virus antigens by CIE.

MATERIALS AND METHODS

Cell Culture

Bovine lung (BL) cells were grown in monolayers in Eagle's minimum essential medium with Earle's salts (MEM) supplemented with 10% fetal bovine serum, 0.1% lactalbumin hydrolysate, 0.16% sodium bicarbonate, 8.3 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and antibiotics (100 I.U. penicillin, 100 ug streptomycin sulfate, 100 ug kanamycin sulfate and 50 ug gentamicin sulfate per ml). Cells were grown in closed systems, on a roller apparatus, at 37°C.

Virus

Plaque-purified PRV strain S62/26 (17) was obtained from D. L. Rock (Veterinary Medical Research Institute, Iowa State University, Ames, Iowa). The virus was passed once in BL cells and a stock pool, containing 7.7×10^8 plaque forming units (PFU) per ml, was frozen at -70°C.

Reference Antibody

Goat anti-PRV serum was provided by W. A. Hagemoser (Department of Veterinary Pathology, Iowa State University, Ames, Iowa). The goat was initially vaccinated intramuscularly with a modified live PRV vaccine (Norden Laboratories, Lincoln, Nebraska), followed by 4 monthly booster inoculations with crude PRV antigen extracts (see Antigen Preparation section). Antibodies were prepared by precipitation with 1.85 M ammonium sulfate, and were dialyzed for 3 days (6 buffer changes) against 0.01 M Phosphate Buffer (PB)

(pH 7.5). The antibodies were mixed with equal volumes of vacuum filtered DEAE Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, New Jersey) (preswollen in the same buffer), and allowed to adsorb for 2 hours with periodic stirring. Immunoglobulins were removed by vacuum filtration. The gel was resuspended in an equal volume of 0.05M PB (pH 7.2), stirred, and vacuum filtered. Antibody containing filtrates were pooled, concentrated to half the original serum volume with polyethylene glycol 6,000 (Fisher Scientific Company, Fairlawn, New Jersey), and dialyzed for 3 days (6 buffer changes) against 0.025 M Tris-Tricine buffer (Biorad Laboratories, Richmond, California) containing 1% (v/v) Triton X-100 (Sigma Chemical Company, St. Louis, Missouri) (TTT buffer, pH 8.6).

Antigen Preparation

Bovine lung cells were grown in 850 cm^2 plastic roller bottles. Monolayers were infected at a multiplicity of 5 PFU/cell and virus inoculum was adsorbed at 37°C for 90 minutes in MEM containing 5% fetal bovine serum. Residual inoculum was then removed, replaced with serum-free MEM, and infected cells were incubated at 37°C for 24 hours. Infected cells at 100% cytopathic effect, were scraped into the medium and centrifuged in an angle head at 100,000 X g at 4°C for 1 hour. The pellet was resuspended in TTT buffer (pH 8.6) at a volume of 2 mls per roller bottle. The suspension was sonicated (Braunsonic 1510) at 100 watts for three 20-second bursts, stirred for 1 hour at 4°C and centrifuged in an angle head at 100,000 X g for 1 hour at 4°C. The supernatant fluid (approximate protein content of 5 mg/ml) was collected, designated crude antigen extract (CA) and stored at 4°C. Crude antigen

control was prepared from uninfected BL cells in an identical manner.

Radiolabelled antigens were similarly prepared with ^{3}H-glucosamine (11 Ci/mMol) or ^{3}H-leucine (1 Ci/mMol), which were used at a concentration of 10 µCi/ml. Isotopes were added to infected cell cultures 3 hours after virus adsorption, and the labelling period lasted 21 hours. Uninfected cells were radiolabelled and served as controls. All labelled preparations were concentrated to one third their original volume with polyethylene glycol 20,000 (Fisher). Isotopes were purchased from Research Products International Corporation, Mount Prospect, Illinois.

Crossed Immunoelectrophoresis

The procedures for crossed immunoelectrophoresis (CIE) were essentially the same as those used by Vestergaard (19). Electrophoresis was performed on 84 x 94 mm plastic plates (LKB, Rockville, Maryland) in 1.4 mm thick, 1% (w/v) agarose gel (Biorad). The agarose was dissolved in TTT buffer (pH 8.6) containing 1% polyethylene glycol 6,000, and TTT buffer (pH 8.6) was present in electrophoresis chambers. First dimension electrophoresis was performed on 15 µl of CA by applying 10 volts per cm for 90 minutes at 10°C. In the second dimension, the separated antigens were electrophoresed against agarose containing 15 or 20% reference antibody by applying 1.5 volts per cm for 18 to 24 hours at 10°C. Co-electrophoresis of two extract preparations was performed in the same manner. Coomassie brilliant blue R250 stain (CBB) (Biorad) was used to visualize precipitates. Autoradiography was performed by placing Ultrofilm ^{3}H (LKB) on the gel side of dried, stained CIE plates. The film was developed after 30 days exposure.

RESULTS

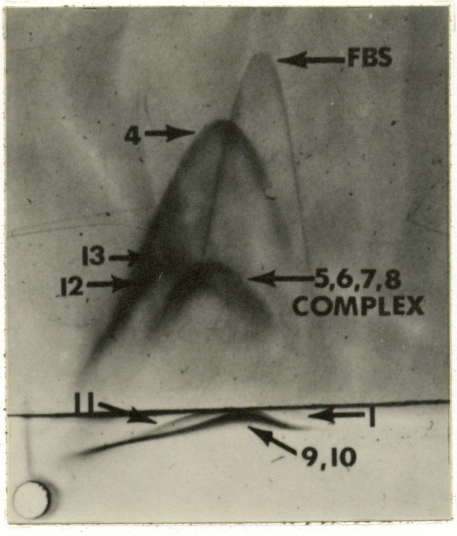
The CBB pattern for unlabelled CA precipitates is given in Figure 1. The CBB and autoradiogram results for ^{3}H-leucine CA alone and co-electrophoresed with unlabelled CA, are shown in Figure 2. Figure 3 gives the CBB and autoradiogram patterns for ^{3}H-glucosamine CA alone and co-electrophoresed with unlabelled CA. A composite illustration of all precipitates detected by CIE is given in Figure 4. The patterns revealed 16 virus-specific antigens, 13 of which were glycosylated. With some exceptions, antigens were numbered by their relative anodic migration in the first dimension. Antigens in different patterns were identified by a combination of peak shape, height, intensity of staining and relative anodic migration in the first dimension.

Antigens (Ags) 5,6,7 and 8 were better separated in the co-electrophoresis patterns than in conventional CIE patterns. Where it was difficult to identify these antigens individually, the collection of peaks in that area was designated the 5,6,7,8 complex.

One host-specific precipitate was detected in autoradiograms of labelled cell controls (not shown), but it did not appear to correspond to any of the virus-specific antigens. The FBS peak was absent in autoradiograms, and co-electrophoresis of unlabelled CA with fetal calf serum increased the height of the FBS peak only (not shown).

Figure 1. Coomassie brilliant blue stain of unlabelled CA (crude antigen) CIE pattern.

Figure 2. ^{3}H-leucine CA (crude antigen) CIE patterns. (A) Coomassie brilliant blue stain of ^{3}H-leucine CA CIE pattern, using 20% reference antibody in the second dimension gel, (B) Autoradiography of same plate, (C) Coomassie brilliant blue stain of ^{3}H-leucine CA (bottom well) co-electrophoresed with unlabelled CA (top well), using 15% reference antibody in the second dimension gel, (D) Autoradiography of same plate.


