Bovine herpesvirus 1 antigens: Susceptibility to inactivation and cross-reactivity with other cattle herpesviruses

by

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GENERAL INTRODUCTION

The large number of herpesviruses associated with a wide variety of diseases in man and other animals and their characteristics of latency and oncogenic potential have contributed to the interest in all members of this group. The bovine herpesviruses have been distinguished as being "associated with a diversity of clinical disease unparalleled by any other virus group affecting cattle" (Gibbs and Rweyemamu, 1977b). They have assumed increased importance in recent years due to their economic impact on the cattle industry.

The recognized significance of bovine herpesviruses has led to extensive studies of immunization against these agents. These studies have included the use of modified live and inactivated virus vaccines. Inactivated virus preparations are recognized as protective, but many of their other characteristics are unknown.

Widespread infection of cattle with bovine herpesviruses, cross-reactions between members of the group, extensive vaccination, and the inability to differentiate uninfected vaccinates from infected animals contribute to difficulties in virus-specific serodiagnosis. The identification of those antigens destroyed by virus inactivation and of those antigens cross-reacting with other bovine herpesviruses is necessary before an antigen specific for live virus of a particular bovine herpesvirus species may be recognized. The identification of this antigen would allow the development of a virus-infection-specific serodiagnostic test. This would be invaluable in simultaneous vaccination-eradication efforts,

epidemiologic investigations, and certification of animals as free of particular diseases.

The objectives of this study were (1) to determine the effects of common virus inactivation procedures on individual bovine herpesvirus 1 (BHV 1) antigens, and (2) to identify those BHV 1 antigens which cross-react serologically with other bovine herpesviruses. BHV 1 inactivation treatments studied included beta-propiolactone, formalin, heat, and ultraviolet irradiation. Infected-treated, infected-untreated, and uninfected-untreated cell preparations were solubilized using nonionic detergent. Individual BHV1 antigens were then recognized and evaluated by testing these preparations alone and in combination by two-dimensional and fused rocket immunoelectrophoresis. This experimentation is described in the section entitled "The Effect of Some Common Inactivation Procedures on the Antigens of Bovine Herpesvirus 1." Cross-reacting bovine herpesviruses studied included BHV 1, BHV 2, BHV 3, and suid herpesvirus 1. Infected and uninfected cell preparations were solubilized using a nonionic detergent. These preparations were tested alone and in combination by two-dimensional immunoelectrophoresis with homologous and heterologous antisera. This experimentation is described in the section entitled "Cross-Reactions of Bovine Herpesvirus 1 Antigens with Those of Other Cattle Herpesviruses."

THESIS FORMAT

This thesis consists of an introduction, a literature review, two separate manuscripts, a general summary, references and acknowledgments. The master's candidate, Randall Lynn Levings, is the senior author and principal investigator for each of the manuscripts.

LITERATURE REVIEW

Herpesviruses

The herpesviruses (Family Herpetoviridae) comprise a large group of viruses (over 100 members) which infect mammals, birds, reptiles, amphibians and bony fish. Organ systems affected include the integument and respiratory, gastrointestinal, lymphoid and nervous systems.

The inclusion of many herpesviruses in the family is based on morphology as observed by electron microscopy (see Herpesvirions-Structure). Three subfamilies of Herpetoviridae have been established on the basis of "host range, duration of reproductive cycle, cytopathology, and characteristics of latent infection" (Roizman et al., 1981). The alphaherpesvirinae (type species = human herpesvirus 1, herpes simplex virus 1) have a short replicative cycle and latent infections occur in ganglia. The betaherpesvirinae (type species = human herpesvirus 5, human cytomegalovirus) have a long replicative cycle and often cause enlargement of infected cells. The gammaherpesvirinae (type species = human herpesvirus 4, Epstein-Barr virus) replicate in lymphoblastoid cells (some also cause infections in epithelioid and fibroblastoid cells) and latent infection has been demonstrated in lymphoid tissue. The nomenclature of the herpesviruses has been variable. The system of Roizman et al. (1981) is used here.

Herpesvirions are made up of three main components; nucleoid, capsid, and envelope. The virion enters the host by viral envelope adsorbtion onto, and fusion with, the host cell membrane. The nucleocapsid is then released into the cytoplasm, and a viral DNA-protein complex is

translocated into the nucleus. Viral DNA is transcribed in the nucleus, the mRNAs migrating to the cytoplasm for translation. The viral DNA is then replicated and nucleocapsids are formed, which bud through altered nuclear membrane and in some cases other membranes of the cell. Virions are released by transport to the cell surface through the modified endoplasmic reticulum.

Many herpesviruses cause diseases of human and veterinary medical importance. Herpes simplex viruses 1 and 2 (human herpesviruses 1 and 2, HHV 1 and 2) are the agents of oral herpes, genital herpes (among the most common of sexually transmitted diseases), herpes keratitis (an important cause of blindness), encephalitis, and herpes neonatorum. Varicella-zoster virus (HHV 3) is the agent of chickenpox and zoster. Epstein-Barr virus (HHV 4) has been associated with infectious mononucleosis, Burkitt's lymphoma, and nasopharyngeal carcinoma. Human cytomegalovirus (HHV 5) usually causes inapparent infection in adults, but is a leading cause of birth defects.