FBS
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13
12
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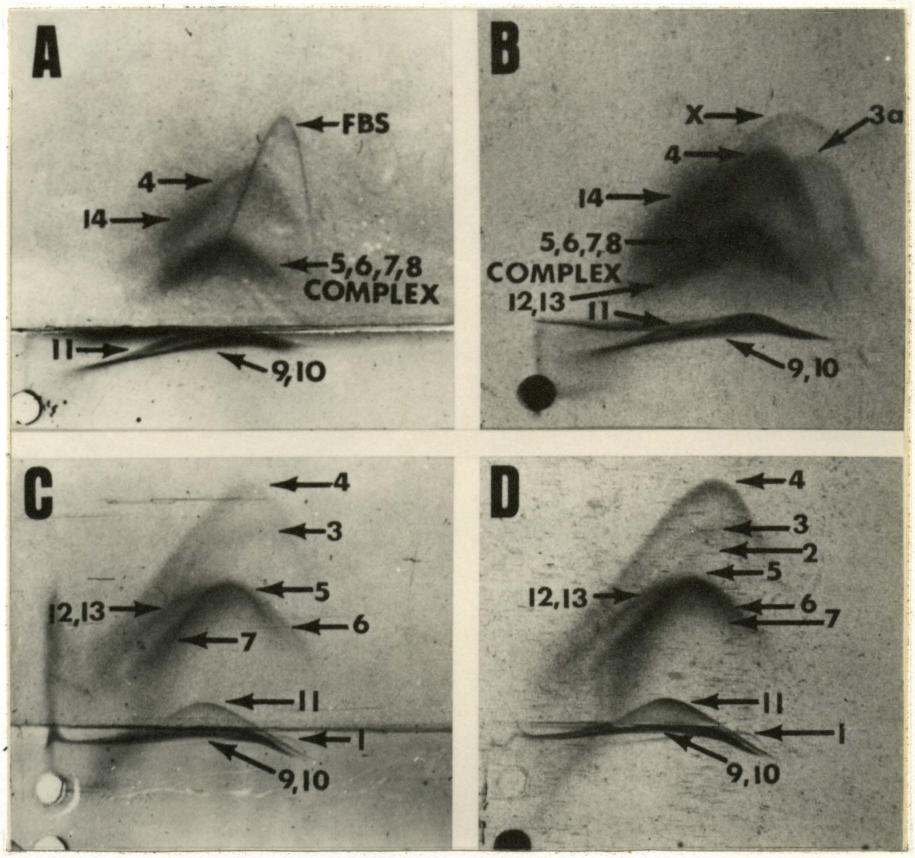
11
1
9,10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9,10
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11
1
9,10

Figure 3. ^{3}H-glucosamine CA (crude antigen) CIE patterns. (A) Coomassie brilliant blue stain of ^{3}H-glucosamine CA CIE pattern, using 20% reference antibody in the second dimension gel, (B) Autoradiography of same plate, (C) Coomassie brilliant blue stain of ^{3}H-glucosamine CA (bottom well) co-electrophoresed with unlabelled CA (top well), using 15% reference antibody in the second dimension gel, (D) Autoradiography of same plate.

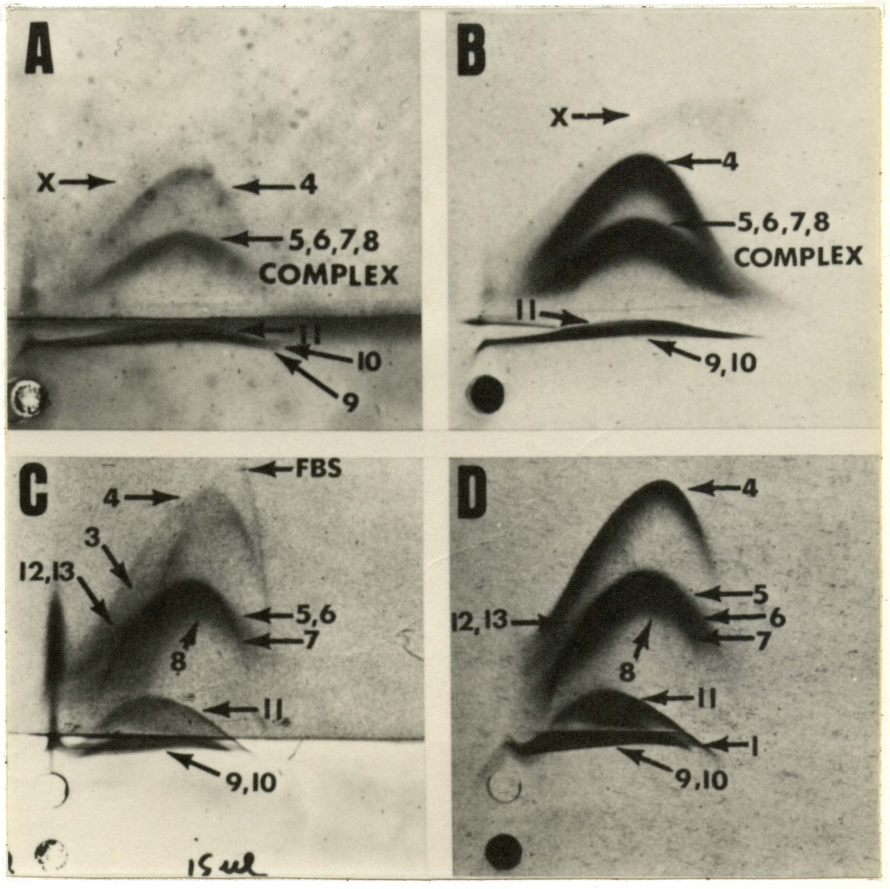
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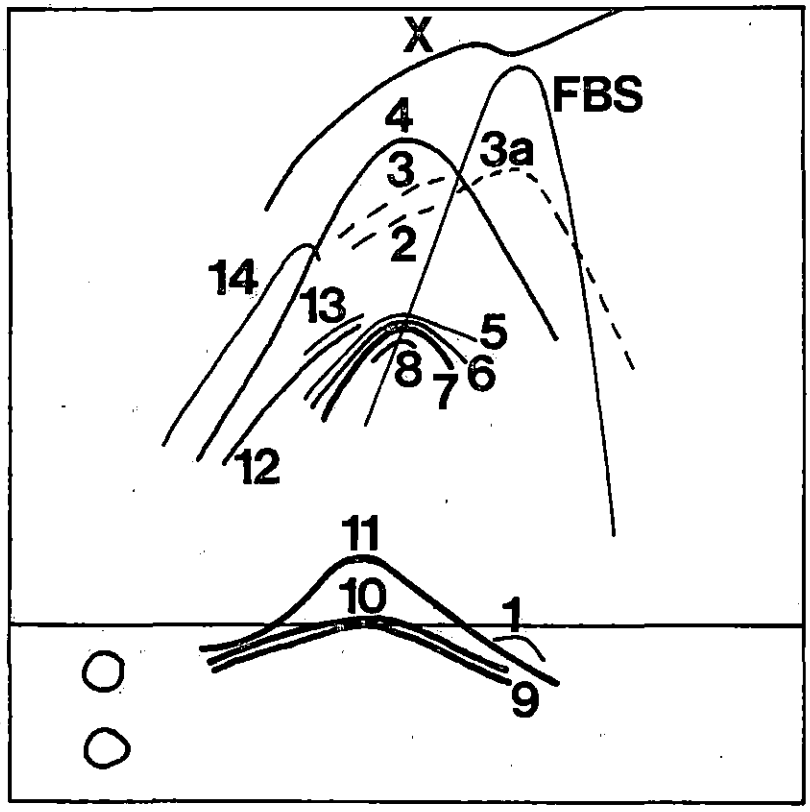
Figure 4. Composite illustration of all precipitates detected in CIE patterns.

Glycosylated antigens ————

Nonglycosylated antigens --------


X
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2
14
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9

DISCUSSION

Despite attempts to standardize experimental procedures, the number and pattern of CIE precipitates varied from batch to batch of prepared antigen. In this study, variability may have been due to the condition of cell cultures, inapparent differences in extraction procedures, concentration of reference antibody in the second dimension gel, differences in migration distances and diffusion in co-electrophoresis, and slight voltage fluctuations during electrophoresis. Batch variation in antigen concentration was the major problem, and this may reflect the difficulty in standardizing cell cultures. A change in concentration will increase or decrease peak height, causing it to become obscured among other peaks, or the antigen concentration may be too low to be detected by CBB or autoradiography. Thus, antigen peaks detected in the CIE pattern of one batch may be missing or obscured in the pattern of another batch. Results were more uniform when unlabelled CA and labelled CA were co-electrophoresed.

The 16 virus-specific antigens were identified from separate patterns and were never found to constitute a single, complete pattern in either a CBB stain or autoradiogram. The FBS peak was identified as a fetal bovine serum component, which was present in virus inocula. Glycoprotein antigens 4,6,7,9,10 and 11 were found most consistently, while antigens 1,2,3,3a,5,8,12,13, 14 and X appeared sporadically. It is possible that Ag 3a may be identical to Ag 2 or Ag 3. This study did not determine whether some antigen peaks were degradation products of others.

Herpes envelope proteins, specifically glycoproteins, are the major targets of neutralizing antibodies and play an important protective role in the infected host (1,6,9,12,15,16,29). Additional studies are needed to determine which of these PRV antigens are structural or nonstructural, and which of the structural antigens are responsible for protection.

ACKNOWLEDGEMENTS

The authors would like to thank Drs. K. B. Platt and B. F. Vestergaard for their technical advice. This work was supported in part by an Iowa State Appropriation through the Iowa Livestock Health Advisory Council.

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PART II. PROTECTIVE ACTIVITY OF GLYCOSYLATED PSEUDORABIES (AUJESZKY'S DISEASE) VIRUS ANTIGENS IN MICE

This manuscript has been submitted for publication to the Archives of Virology.

PROTECTIVE ACTIVITY OF GLYCOSYLATED PSEUDORABIES (AUJESZKY'S DISEASE) VIRUS ANTIGENS IN MICE

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SUMMARY

Pseudorabies (Aujeszky's disease) virus infected cell antigens were solubilized with Triton X-100. The crude antigen extract was separated by concanavalin A, affinity chromatography into bound and unbound fractions. The antigenic compositions of crude antigen, bound and unbound fractions were analyzed by fused rocket immunoelectrophoresis and crossed immuno-electrophoresis. The preparations were then mixed with adjuvant and inoculated into mice. Mice vaccinated with crude antigen and the bound fraction (containing viral glycoprotein antigens 4 and the 5, 6, 7, 8 complex) developed high serum neutralizing antibody titers and a high degree of protection against lethal challenge with virulent pseudorabies virus. Mice vaccinated with the unbound fraction (containing viral glycoprotein antigens 9 and 10) developed low serum neutralizing antibody titers and a low degree of protection. We conclude that concanavalin A bound pseudorabies virus glycoproteins are important in establishing humoral and protective immunity in infected mice.

INTRODUCTION

Herpesvirus glycoproteins are considered the major targets of neutralizing antibodies and play an important, protective role in the infected host (2,4,5,6,8,9,11, 12,15,19,20).

Purification of glycopeptides from turkey herpesvirus infected cell extracts has been accomplished with affinity chromatography, followed by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (19). The glycopeptides were inoculated into chickens and gave partial protection against challenge with virulent Marek's disease virus. Incomplete protection may have been a result of the SDS treatment, which denatures protein antigens with subsequent reduction in immunologic function (3,10,18,19).

Membrane bound glycoproteins may be extracted efficiently from virus infected cells with the nonionic detergent, Triton X-100 (18). The resulting extract can be separated by affinity chromatography, yielding immunologically active glycoprotein antigens in the bound fraction (3,17). The aim of the present study was to isolate pseudorabies virus glycoprotein antigens in this manner and evaluate their role in humoral and protective immunity in mice.

MATERIALS AND METHODS

Virus Propagation

Plaque-purified pseudorabies virus (PRV) strain S62/26 (13) was obtained from D. L. Rock (Veterinary Medical Research Institute, Iowa State University, Ames, Iowa). Stock virus was propagated in low passage bovine lung (BL) cell cultures that were grown in 850 cm^2 roller bottles. The culture medium consisted of Eagle's minimum essential medium with Earle's salts (MEM) supplemented with 10% fetal bovine serum, 0.1% lactalbumin hydrolysate, 0.16% sodium bicarbonate, 8.3 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and antibiotics (100 I.U. penicillin, 100 ug streptomycin sulfate, 100 ug kanamycin sulfate and 50 ug gentamicin sulfate per ml).

Reference Antibody

Goat anti-PRV serum was provided by W. A. Hagemoser (Department of Veterinary Pathology, Iowa State University, Ames, Iowa). The immunizing procedures and preparation of reference antibody were as described previously (7).

Antigen Preparation

Crude antigen (approximate protein content of 5 mg/ml) was prepared by solubilizing PRV infected cells in 1% Triton X-100 as reported previously (7). Crude antigen control was prepared from uninfected cells in an identical manner. For affinity chromatography, a 100 ml bed volume of concanavalin A (con A) Sepharose (Pharmacia Fine Chemicals, Piscataway, New Jersey)

was equilibrated in 0.025 M Tris-Tricine buffer (Biorad Laboratories, Richmond, California) containing 1% (v/v) Triton X-100 (Sigma Chemical Company, St. Louis, Missouri) (TTT buffer, pH 8.6). Twenty mls of crude antigen were allowed to adsorb into the column for 30 minutes. The unbound fraction was eluted with 200 mls of buffer and designated UB. The bound fraction was eluted with 200 mls of ∝-methyl-D-mannoside (Sigma) (25 mg/ml) and designated B. Both B and UB fractions were concentrated by ultrafiltration (10,000 MW) to 20 mls and dialyzed for 3 days (6 buffer changes) against TTT buffer (pH 8.6). Fractions designated B control and UB control were prepared in an identical manner starting with 20 mls of crude antigen control. Preparations were assayed for virus infectivity by inoculating 25 mls of 1:100 dilutions onto 75 cm^2 flask, BL cell monolayers. Cultures were incubated at 37°C for 1 hour. Inocula were then removed, replaced with maintenance media and cells were observed for cytopathic effects for a minimum of 2 weeks. Radiolabelled crude antigen was prepared as described previously (7) by the addition of ^{3}H-glucosamine to infected cells 3 hours after adsorption with virus. The labelling period lasted 21 hours.

Immunization and Bleeding Schedules

Five-week old, female Swiss Webster mice (Biolab Corporation, St. Paul, Minnesota) were acclimatized for 4 weeks prior to use in experiments. Three 9-week old mice were sacrificed to obtain preinoculation sera. Six antigen preparations, crude antigen, crude antigen control, B, B control, UB and UB control, were emulsified with equal amounts of Freund's incomplete adjuvant and were used to immunize 9-week old mice. A total of two-hundred

nineteen mice were inoculated subcutaneously with 0.2 ml doses of emulsified antigens. Groups of 40 mice were inoculated with B, UB or crude antigen preparations. Groups of 33 mice were inoculated with B control, UB control or crude antigen control. Thirty-three mice served as uninoculated controls. At 21 days post-inoculation (PI), all control vaccinates and 33 mice from each group of 40 inoculated with viral antigen preparations received a second, identical vaccination. Twenty-one mice (groups of 3 mice that were inoculated with B, UB or crude antigen preparations) were sacrificed for serum and 33 mice remained unvaccinated. At 42 days PI, 42 mice (groups of 7 mice that were uninoculated or twice-inoculated with control or viral antigen preparations) were sacrificed for serum.

Challenge Procedures

Fifteen-week old (the age of experimental animals at challenge) mice were used to determine the 50% lethal dose (LD_{50}) endpoint, which was calculated by the Reed Muench method (14) to be $10^{4.4}$ per 0.1 ml of stock PRV. At 42 days PI, 156 vaccinated (control or viral antigen vaccinates) and 33 unvaccinated mice were challenged intravenously (tail vein) with a 10, 100 or 1000 LD_{50} dose contained in 0.1 ml serum-free MEM. Mice were observed for clinical signs and death for a 2 week period.

Immunoelectrophoresis

Crude antigen peaks present in the fused rocket immunoelectrophoresis pattern were tentatively identified by comparison of relative peak heights, intensity of staining and fusion with individual crude antigen CIE reference

peaks. The antigenic compositions of inocula were then evaluated by comparison of their CIE and fused rocket patterns with those of the crude antigen. Crossed immunoelectrophoresis of antigen preparations was performed as described previously (7) using 15% reference antibody in the second dimension gel. Fused rocket immunoelectrophoresis was performed according to Svendsen (16). Fifteen-ul samples of antigen preparations were allowed to diffuse for 1 hour in non-antibody containing gel, before electrophoresis into a gel containing 15% reference antibody. Intermediate gel CIE, performed according to Axelson (1), was used to characterize the reaction of mouse PI antisera to precipitating antigens in the crude antigen preparation. Fifteen-ul samples of crude antigen and ^{3}H-glucosamine labelled crude antigen were co-electrophoresed. Serum samples from vaccinates inoculated with the same antigen preparation were pooled and incorporated at 20%, into intermediate gels placed between the first and second dimension gels. The second dimension gel contained 15% reference antibody. Reactions of intermediate gel-incorporated, mouse antisera with individual antigens were quantitated by measuring the area under each antigen peak. The borderline between the first dimensional gel and the intermediate gel was used as a baseline. Antigen peak areas from test plates were then compared with corresponding antigen areas from a simultaneously run control plate. A minimum of 10% decrease in area from that of the control plate arbitrarily was considered to indicate a specific reaction of a given antigen with the intermédiate gel antisera. Precipitates were visualized by Coomassie brilliant blue R250 stain (CBB) (Biorad) and autoradiography. For autoradiography, Ultrofilm ^{3}H (LKB, Rockville, Maryland) was placed on the gel side of dried, stained plates and the film was developed

after 30 days exposure.