The herpesviruses of cattle and their associated diseases will be discussed in detail elsewhere (see Cattle Herpesviruses), but are briefly summarized here. Infectious bovine rhinotracheitis-infectious pustular vulvovaginitis virus (bovine herpesvirus 1, BHV 1) is a cause of upper respiratory tract disease, reproductive tract disease, abortion, conjunctivitis and calf encephalitis. Bovine mammillitis virus (BHV 2) is a cause of ulcerative mammillitis or generalized skin disease. Movar virus (BHV 3) infection is often inapparent, but may cause respiratory disease. Pseudorabies virus (suid herpesvirus 1, SHV 1) causes a fatal nervous

system disease in cattle. Malignant catarrhal fever virus (alcephaline herpesvirus 1) in cattle causes inflammation of the mucous membranes of the mouth, nose and eyes, and nervous system signs.

In swine, pseudorabies virus (SHV 1) may cause respiratory and nervous system disease, and swine cytomegalovirus (SHV 2) is the agent of inclusion body rhinitis. In sheep, an association between a herpesvirus (sheep herpesvirus, caprine herpesvirus 1) and pulmonary adenomatosis ("Jaagsiekte"), a transmissible lung neoplasm, was suggested (Mackay, 1969, Malmquist et al., 1972, Martin, 1976) but has more recently been discounted (Villiers and Verwoerd, 1980). Caprine herpesvirus 2 infects young goats resulting in respiratory and gastrointestinal disease.

Equine rhinopneumonitis virus or equine abortion virus (equine herpesvirus 1, EHV 1) causes respiratory disease or abortion in the sixth through eleventh month of gestation and a neurological syndrome in pregnant mares. Pharyngitis caused by EHV 2 has been reported in young foals. Equine coital exanthema virus (EHV 3) causes vesicular lesions of the vulva of the mare and the penis of the stallion, and is transmitted by coitus.

Dog herpesvirus (canid herpesvirus 1) causes a fatal viremic disease in infant puppies; in adults it causes mild vaginitis or posthitis and occasionally conjunctivitis. Feline rhinotracheitis virus (feline herpesvirus 1, FHV 1) causes an upper respiratory infection and may cause glossitis, conjunctivitis, keratitis and abortion. Urolithiasis in experimentally-infected cats has been associated with FHV 2.

Marek's disease virus (gallid herpesvirus 2) is the agent of an inflammatory and neoplastic disease of the nervous system and other organs of

chickens, turkeys, and other birds. The disease, also known as neurolymphomatosis or range paralysis, is manifested by flaccid or spastic paralysis of the leg, wing or neck due to nerve trunk involvement, by fading of the iris and irregularity of the pupil (ocular lymphomatosis) and by tumors in the gonad, liver, lung and skin. Avian infectious laryngotracheitis virus (gallid herpesvirus 1) causes rhinitis, upper respiratory disease and conjunctivitis in chickens and pheasants, resulting in high mortality. Pigeon herpesvirus (columbid herpesvirus 1) causes coryza in pigeons. Duck virus enteritis, caused by anatid herpesvirus 1, is characterized by disease of the gastrointestinal, respiratory, and nervous systems.

Channel catfish virus (ictalurid herpesvirus 1) causes a hemorrhagic, systemic infection in fry and fingerling channel catfish.

Cattle Herpesviruses

Bovine herpesvirus 1

Bovine herpesvirus 1 is worldwide in distribution (Gibbs and Rweyemamu 1977a) and is ubiquitous in the United States (Rosner, 1968, Sheffy and Krinsky, 1973, Kahrs, 1974). Cattle are the principal reservoir, although goats (Mohanty et al., 1972a), swine (Nelson et al., 1972) and other species can be infected. Infectious bovine rhinotracheitis was described in feedlots in the western U.S. in 1955 by Miller (1955), and the associated herpesvirus (BHV 1) was isolated the following year (Madin et al., 1956), although serologic evidence shows the virus had been present in the U.S. much earlier. The virus was later associated with infectious

pustular vulvovaginitis (IPV, Kendrick et al., 1958, McKercher, 1963), so one common name for BHV 1 is IBR-IPV virus (Baker et al., 1960). Abortion (Lukas et al., 1963, McKercher and Wada, 1964) and other clinical effects of infection were subsequently described.

Transmission is by direct contact, large quantities of virus being shed in respiratory, ocular, and reproductive secretions during primary infections and following reactivation of latent infections (Sheffy and Davies, 1972, Sheffy and Rodman, 1973, Davies and Duncan, 1974). The incubation period varies from two to six days. Latency is a characteristic of herpesviruses and has been reported for BHV 1 infections involving a variety of clinical syndromes. BHV 1 DNA has been demonstrated in the trigeminal ganglia of intranasally-infected calves (Ackermann et al., 1982) and the sacral ganglia of intravaginally-infected calves (Ackermann and Wyler, 1984) during the latent stage of infection.

Although antigenic differences between BHV 1 isolates have been reported (Buening and Gratzek, 1967, House, 1972, Crandell, 1973, Potgieter and Maré, 1974a), distinct antigenic groups have not been demonstrated (Ludwig, 1983). However, isolates from reproductive disease have been demonstrated to differ from isolates of other clinical manifestations by restriction enzyme analysis (Engels et al., 1980, 1981). Reviews of BHV 1 literature include those written by McKercher (1959 and 1973), Gibbs and Rweyemamu (1977a), Kahrs (1977), and Ludwig (1983).