Serum Neutralization Test

The plaque-reduction serum neutralization (SN) test was used to determine mouse anti-PRV serum titers. One-tenth ml volumes of serum dilutions were mixed with an equal amount of MEM containing approximately 150 plaque forming units of PRV. The virus/serum mixtures were incubated for 1 hour at 37°C, and 0.1 ml of these mixtures were inoculated onto monolayers of BL cells in 16 mm, 24 well plastic tissue culture plates. Cultures were adsorbed for 1 hour and overlain with 1% agarose containing MEM, 1% diethyl-aminoethane dextran (DEAE), 2% fetal bovine serum, and antibiotics. Cultures were incubated at 37°C for 24 hours before fixing and staining. Serum neutralization titers were expressed as the reciprocal of the highest serum dilution reducing the plaque count by at least 50%.

Statistical Analyses

A split-plot experimental design was used, where the challenge doses were the whole-plot treatments and vaccinations were the split-plot treatments. The whole-plot units were cages containing 4 mice each and mice within a cage were given different vaccination treatments (B, UB, crude antigen and no vaccination). Analysis of variance was used to analyze SN antibody titers and challenge data. For purposes of statistical analyses, effects due to vaccination with crude antigen were considered to represent the combined effects due to vaccination with B and UB. The four vaccination treatments formed a 2 by 2 factorial arrangement based upon the presence and absence of B and UB.

RESULTS

Inocula

Attempts to isolate virus from inocula were unsuccessful.

Antigens present in inocula are shown in Figure 1. Precipitating antigens in the CIE pattern were numbered according to an earlier study, in which the FBS peak was found to be a fetal bovine serum component (7). Crude antigen peaks in the fused rocket pattern were tentatively identified by comparison of relative peak heights, intensity of staining and fusion with individual crude antigen CIE reference peaks (not shown). Antigens present in fused rocket patterns of individual inocula were identified by fusion with peaks in the crude antigen pattern. The crude antigen preparation contained the viral antigens 1, 3, 4, the 5, 6, 7, 8 complex, 9, 10, the 11/11a complex 12, 13, Y and Z. The B preparation (consisting of antigens that were bound to the affinity column) contained the viral antigens 4, the 5, 6, 7, 8 complex, the 11/11a complex, Y and Z. The UB preparation (consisting of antigens that were not bound to the affinity column) contained antigens 9 and 10 and the 11/11a complex. Antigens 3, Y and Z were detected only in fused rocket immunoelectrophoresis patterns. With the exception of antigens Y and Z, the identities of antigens present in fused rocket patterns of B and UB preparations were confirmed by CIE (not shown).

Serologic Response

Figure 2 gives the standard intermediate gel CIE reference pattern for evaluating reactions of antigens with intermediate gel-incorporated mouse antisera. The intermediate gel contained mouse preinoculation serum. To

Figure 1. Identification of antigens present in inocula. Fused rocket immunoelectrophoresis was performed on crude antigen (1), crude antigen control (2), B (3), B control (4), UB (5), and UB control (6) preparations. (A) Coomassie brilliant blue stain of standard crude antigen CIE pattern, (B,C,) Fused rocket immunoelectrophoresis of viral antigens using crude antigen as a reference pattern, (D) Fused rocket immunoelectrophoresis of control antigens using crude antigen as a reference pattern.

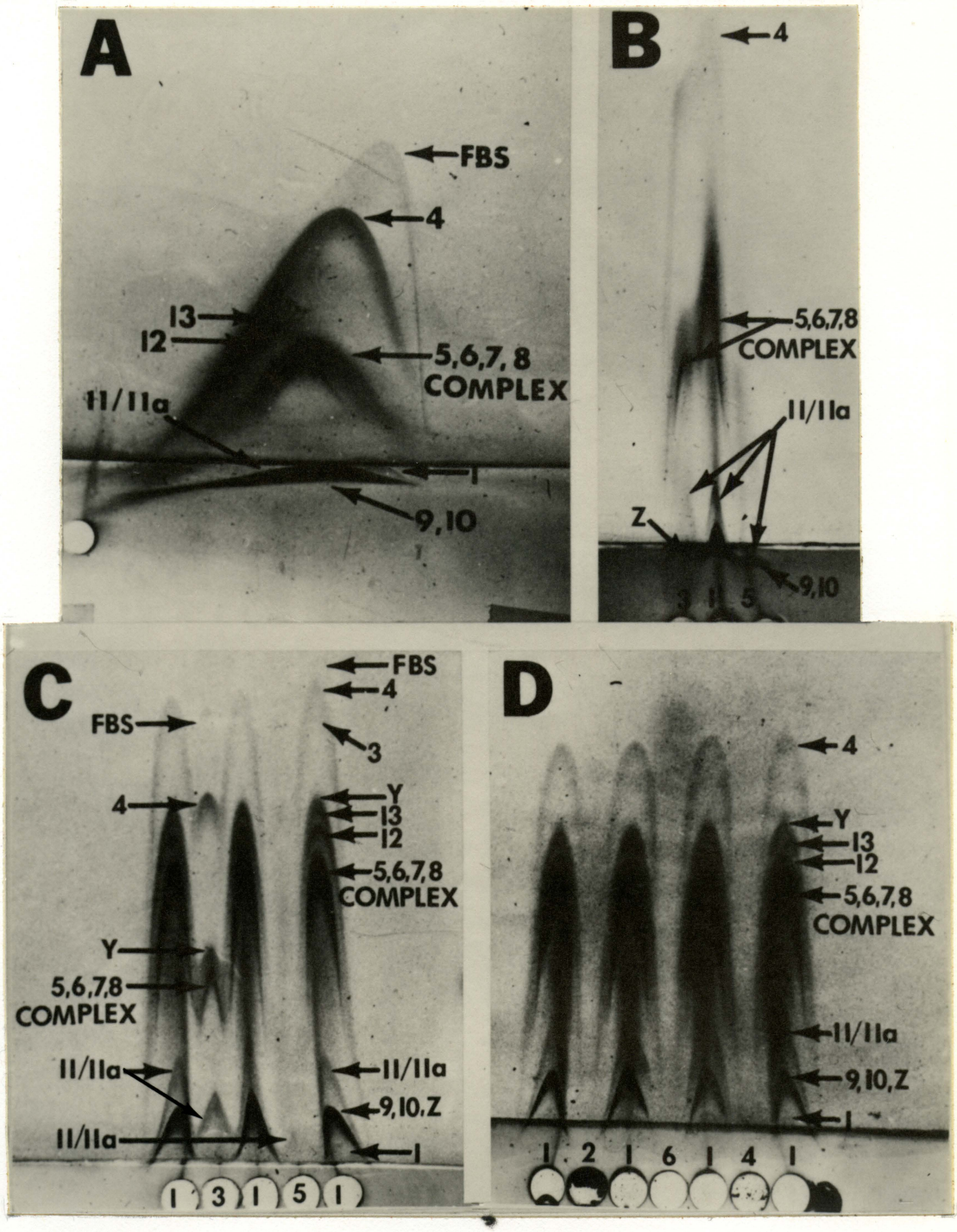
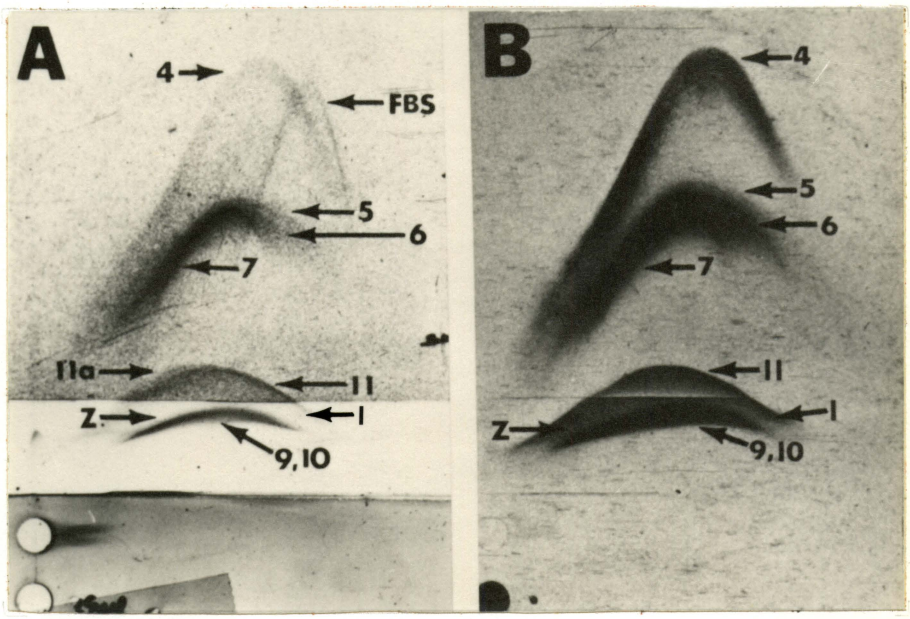

A
FBS
4
13
12
5,6,7, 8
COMPLEX
11/11a
1
9,10
B
4
5,6,7,8
COMPLEX
11/11a
Z
9,10
3 11 5
C
FBS
4
FBS
3
4
Y
13
12
5,6,7,8
COMPLEX
Y
5,6,7,8
COMPLEX
11/11a
11/11a
9,10.Z
11/11a
1
1 3 1 5 1
D
4
Y
13
12
5,6,7,8
COMPLEX
11/11a
9,10,Z
1
1 2 1 6 1 4 1

Figure 2. Intermediate gel CIE reference pattern for evaluating reactions of antigens with intermediate gel-incorporated mouse antisera. (A) Coomassie brilliant blue pattern with intermediate gel containing 20% preinoculation serum, (B) Autoradiogram of the same plate.


A
4
FBS
5
6
7
IIa
II
Z
I
9,10
B
4
5
6
7
II
I
Z
9,10

reduce day to day variation in peak areas, reactions with mouse antisera were evaluated also by comparison with simultaneously run controls (not shown). Incorporation of the intermediate gel revealed an additional antigen (Ag), 11a, which was not observed in Figure 1. This antigen comigrated with Ag 11 but was not glucosamine labelled. Antigen 11a was not detected easily in CBB patterns. Because CBB patterns in Figure 1 did not have corresponding autoradiograms to distinguish Ag 11 from Ag 11a, the peak corresponding to one or both of these antigens was designated the 11/11a complex. With the exception of Ag 11a, all antigens detected in the intermediate gel CBB pattern could be detected also in the corresponding autoradiogram. Antigens 3, 12, 13, and Y could not be identified positively by intermediate gel CIE. Antigen Z was visible more clearly in intermediate gel CIE than it was by conventional CIE.

A summary of intermediate gel CIE serologic data is given in Table 1. Precipitating antigens are characterized by glucosamine incorporation and their reaction with mouse antisera incorporated into intermediate gels.

Results from the incorporation of 21-day PI mouse antisera into CIE intermediate gels are shown in Figure 3. Viral reference peak areas were not affected by mouse antisera produced against either B or UB preparations. Twenty-one day PI antisera to crude antigen reacted with viral glycoprotein antigens 4, the 5, 6, 7, 8 complex, 9, 10, 11 and X; the nonglycoprotein Ag 11a also was depressed (not shown).

Results from the incorporation of 42-day PI mouse antisera into CIE intermediate gels are shown in Figure 4. The antisera produced against the

Table 1. Identification of antigens reacting with mouse antisera in intermediate gel CIE

Viral Antigen	Glycosylation[a]	21-day mouse PI antisera to: UB	B	Crude Antigen	42-day mouse PI antisera to: UB	B	Crude Antigen
4	Yes	-[b]	-	+	-	+	+
5,6,7,8 complex	Yes	-	-	+	-	+	+
9	Yes	-	-	+	+	+	+
10	Yes	-	-	+	+	+	+
11	Yes	-	-	+	+	+	+
11a	No	-	-	+	+	+	+

[a]Based upon ^{3}H-glucosamine incorporation.

[b](+) indicates antigen reaction with anti-UB, anti-B or anti-crude antigen antisera incorporated into CIE intermediate gels, (-) indicates no reaction.

Figure 3. Incorporation of 21-day PI mouse antisera into CIE intermediate gels. Autoradiogram patterns were formed by co-electrophoresis of unlabelled crude antigen (top wells) with ^{3}H-glucosamine labelled crude antigen (bottom wells). Intermediate gels contained mouse antisera produced against (A) UB, (B) B and (C) crude antigen preparations.

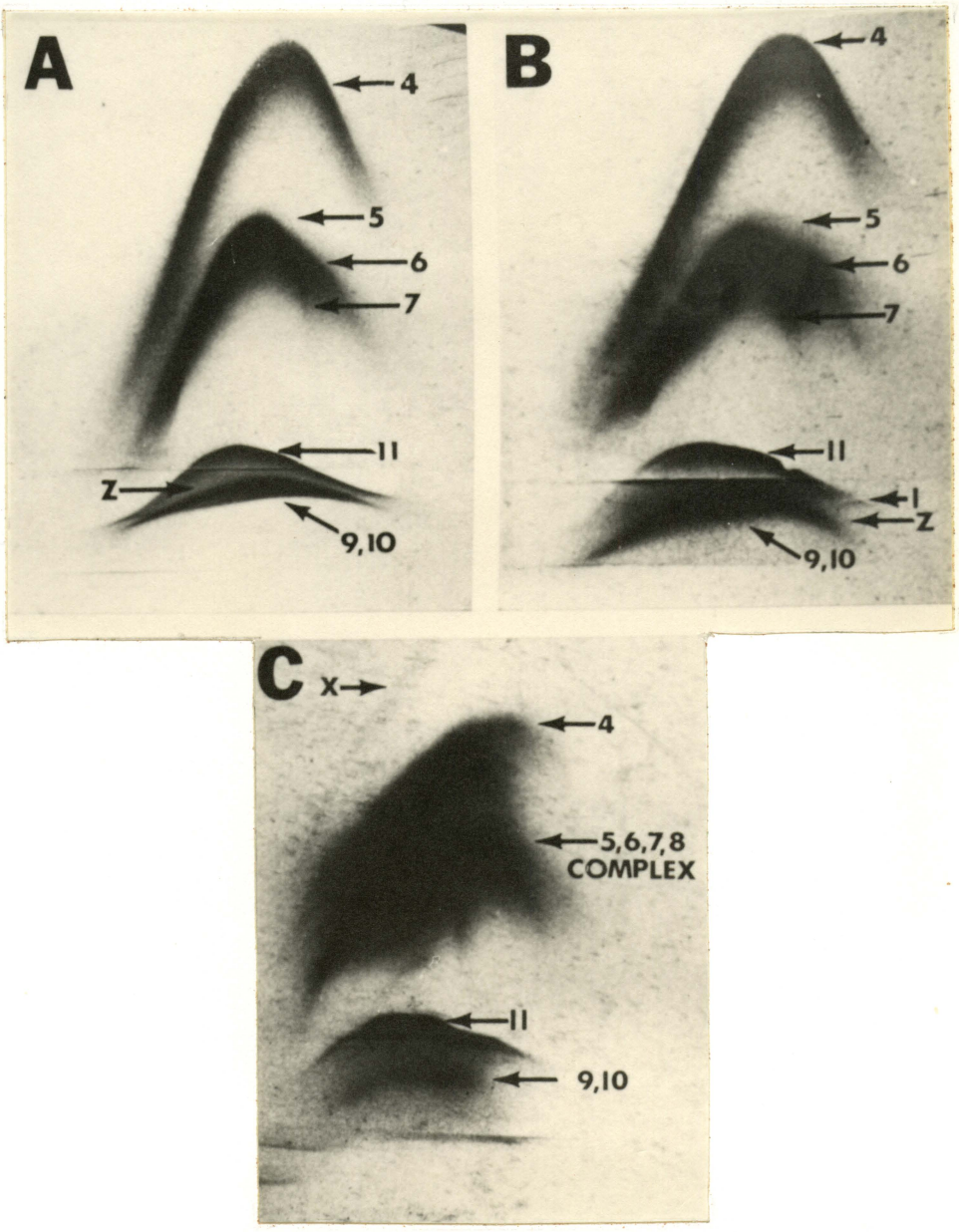
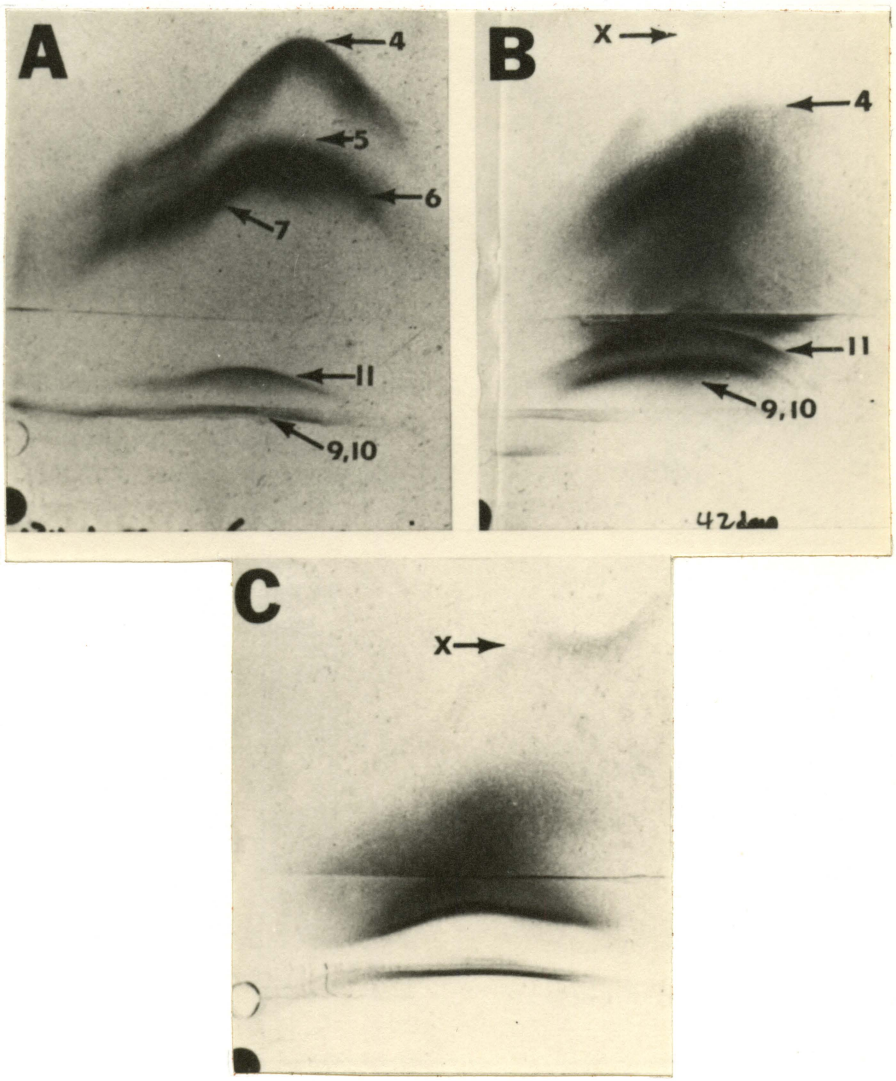