Respiratory tract disease The respiratory disease associated with BHV 1 infection (IBR) is characterized by fever, depression, inappetance, and dyspnea. Catarrhal, progressing to mucopurulent, rhinitis may be

associated with hyperemia of the nasal turbinates and muzzle ("red nose") or foci of necrosis with adherent white material ("plaques") resulting from the coalescence of pustules (Studdert et al., 1964). This plaque material consists of leukocytes, fibrin and necrotic epithelial cells. Intranuclear inclusion bodies may be observed (Crandell et al., 1959). A severe generalized necrotic tracheitis with diphtheritic membrane ("sewer pipe trachea") may be seen. The case-fatality rate of IBR is low unless secondary bacterial infection occurs, e.g., pulmonic pasteurellosis or shipping fever. Catarrhal to mucopurulent conjunctivitis may accompany the respiratory disease (Ferris et al., 1964, McKercher and Wada, 1964). Abortion may also accompany respiratory disease, beginning in a herd while clinical signs are evident and continuing for 90-100 days.

Conjunctivitis Conjunctivitis may accompany the respiratory manifestation of BHV 1, or it may be the principal clinical sign of the infection (Timoney and O'Connor, 1971, Rebhun et al., 1978). It may consist of inflammation of the conjunctiva, clear to mucopurulent ocular discharges, pustules or plaques of necrotic debris on the conjunctiva, and occasionally corneal opacity, which starts at the corneoscleral junction and proceeds centripetally. Abortion may accompany the conjunctival form of BHV 1 infection.

Reproductive tract disease Infectious pustular vulvovaginitis, or Bläschenausschlag, caused by BHV 1, is manifested by pustules or white necrotic plaques on the vulvar and vaginal mucosa, and mucopurulent discharge. The disease is transmitted by natural breeding and sniffing cattle (Kahrs and Smith, 1965). The disease usually develops one to three

days after mating. IBR and IPV may, but do not usually, occur together. Bulls infected with BHV 1 through breeding of cows with IPV infection may develop balanoposthitis with foci of necrosis (Kendrick and McEntee, 1967). These bulls may transmit the agent by natural breeding or by contamination of semen to be used for artificial insemination (Saxegaard, 1970, White and Snowdon, 1973, Schultz et al., 1976). Insemination of cows with BHV 1-contaminated semen may result in endometritis, shortened estrus periods (Kendrick and McEntee, 1967) and lower conception rates (Parsonson and Snowdon, 1975).

Abortion and neonatal disease Abortion due to BHV 1 may be associated with any or none of the other clinical manifestations of infection. In field conditions, about 25% of pregnant cattle may abort in association with an outbreak; higher percentages are reported in experimental circumstances (Owen et al., 1964). Although fetuses exposed in any stage of gestation may be aborted, most are in the third trimester (Owen et al., 1964, Kahrs et al., 1973). The exposure-abortion interval may range from eight days to several months, with abortions during the herd outbreak or up to 100 days afterward (Saunders et al., 1972, Wilson, 1974, Dellars, 1975). Microscopic focal necrosis of liver, stomach, kidneys, and adrenal glands as well as intranuclear inclusion bodies may be observed in the aborted fetus, which may or may not be autolysed (Dellars, 1975).

Infection in utero in late gestation or shortly after birth results in an acute febrile systemic disease of the newborn with high mortality (Baker et al., 1960, Reed et al., 1973). It may result in respiratory distress,

diarrhea, diffuse peritonitis, and white necrotic lesions on the mucosa of the mouth, tongue, esophagus and all four stomach compartments.

Encephalomyelitis Infection of young cattle with BHV 1 may result in nonpurulent leptomeningitis and encephalitis (Barenfus et al., 1963). This is manifested by incoordination, circling or licking at the flanks (Beck, 1975), recumbency, and death (McKercher et al., 1970). Gross lesions are not remarkable. Histologic lesions are most severe in the cerebrum, thalamus and basal ganglia. Lymphocytic meningitis and perivascular cuffing with mononuclear cells are the principle lesions.

Other clinical syndromes Bovine mastitis has been experimentally produced by udder inoculation of BHV 1 (Greig and Bannister, 1965), and the virus has been isolated from mastitis cases (Roberts and Carter, 1974, Gourlay et al., 1974). One report exists of BHV 1 isolation from perineal skin of chronic dermatitis cases in bulls (Bwangamoi and Kaminjolo, 1971).

Bovine herpesvirus 2

BHV 2, originally isolated by Alexander et al. in 1957, has been reported in North America, Europe, Africa and Australia (Gibbs and Rweyemamu, 1977b), and is the cause of nonfatal diseases with various clinical signs and names in those regions. The bovine dermotrophic viruses of the United States, the virus causing bovine herpes mammillitis, ulcerative mammillitis and skin gangrene of the udder in the United Kingdom, and the "Allerton virus" causing pseudolumpyskin disease in South Africa are believed to belong to the same species (Gigstad et al., 1971, Dardiri, 1973, Cilli and Castrucci, 1976, Gibbs and Rweyemamu, 1977b, Gigstad and

Stone, 1977), although minor antigenic differences have been noted between strains (Castrucci et al., 1979).

Cattle are considered the natural reservoir although wild herbivores in Africa can apparently be infected (Gibbs and Rweyemamu, 1977b). The method of transmission is not definitely known, but insect vectors and milking have been suggested (Cilli and Castrucci, 1976). The incubation period is two to ten days. Martin et al. (1975) demonstrated persistent infection with BHV 2 and recrudescence following corticosteroid injections. Although some infections may be inapparent (Dardiri and Stone, 1972), pyrexia may be present and edema, vescicles, ulcers and scabs or poxlike lesions may occur on infected teats and udders, with mastitis as a frequent sequel (Martin et al., 1966). Reduced milk production is observed in affected herds. The uncomplicated disease is self-limiting, and lesions resolve in several weeks (Gibbs and Rweyemamu, 1977b). Calves nursing affected cows may develop oral or muzzle ulcers. The generalized skin disease described in South Africa is characterized by pyrexia and firm round nodules most prevalent on the head, neck, back and perineum (pseudolumpyskin disease). This may, but usually does not, occur with udder infection. Epidermal lesions consist of intracellular edema in the stratum germinativum and stratum spinosum, infiltration of mononuclear and polymorphonuclear cells, and formation of syncytia with intranuclear inclusion bodies.

The literature concerning this virus has been reviewed by Martin (1973), Cilli and Castrucci (1976), Gibbs and Rweyemamu (1977b), and Ludwig (1983).

Bovine herpesvirus 3

BHV 3 strains have been isolated from a variety of clinical syndromes and from clinically normal cattle in North America, Europe and Africa (Gibbs and Rweyemamu, 1977b). In most cases, experimental inoculation with the agents has failed to elicit disease, although inoculation into the nose and trachea of calves has resulted in fever, respiratory disease and conjunctivitis in a few instances (Bodon et al., 1971, Mohanty et al., 1972b, Mohanty, 1973). Virus has been reisolated from the thoracic lymph node, spinal cord and trigeminal ganglion of corticosteroid-treated, experimentally infected cattle (Krogman, 1980) and the trigeminal ganglia of slaughtered cattle (Homan and Easterday, 1980). Evidence has been presented for a lymphoid-associated persistent virus infection in cattle (Osorio and Reed, 1983). The literature concerning this virus has been reviewed by Gibbs and Rweyemamu (1977b), Mohanty (1980), and Ludwig (1983).

Suid herpesvirus 1

SHV 1 is the etiologic agent of Aujesky's disease or pseudorabies, which is usually considered a disease of swine, its reservoir host (Shope, 1935). In swine latent, subclinical, or clinical infections may occur, with syndromes including abortion or acute death in baby pigs. Cattle and other dead-end hosts are infected by the bite or nasal discharge of infected swine. In cattle, the disease has been called "Mad Itch." Intense pruritis leading to self-mutilation is frequently observed, with the hindlimbs and vulva most frequently involved, though other areas may be affected (perhaps reflecting different sites of inoculation). Fever, rapid respiration and behavioral changes, leading to convulsions, recumbency and

death are often observed. The lymphocytic infiltration of the peripheral nerves supplying the affected area support the neurogenic origin of the self-mutilation. In the central nervous system, a nonsuppurative encephalomyelitis, meningitis and ganglioneuritis may be seen, with intranuclear inclusion bodies present. The literature concerning this virus has been reviewed by Baskerville et al. (1973), McKercher (1973) and Gustafson (1981).

Alcelaphine herpesviruses 1 and 2

Alcelaphine herpesviruses 1 and 2 are the etiologic agents of African malignant catarrhal fever (MCF or snotsiekte in South Africa) and MCF-like disease, the reservoirs and asymptomatic carriers of which are the blue and black wildebeest and the hartebeest, respectively. Sheep-associated or American MCF may also be caused by one of these agents, but this has not been confirmed. The diseases are very similar in clinical and pathologic respects (Plowright, 1968), and so will be discussed together. MCF occurs worldwide, and is a highly fatal pansystemic disease characterized by mucosal lesions, a severe rhinitis, panopthalmia, lymphadenopathy, encephalitis, and often diarrhea. Transmission may occur from the reservoir host by ingestion of contaminated secretions and excretions (Plowright, 1968, Storz et al., 1976). Cow-to-cow transmission is believed not to occur. Clinically inapparent infection in the wildebeest with induction of virus excretion by betamethasone has been reported (Rweyemamu et al., 1974). There is evidence of a latent infection in the bovine lymphocyte (Patel and Edington, 1981).

Fever, depression, photophobia and conjunctivitis with excess lacrimation may be early signs. Severe panopthalmitis, including corneal opacity progressing centrally from the limbus, may follow. The nasal discharge is mucopurulent, and the nasal mucosa may be crusted, hyperemic and necrotic. A diphtheritic membrane on the trachea may occur. Hyperemia and ulceration of the entire gastrointestinal tract may be noted. Raised skin lesions may slough or scab in hairless areas and coronary band lesions may be observed. White foci are noted in the kidney cortex. The brain and meninges may be edematous, and neurologic signs may be seen. Necrotizing vasculitis is characteristic histologically.

The literature on MCF has been reviewed by Plowright (1968) and Maré (1977).

Other cattle herpesviruses

A herpesvirus has been isolated from the reproductive tissues of cattle with "Epivag Syndrome" (Epididymitis-vaginitis, Maré and Van Resnburg, 1961, Theodoridis, 1978). Because many enlarged cells were produced in tissue cultures infected with the virus, it has been described as a bovine cytomegalovirus (Mohanty, 1980).

A virus of herpesvirus structure was isolated by Van Der Maaten and Boothe (1972) from the lymphocytes of two cattle with lymphosarcoma. The virus was strongly cell-associated and caused syncytia is tissue culture, but intranuclear inclusion bodies were not observed. Calves infected with the agent manifested no clinical signs, but the virus was reisolated from the circulating leukocytes of these calves 4-12 months post-inoculation.