A
4
5
6
7
11
Z
9,10
B
4
5
6
7
11
1
Z
9,10
C
X
4
5,6,7,8
COMPLEX
11
9,10

Figure 4. Incorporation of 42-day PI mouse antisera into CIE intermediate gels. Autoradiogram patterns were formed as in Figure 3. Intermediate gels contained mouse antisera produced against (A) UB, (B) B, and (C) crude antigen preparations.


A
4
5
6
7
11
9,10
B
X
4
11
9,10
42dpa
C
X

UB preparation reacted with the glycoprotein antigens 9, 10 and 11, and the nonglycoprotein Ag 11a (not shown), but did not react with Ag 4 or the 5, 6, 7, 8 complex. The 42-day PI mouse anti-B sera reacted with the glycoprotein Ags 4, the 5, 6, 7, 8 complex, 9, 10, 11 and X, and the nonglycoprotein Ag 11a (not shown). The 42-day PI antisera produced against crude antigen reacted with all antigens. The crude antigen control, B control, UB control and unvaccinated control antisera had no effect on peak areas (not shown).

The statistical analysis and factorial arrangement of mouse 21-day PI serum neutralizing antibody response data are given in Table 2. The average difference between B-present and B-absent was to increase dilutions by 4.8. This effect was statistically significant ($p = 0.0001$). The average difference between UB-present and UB-absent showed a dilution increase of 0.2, which was not significant. The effect on antibody response with and without B was 24 times the effect with and without UB. Interaction (synergism) between B and UB was not significant.

The statistical analysis and factorial arrangement of mouse 42-day PI serum neutralizing antibody response data are given in Table 3. The average difference between B-present and B-absent was to increase dilutions by 3.8. This effect was statistically significant ($p = 0.0001$). The average difference between UB-present and UB-absent showed a dilution increase of 1.3, which also was statistically significant ($p = 0.025$), but considerably less so than with B. The effect on dilutions with and without B was 2.9 times the effect with and without UB. Interaction between B and UB was not significant.

Table 2. Statistical analysis and factorial arrangement[a] of mouse 21-day PI serum neutralizing antibody response based on the presence or absence of B or UB antigens in the inoculum

	UB-absent	UB-present	B-means	Mean difference (B)
B-absent	0[b]	0.3	0.2	4.8[c]
B-present	5.0	5.0	5.0	
UB-means	2.5[d]	2.7		
Mean difference (UB)	0.2[e]			

[a]Values given under B-absent/UB absent, B absent/UB present, B present/UB absent and B present/UB present represent antibody responses for unvaccinated animals, UB, B and crude antigen vaccinates, respectively.

[b]Antibody response is expressed as the average number of two-fold dilutions of 21-day mouse PI antisera required to reach the serum neutralizing endpoint. Values are rounded to the nearest tenth and are based on a total of 7 mice.

[c]Statistically significant at $p = 0.0001$.

[d]Means of average values (UB-means and B-means) are rounded to the nearest tenth.

[e]Not statistically significant.

Table 3. Statistical analysis and factorial arrangement[a] of mouse 42-day PI serum neutralizing antibody response based on the presence or absence of B or UB antigens in the inoculum

	UB-absent	UB-present	B-means	Mean difference (B)
B-absent	0[b]	0.8	0.4	3.8[c]
B-present	6.0	7.7	4.2	
UB-means	3.0[d]	4.3		
Mean difference (UB)	1.3[e]			

[a]Values given under B-absent/UB absent, B-absent/UB present, B present/UB absent and B present/UB present represent antibody responses for unvaccinated animals, UB, B and crude antigen vaccinates, respectively.

[b]Antibody response is expressed as the average number of two-fold dilutions of 42-day mouse PI antisera required to reach the serum neutralizing endpoint. Average values are rounded to the nearest tenth and are based on a total of 6 mice.

[c]Statistically signifcant at p = 0.0001.

[d]Means of average values (UB-means and B-means) are rounded to the nearest tenth.

[e]Statistically signifcant at p = 0.025.

Combined antibody response data are given in Figure 5. The highest antibody responses were found at 42 days PI, in mice inoculated with B and crude antigen preparations. The geometric mean titers of 42-day PI antisera produced against UB, B and crude antigen preparations were 1.7, 64 and 208, corresponding to 0.8, 6.0 and 7.7 average two-fold dilutions, respectively. Statistical analyses of the serologic data (Table 3) indicated that high SN response was due to vaccination with B-containing inocula (con A isolated or crude antigen inocula). Vaccinated and unvaccinated control sera gave no SN antibody response.

Protection

Percent survival data and statistical analyses are given in Table 4. Twenty-two percent (2 of 9) of the mice vaccinated with crude antigen control survived at the lowest challenge dose. All other controls did not survive. At all challenge doses, crude antigen induced the highest percent survival followed by B and then UB. For all inocula, survival of vaccinates decreased as challenge dose increased. Percent survival data for unvaccinated controls and the UB, B and crude antigen vaccinates averaged over all challenge doses (10, 100 and 1000 LD_{50}) are given in Table 5. The average difference between B-present and B-absent was to increase percent survival by 35.5. This effect was statistically significant ($p = 0.0001$). The average difference between UB-present and UB-absent showed a percent survival increase of 20.5. This effect was also statistically significant, ($p = 0.0049$), although considerably less so than with B. The effect on percent survival with and without B was approximately 1.7 times the effect with and without UB. Interaction between

Figure 5. Serum neutralizing antibody response at 0, 21 and 42 days PI. Response was expressed as the average number of two-fold dilutions required to reach the serum neutralizing endpoint. The preinoculation value (0-day PI) is a mean based on a total of 3 mice, 21-day PI values are means based on a total of 7 mice, and 42 day PI values are means based on a total of 6 mice. Serum neutralizing response results are given for unvaccinated controls (●—●) and UB (⊙—⊙), B (□—□), and crude antigen (▲—▲) vaccinates. Minimum and maximum SN values (⌶) also are given.