Herpesvirions

Structure

The herpesvirion (120-200nm diameter) consists of three major components; the core, the capsid, and the envelope. The core or nucleoid is found inside the capsid, and in HHV 1 is an electron-dense toroid structure 50nm high, with an outside diameter of 70nm and an inside diameter of 18nm (Furlong et al., 1972). It was proposed that the core is composed of a cylindrical protein structure around which the DNA is wound with a spacing of 4-5nm. The herpesvirus DNA is a linear, double-stranded molecule of 80-150 megadaltons, 32-75% G+C. The structures of herpesvirus DNAs have been reviewed by Gentry and Randall (1973) and Roizman and Furlong (1974). Between the core and capsid lies the pericore (Roizman and Furlong, 1974), which may contain proteins that interact with both core and capsid.

The icosahedral capsid is approximately 100nm in diameter, and is made up of 162 capsomers, including 150 hexagonal and 12 pentagonal units (Wildy et al., 1960). The hexamers measure 8-10.2nm in diameter and 12.5nm in length, with a 4nm diameter hole halfway through the long axis (Roizman and Spear, 1973). The tegument is a layer of material which lies between the capsid and envelope (Roizman and Furlong, 1974).

The envelope is a bi-layered nonrigid membrane which is structurally similar to a cellular nuclear membrane (Epstein, 1962) and may occasionally bear projections 8-10nm long and spaced 5nm apart (Wildy et al., 1960, Watson, 1973).

The bouyant density (CsCl) of the particle is 1.20-1.29 g/cm^3 and the virion is sensitive to lipid solvents.

This subject has been reviewed by Wildy et al. (1960), Gentry and Randall (1973), and O'Callaghan and Randall (1976).

Antigen analysis

Virus antigens can be analyzed by immunological, biochemical, and combined methods. These may be applied to isolated elements of the virion, whole virion, or infected cells to yield a variety of information.

Immunological The immunological techniques include virus neutralization (VN), virus sensitization, complement fixation (CF), immunodiffusion (ID), immunoagglutination, and the use of labeled antibodies, including immunofluorescence (IF), immunoperoxidase (IP), immunoenzyme, and radioimmunoassays (RIA). Virus neutralization may be used to distinguish antigenic differences between herpesvirus strains, either by differences in titers or in neutralization kinetics. This technique can also be extended to the use of inactivated virus antigen preparations to inhibit the neutralization of a known live virus preparation. Virus sensitization exploits the ability of nonneutralizing antibodies to lead to virus neutralization by antiglobulin, complement, or Staph A. Complement fixation makes use of the ability of an antigen-antibody complex to tie up complement. The most common immunodiffusion techniques employ immunoprecipitation in agar gels, including radial immunodiffusion and double immunodiffusion. One limitation of these techniques is that only precipitating antigen-antibody complexes are detectable. Other immunodiffusion techniques include radial immune hemolysis. Immune agglutination

techniques include HA and HI for a few herpesviruses (including EHV 1), passive hemagglutination, and agglutination of antigen-bearing latex particles.

The use of labeled antibodies has dramatically increased recently due to their sensitivity and specificity. Direct, indirect, double antibody sandwich, competitive, biotin-avidin, and combinations of these with antibody-antibody or antibody-label complexes have found use in antigen identification, quantitation and localization.

In vitro techniques using elements of the cell mediated immune subsystem include lymphocyte activation, cytotoxicity, and other assays. In vivo immune phenomena employed include immediate hypersensitivity, delayed hypersensitivity, and active and passive immunization. Immunological analyses are reviewed by Van Regenmortel (1981).

Biochemical/biophysical The biochemical and biophysical techniques used include electron microscopy, the use of various disrupting and labeling agents, chromatograpy, electrophoresis, peptide analysis, and amino acid sequencing. Electron microscopy allows visualization and localization of the antigens of virions and virus-infected cells, but may not allow identification. Treatment of virions with nonionic detergents, freezing and thawing, osmotic shock, chaotropic agents, proteolytic enzymes, non-penetrating dyes, and labeling with I^{125} by the lactoperoxidase reaction may aid in the identification of envelope antigens. The interuption of viral infection in vitro (e.g., with Actinomycin D) allows isolation of early vs. late viral proteins. The use of chromatography allows identification, characterization, and purification of antigens.

Molecules may be separated according to size, affinity for certain compounds (e.g., lectins) or according to their ionic character (e.g., anion exchange, cation exchange).

Electrophoresis of proteins may be done on paper, cellulose acetate, or polyacrylamide gels. These can in turn be stained or used for autoradiography if the preparation is labeled with radioisotope-labeled amino acids. By producing the virus preparation in the presence of specific labeled precursors (e.g., C^{14} glucosamine), further information may be obtained. Electrofocusing can be used for identification and purification of protein antigens, and this technique may be combined with polyacrylamide gel electrophoresis (PAGE) for increased resolution.

Peptide analysis and amino acid sequencing of viral proteins is not yet commonly utilized for herpesviruses, as it is for some other groups (e.g., some members of the picornaviridae). The biochemical/biophysical methods are reviewed by O'Callaghan and Randall (1976), Altstein and Zhdanov (1979), Lauffer (1981), and Fraenkel-Conrat (1981).

Combination The combinations of the two categories discussed above are obviously many, but some of the more commonly used include immune electron microscopy, immune affinity chromatography, and immunoelectrophoresis. Immune electron microscopy can be used on tissues (using the antibodies linked to ferritin or peroxidase as a stain) and on antigens in suspension. The applications of the latter include precipitation, decoration, trapping, and combinations thereof. Immune affinity chromatography involves passing a mixture containing the unknown antigen or antibody through a column with known antibody or antigen bound to inert

substrate. By testing the unbound fraction, or eluting and testing the bound fraction, purification and identification of antigens is possible.

Immunoelectrophoresis techniques are used to identify and quantify viral antigens. Modifications of the original agar gel technique include counterimmunoelectrophoresis, in which the antigen and antibody preparations migrate toward each other (at pH 7.0-8.0), rocket immunoelectrophoresis, in which the antigen migrates under an electrical potential into a field of antibody, line immunoelectrophoresis, similar to rocket, except the antigen migrates from a trough or horizontal gel instead of a well, and crossed or two-dimensional immunoelectrophoresis. The latter technique has been found to yield higher resolution than classic immunoelectrophoresis and allows quantitation and greater fine tuning of identification through its numerous modifications. These include tandem crossed, addition of antigen or antibody, inclusion of lectin, antigen or antibody in intermediate gel, as well as others.

The literature concerning this subject has been reviewed by Van Regenmortel (1981) and Axelson and Bock (1972).

Proteins and antigens

Structural Studies using PAGE of highly purified HHV 1 have revealed 33 polypeptides ranging in size from 25,000 to 275,000 daltons (Heine et al., 1974, Roizman and Furlong, 1974). The major capsid protein was approximately 150,000 daltons. Empty capsids revealed four structural polypeptides (Gibson and Roizman, 1972, 1974, Roizman and Furlong, 1974), with a 47,000 dalton protein associated with the core. At least 13 glycoproteins were observed in the virions and are thought to be confined

to the envelope. Structural (and perhaps non-structural) glycoproteins are inserted in the cellular membranes of infected cells (Ben-Porat and Kaplan, 1973, Spear and Roizman, 1970, Heine et al., 1972, Heine and Roizman, 1973). Thus, the glycoproteins may be important in such phenomena as viral attachment and antigenicity, and the behavior of infected cells, including oncogenicity (Tevethia et al., 1972, Levitan and Blough, 1975, Nahmias and Roizman, 1973, Duff and Rapp, 1971a,b). The protein moiety is inserted into that section of membrane being modified for budding through and glycosylation occurs in situ (Ben-Porat and Kaplan, 1973, Spear and Roizman, 1970) with at least six constituents present; glucosamine, galactosamine, fucose, mannose, sialic acid, and galactose (Roizman and Furlong, 1974). Virus particles, but not infectious virions, are produced in the presence of 2-deoxyglucose, an inhibitor of glycosylation (Courtney et al., 1973, Ludwig et al., 1974).

Similar results in number and sizes of polypeptides, including the major capsid protein, were obtained in studies of other herpesviruses using similar techniques. Studies of BHV 1 have revealed 18 polypeptides, 29,000-250,000 MW, 8 glycosylated (Sklyanskaya et al., 1977); 21 polypeptides, 31,000-275,000 MW, 10 glycosylated (Pastoret et al., 1980); 25-33 polypeptides, 12,000-330,000 MW, 11 glycosylated (Misra et al., 1981); and 33 polypeptides, 13,000-275,000 MW (Bolton et al., 1983). Dolyniuk et al. (1976) demonstrated 33 polypeptides in the virions of HHV 4. Work on EHV 1 revealed 20 (Abodeely et al., 1971, Killington et al., 1977) to 28 (Perdue et al., 1974, Kemp et al., 1974) structural proteins ranging from 16,000 to 276,000 daltons. Studies on mouse cytomegalovirus

demonstrated 26 polypeptides, while SHV 1 is reported to contain 20 (Stevely, 1975, Killington et al., 1977).

Nonstructural Forty-nine viral-specific proteins, ranging in molecular weight from 15,000 to 280,000, have been detected in HHV 1-infected human cells, at least 16 of these being nonstructural (Honess and Roizman, 1973). Fifty-one proteins were observed in HHV 2-infected cells, of molecular weight 20,000 to 281,000 (Powell and Courtney, 1975). These proteins were grouped into α, β and γ groups, reflecting an interlocking cascade of viral protein synthesis phases (Honess and Roizman, 1974, 1975). The α group seems to consist of nonstructural proteins, and most β proteins are nonstructural as well. Some of the nonstructural proteins have been associated with virus-specific enzymes (Dubbs and Kit, 1964, Powell and Purifoy, 1977), and one HHV 2-induced polypeptide has been detected in HHV 2-transformed cell lines (Flannery et al., 1977). Antisera against HHV 1-infected cells have been shown to inhibit viral enzyme activities in infected cell extracts (Klemperer et al., 1967, Keir et al., 1966). In addition to the 25-33 structural proteins detected in BHV 1, 15 nonstructural proteins were noted in BHV 1-infected cells (Misra et al., 1981).

Inactivation

Although many physical and chemical treatments have been shown to inactivate herpesviruses, those receiving the most study have been heat, ultraviolet irradiation, photodynamic methods, formaldehyde and beta-propiolactone because of their straightforward nature, the ability to vary the treatment dose, and the possibility of genome survival in spite of loss

of lytic infection capability. The problem of genome survival takes on added significance considering the oncogenic potential of herpesviruses.