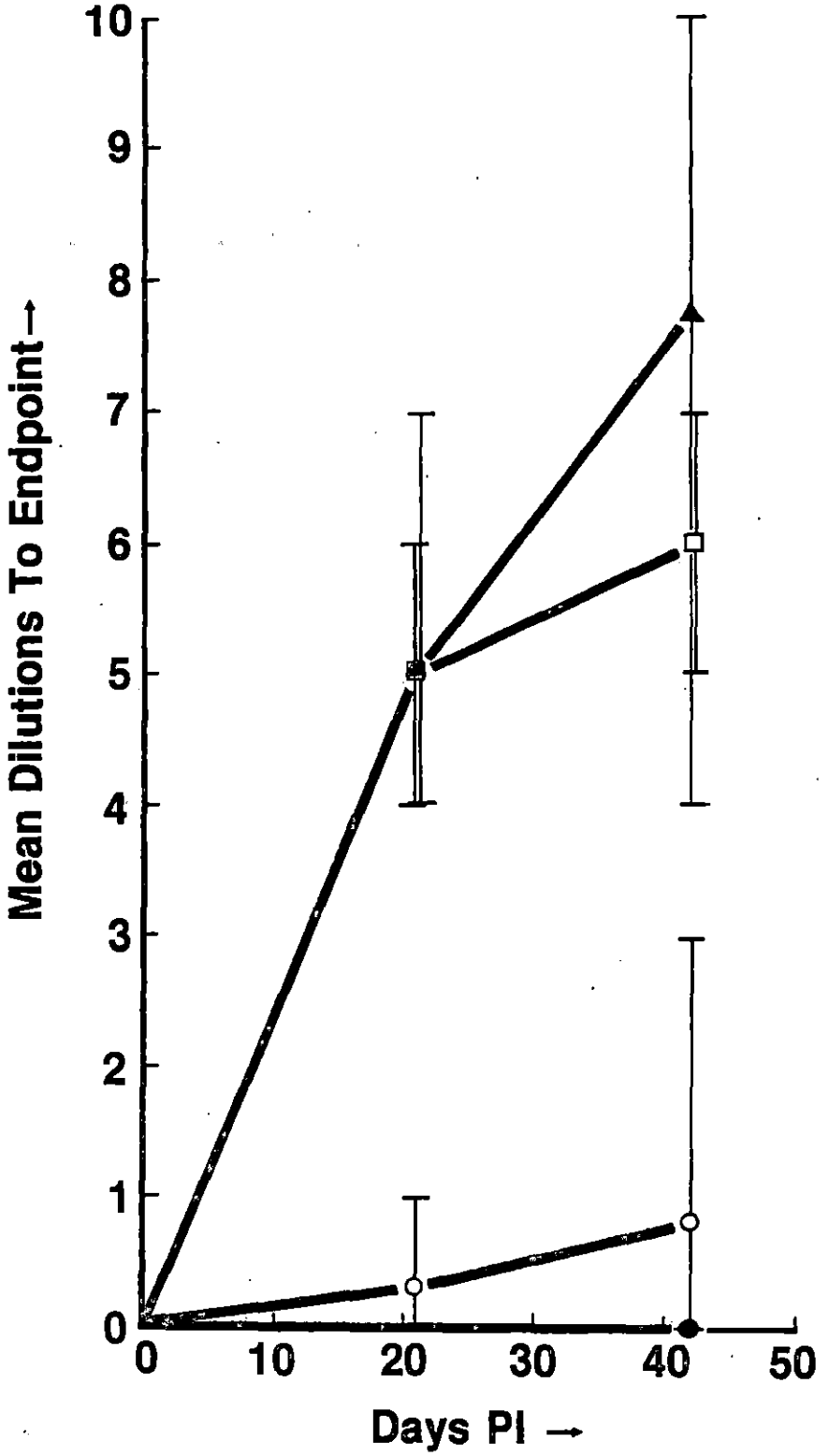

Mean Dilutions To Endpoint →
10
9
8
7
6
5
4
3
2
1
0
0
10
20
30
40
50
Days PI →

Table 4. Percent survival of vaccinates and controls

	CHALLENGE DOSE								
	10 LD_{50}			100 LD_{50}			1000 LD_{50}		
	UB	B	Crude Antigen	UB	B	Crude Antigen	UB	B	Crude Antigen
Control[a]	0[b]	0	22	0	0	0	0	0	0
Treated[c]	56	78	100	11	22	33	0	11	33

[a]Control mice were vaccinated with UB control, B control or crude antigen control preparations.

[b]Percent survival values at all challenge doses are rounded to the nearest whole number and are based on a total of 9 mice.

[c]Treated mice were vaccinated with UB, B or crude antigen preparations.

Table 5. Statistical analysis and factorial arrangement[a] of percent survival of vaccinates and controls averaged over all challenge doses (10, 100 and 1000 LD_{50}) based on the presence or absence of B or UB antigens in the inoculum

	UB-absent	UB-present	B-means	Mean difference (B)
B-absent	0[b]	22.0	11.0	35.5[c]
B-present	37.0	56.0	46.5	
UB-means	18.5[d]	39.0		
Mean difference(UB)		20.5[e]		

[a]Values given under B-absent/UB absent, B-absent/UB present, B present/UB absent and B present/UB present represent antibody responses for unvaccinated animals, UB, B and crude antigen vaccinates, respectively.

[b]Percent survival values are rounded to the nearest whole number and are based on a total of 27 mice.

[c]Statistically significant at p = 0.0001.

[d]Means of percent survival values (UB-means and B-means).

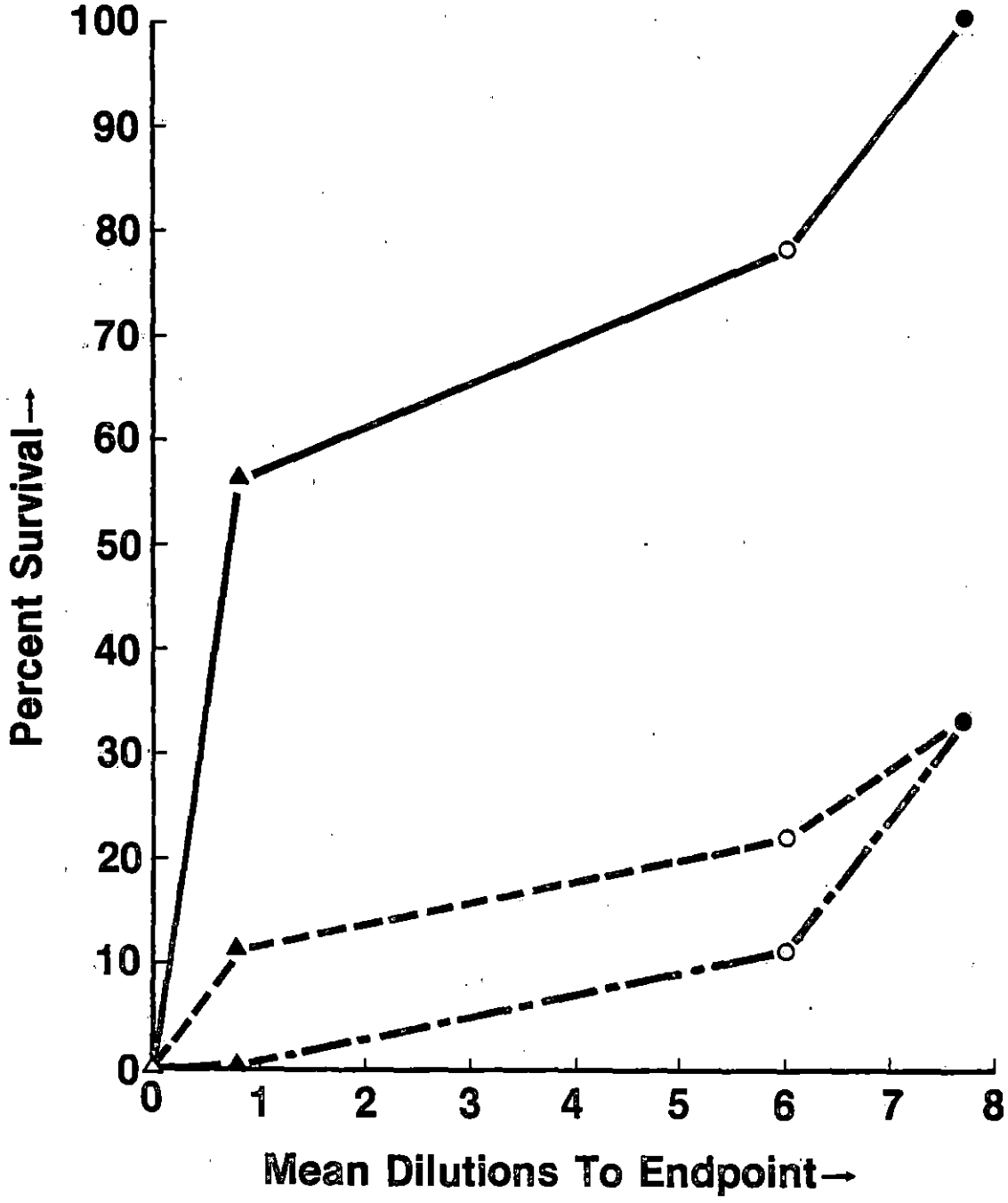
[e]Statistically significant at p = 0.0049.