A biphasic curve of inactivation (i.e., beginning with a plateau or lag phase) of HHV 1 at 37°C has been reported (Kaplan, 1957, Hoggan and Roizman, 1959) and was confirmed for HHV 1 and 5 (Plummer and Lewis, 1965). HHV 1 physical integrity and ability to absorb to mammalian cells were unaffected by 36°C inactivation (Lancz, 1980). Infectivity of HHV 1 and 2 inactivated by heating at 56°C was revealed when cell entry was enhanced by Ca-DMSO ("transfection," Fenyves, 1982). Inactivation of HHV 1 and 2 at 56°C effectively destroyed transforming ability, with lower temperatures being less effective (Rapp and Turner, 1978).

UV-inactivated HHV 1 was shown to produce smaller plaques in tissue culture than unirradiated virus. Pyrimidine dimers and the delay involved in repair was suggested to account for this phenomenon (Ross et al., 1972a). Irradiated virus adsorbs to and penetrates cells as efficiently as unirradiated virus. UV-inactivated HHV 1 was shown to undergo host cell reactivation and multiplicity reactivation (Ross et al., 1972a, Roubal and Vonka, 1973), as expected if pyrimidine dimers underlie the inactivation. Oncogenic transformation by UV-inactivated HHV 1 has been observed *in vitro* (Duff and Rapp, 1971b).

It has been suggested that photodynamic treatment of HHV 1 prevents virus maturation (Khan et al., 1977). Cervical carcinomas were observed in mice following vaginal application of formalin-inactivated HHV 2 (Wentz et al., 1975). SV-40 or polyoma virus was shown to be capable of trans-

formation or tumor formation, respectively, following beta-propiolactone inactivation (Brown et al., 1974, Seemayer and Defendi, 1974).

Immunologic Relationships Among the Herpesviruses

The immunologic relationships between the members of the herpesvirus group have been investigated by a variety of techniques. These investigations have been undertaken for many reasons, including proof of non-identity, construction of taxa, identification of structural elements common to herpesviruses, and identification of type-specific antigens to aid diagnosis.

Figure 1 represents a selection of the positive and negative cross-reaction results reported for herpesviruses. In each case, the homologous system yielded a positive result. No attempt has been made to explore the intricacies of each test system or the possible interpretations of the myriad of results. This subject has been reviewed by Plummer (1964) and Honess and Watson (1977).

Figure 1: Immunologic relationships of herpesviruses

Ag = Antigen preparation; IR = Immune response to virus

HV = Herpesvirus; H = Human; NHP = Nonhuman primate; B = Bovine; A = Alcelaphine; Cp = Caprid;
S = Suid; E = Equid; Cn = Canid; F = Felid

N = Virus neutralization; F = Immunofluorescence; C = Complement fixation;
D = Immunodiffusion; E = Immunoelectrophoresis; X = Other technique

References	Additional Information
1. Aguilar-Setién et al. 1979b	N = Hyperimmunizing BHV 1-N+ cattle with SHV1 increased titers to both
2. Aguilar-Setién et al. 1979a	X = Delayed-type hypersensitivity reactions to BHV 1 and SHV 1 were similar in BHV 1-N+ cattle
3. Aguilar-Setién et al. 1980	X = Counter-immuno-electro-osmophoresis
4. Aurelian 1968	
5. Bartha et al. 1967	
6. Bartha et al. 1966	
7. Belák and Pálfi 1974	
8. Biront et al. 1982	X = Protection against SHV 1 in cattle with BHV 1
9. Blue and Plummer 1973	NHPHV = Saimiriine herpesvirus 2 (Squirrel monkey herpesvirus)
10. Carlson and Scott 1978	
11. Carmichael and Barnes 1961	
12. Castrucci et al. 1981	X = decrease in clinical signs and virus titer upon BHV 2 intravenous or intradermal challenge in cattle
13. Evans et al. 1973	NHPPHV = Saimiriine herpesvirus 2; X = counterelectrophoresis
14. Fabricant and Gillespie 1974	NHPHV = Cercopithecine herpesvirus 1 (B virus); HHV 1 = "HSV"-no further identification given
15. Gutekunst et al. 1978	F = direct and indirect
16. Johnson and Thomas 1966	HHV 1 = "HSV"-no further identification given; BHV 2 = "a bovine teat virus"
17. Kalunda 1975	
18. Killington et al. 1977	
19. Krjukov et al. 1971	
20. Liebermann et al. 1967	
21. Luther et al. 1971	
22. Pauli and Ludwig 1977	X = Immunoprecipitation polyacrylamide gel electrophoresis (IPPAGE)
23. Plummer 1964	NHPHV = Cercopithecine herpesvirus 1
24. Rawls et al. 1968	
25. Rawls et al. 1970	
26. Ross et al. 1972b	F = Indirect; X = virion agglutination
27. Sterz et al. 1974	X = decrease in clinical signs upon HHV 1 challenge in mice
28. Stewart et al. 1965	
29. Studdert 1974	
30. Verwoerd et al. 1979	
31. Watson et al. 1967	NHPHV = Cercopithecine herpesvirus 1
32. Wolff et al. 1979	X = ELISA
33. Yeo et al. 1981	X = IPPAGE; All tests with antiserum against a major DNA-binding protein of HHV 2