B and UB was not significant. There was some interaction between challenge dose and vaccination with B-containing preparations (B and crude antigen) ($p = 0.038$). This interaction was caused by the greater protective effect induced by the B-containing inocula at the lowest challenge level than at both higher levels.

Relationship Between Serologic Response and Protection

The neutralizing antibody response as it relates to protective activity of antigens is shown in Figure 6. The data indicated that at all challenge doses, higher neutralizing antibody titers and vaccination with B-containing preparations were associated with a higher percent survival, while lower neutralizing antibody titers and vaccination with the UB preparation were associated with a lower percent survival.

Figure 6. Relationship between neutralizing antibodies and protective activity. Percent survival values are based on a total of 9 mice. Mean dilution values are based on a total of 6 mice bled at 42 days PI. Results are given for unvaccinated controls (Δ), UB (▲), B (O), and crude antigen (●) vaccinates, at 10 LD_{50} (——), 100 LD_{50} (– – –), and 1000 LD_{50} (— – —) challenge doses.


100
90
80
70
60
50
40
30
20
10
0
Percent Survival→
0
1
2
3
4
5
6
7
8
Mean Dilutions To Endpoint→

DISCUSSION

The reaction of antigens with intermediate gel antisera caused a decrease in affected peak areas (compared to controls), often accompanied by a generalized blurring of the precipitate pattern. In some instances, blurring made identification of affected peaks difficult. It is not known whether blurring was a property of affected peaks only, or if non-reacting antigen peaks appeared blurred because of the incomplete formation of a higher, affected peak. For the purposes of this study, only antigens with decreased peak areas were considered to have reacted with intermediate gel antisera.

We detected viral glycosylated Ags 1, 4, the 5,6,7,8 complex, 9,10, 11 and Z, and the nonglycosylated Ag 11a in the crude antigen reference pattern, which was formed by intermediate gel CIE. Of these antigens, Ags 4, the 5, 6, 7, 8 complex, the 11/11a complex, and Z were detected in the B preparation, while Ags 9, 10 and the 11/11a complex were detected in the UB preparation. Antigens 1 and Z could not be identified consistently in intermediate gel CIE patterns. Antigens 12 and 13 probably were not easily detected because of the addition of an intermediate gel in the standard CIE reference pattern of crude antigen. Increased migration distance and diffusion through CIE gels may have reduced antigen concentrations so that observable peaks to Ags 3 and Y were not formed.

Antisera produced against B and UB preparations reacted with both antigens in the 11/11a complex. Anti-B sera also reacted with antigens 9 and 10, which were not detected in the B preparation. The reaction of antisera to antigens not detected in their corresponding inocula may be due to serological

cross-reactivity between antigens 11 and 11a and between antigens 9, 10 and Z, indicating degradation or precursor relationships among antigens. It is possible that Ag 11a is a nonglycosylated precursor to the glycosylated Ag 11; antigens 9, 10 and Z also may represent partially and fully glycosylated forms of the same antigen. It is also possible that antigens 9, 10, and 11a in the B preparation, and antigen 11 in the UB preparation were present in concentrations too low to be detected by immunoelectrophoresis, but adequate to evoke an immune response.

Separation of crude antigen by con A chromatography resulted in incomplete separation of glycosylated and nonglycosylated antigens. The glycoprotein antigens 9, 10, and possibly 11 were eluted with the UB fraction. A glucosamine labelled antigen that did not bind to con A has been reported previously by Vestergaard et al. (18). Incomplete recovery of con A separated antigens from the affinity column was indicated by fused rocket immunoelectrophoresis, which showed that peaks present in both B and UB preparations were lower than the corresponding peaks in the crude antigen preparation.

All control animals died, except for 2 of 9 crude antigen control vaccinates, which survived at the lowest challenge dose. Their survival may have been the result of misvaccination with crude antigen, injection of an insufficient amount of challenge dose, or nonspecific stimulation of a generalized immune response by adjuvant.

Combined results indicate that the glycoprotein antigens 4 and the 5, 6, 7, 8 complex were responsible for the higher humoral and protective immunity induced in vaccinated mice. Mice vaccinated with these antigens (B and crude antigen vaccinates) demonstrated 89, 28 and 22% survival with

increasing challenge doses, while mice not receiving these antigens (UB vaccinates and unvaccinated controls) demonstrated 28, 5.6 and 0% survival. Survival results reflected both the quality and quantity of viral antigens present in the different inocula. It is likely that the predominance of glycoprotein antigens, the greater antigen mass and the greater number of different antigens present in B-containing inocula all contributed to the higher percent survival of B and crude antigen vaccinates. Vaccination with glycoprotein antigens 9 and 10 may have been responsible for the low humoral response and the slight protective immunity induced in some UB vaccinates. This would support results from previous studies, which indicate the importance of herpesvirus glycoproteins in both humoral and protective immunity (4,6,9,12).

ACKNOWLEDGEMENTS

The authors would like to thank Luciano Talens and Mary Tymeson for their assistance with experimental animals. This work was supported in part by an Iowa State Appropriation through the Iowa Livestock Health Advisory Council.

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SUMMARY AND CONCLUSIONS

This study involved the characterization of Triton X-100 solubilized, PRV infected cell antigens. The crude antigen extract was analyzed by fused rocket immunoelectrophoresis, CIE, and intermediate gel CIE. Coomassie brilliant blue and autoradiogram patterns revealed 19 virus-specific antigens, of which at least 15 were glycosylated.

The crude antigen was separated into bound and unbound fractions by concanavalin A, affinity chromatography. The antigenic compositions of crude antigen, bound and unbound fractions were analyzed by fused rocket immunoelectrophoresis and CIE. The preparations were mixed with adjuvant and inoculated into mice. Mice vaccinated with crude antigen and the bound fraction (containing viral glycoprotein antigens 4 and the 5, 6, 7, 8 complex) developed high serum neutralizing antibody titers and a high degree of protection against lethal challenge with virulent pseudorabies virus. Mice vaccinated with the unbound fraction (containing viral glycoprotein antigens 9 and 10) developed low serum neutralizing antibody titers and a low degree of protection. The following conclusions may be made:

1. The majority of precipitating antigens in the crude antigen extract is glycosylated.
2. Concanavalin A bound PRV glycoproteins are important in establishing humoral and protective immunity in mice.

Further research is needed in the following areas:

1. Pseudorabies virus infected cell antigens should be compared to those present in the virion to identify structural and nonstructural components.

2. The ability of the concanavalin A bound fraction to induce cellular immunity should be evaluated.
3. The protective activity of the concanavalin A bound fraction should be verified in swine, cattle, and other domestic animals at risk. A pseudorabies vaccine prepared using concanavalin A Sepharose could be valuable in eradication. Immunoprecipitation methods detected antigens 4, the 5,6,7,8 complex, the 11/11a complex, Y and Z in the bound fraction, while antigens 9 and 10 were not detected. Serologic tests developed to detect immune sera reactions to antigens 9 and 10 would facilitate differentiation between vaccinates and infected animals.

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ACKNOWLEDGEMENTS

The completion of this thesis project would not have been possible without the help of many individuals.

I am especially grateful to my major professor, Dr. D. E. Reed, for his patience, time, guidance, and contribution to all aspects of the thesis project.

My sincere thanks go to Dr. P. N. Hinz for his assistance with statistical analyses, Dr. J. D. McKean for locating current, unpublished information, Drs. K. B. Platt and B. F. Vestergaard for their technical advice, and Dr. C. L. Tipton for his input during the planning stages of this project.

Technical assistance given by Luciano Talens, Mary Tymeson, Judi Wheeler, Elise Huffman, Denise Warriner and Mickey Hersey was greatly appreciated.

Special thanks go to my husband, Doug Warner, for the moral support and encouragement he provided throughout this project.