IR / Ag	HHV 1	HHV 2	HHV 3	HHV 4	NHPHV	BHV 1	BHV 2	BHV 3	AHV 1	CpHV 1	SHV 1	EHV 1	EHV 2	EHV 3	CnHV 1	FHV 1	FHV 2
HHV 1		+18ND,24N 25N,27NC 26F,33FDX	-32NFCX	+13DEX	+9NF,23N 31ND -9N	+9NF,13D -9N,23NC 27NC	+18ND 27NFCX				+18D,26FX +33X -18N -23N,27NC	+9F,33X -9N,18ND 23NC	-23NC		+4N	-16N	
HHV 2	+18ND,24N 25N,26F 33X					+18N -27	+18D,27NF				+18D,26F 33X -18N	+33X -18ND					
HHV 3	-32NFCX																
HHV 4	+26F		+26F			+13DE						+26F					
NHPHV	+9NF,23N 31ND -9N			+13D		+9F,13D -9N						+9F -9N					
BHV 1	+9NF -9N,22NCX 23NC,27NC			+13DEX	+9F -9D,23D		-27NC	-5N,7N,19N	-17N	-30N	+1N,3X -3ND,5N 23N,27NC	+11CD -9F,11N 23NC	-23NC			-10F,16N	
BHV 2	+12X,18ND 17NCX 27NCD,33X	+12X,18ND 27NCD 33FDX				-27NCD		-7N	-17N	-30N	+18D,33X -18N, 27NCD	+33X -18ND				-26N	
BHV 3	-21N					-5N,6N,7N 19N,20N 21N	-7N,21N		-5N,6N, 20N,21N		-5N,6N,7N 20N,21N	-7N					
AHV 1						-17N	-17N										
CpHV 1	+18D,22X26 FX,31D,33X -18N,22N 23NC,27NC					-30N +2X,3NX 13DE,23C -3D,5D,8X 23N,27NC	-30N					-30N					
SHV 1		+18D,26F 33FDX -18N,31N		+13DEX	+31D -23N,31N		-18ND, 27NC	-5N,7N				+18D,33X -18N,23N	-23NC				
EHV 1	+9F,18D +33X -9N -18N,23N	+18D, 33FDX -18N		-13D	+9F -9N,23N	+11CD,23C -9NF,11N 13D,23N	-18ND	-7N		-30N	+18D,33X -18N,23N		+23C -15NFCD 23N,29N	+15FCD -15N,29N		-10F	
EHV 2	-23NC				-23N	+23C -23N					-23N	-15NCD 23NC,29N		-15NFCD, 29N		-10F	
EHV 3												+15NFCD -15N,29N	-15NCD, 29N			-10F	
CnHV 1	+4N					-28N					-28N	-28N					
FHV 1																	
FHV 2	-14N				-14N	-14NF	-14N				-14NF				-14N	-10E,14NF	

PART I. THE EFFECT OF SOME COMMON INACTIVATION PROCEDURES ON THE ANTIGENS OF BOVINE HERPESVIRUS 1

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THE EFFECT OF SOME COMMON INACTIVATION PROCEDURES ON THE ANTIGENS OF BOVINE HERPESVIRUS 1

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ABSTRACT

Bovine embryonic kidney cells were infected with bovine herpesvirus 1 (BHV 1) or were sham-inoculated. When cytopathic effect was apparent, the cells were treated with beta-propiolactone, formalin, heat (56°C), or ultraviolet irradiation until the virus was inactivated. Infected-treated, infected-untreated (IU), and sham-inoculated cultures were solubilized using Triton X-100 detergent. Resulting preparations were tested by two-dimensional and fused rocket immunoelectrophoresis and were evaluated for their ability to inhibit virus neutralization by BHV 1 antiserum.

Eleven viral antigens were detected consistently in IU preparations, which strongly inhibited virus neutralization. Eight or more IU antigens were detected in beta-propiolactone-treated, formalin-treated, and heat-treated preparations; these inhibited virus neutralization less strongly than the IU preparations. No IU antigens were detected in ultraviolet-treated preparations, nor did this material inhibit virus neutralization. One of the IU antigens was reduced preferentially by all treatments. The selective destruction of antigens by the various treatments might allow antigen-specific serologic testing to distinguish vaccinated from naturally-exposed cattle.

INTRODUCTION

A variety of chemical and physical treatments, including beta-propiolactone (BPL), formalin, heat, and ultraviolet (UV) irradiation is used to inactivate viruses. Inactivated virus is often used for viral characterization, in vitro immunologic tests, and preparation of vaccines because many treatments eliminate infectivity, but not antigenicity or other characteristics.

The immunogenicity of inactivated bovine herpesvirus 1 (BHV 1) has been studied extensively in cattle. Formalin-inactivated (Schipper and Kelling, 1975) and nonionic detergent-solubilized (Lupton and Reed, 1980) BHV 1 vaccines without adjuvant lacked efficacy as measured by induction of virus neutralizing (VN) antibodies. However, vaccination of cattle with two doses of adjuvanted BHV 1 which had been inactivated with formalin (Matsuoka et al., 1972), nonionic detergent (Lupton and Reed, 1980), ethanol (Haralambiev, 1976), and heat or UV (Hristov and Karadjov, 1975) induced production of VN antibodies. In these studies, formalin or nonionic detergent-inactivated (parenteral) vaccines protected cattle from virulent BHV 1 intranasal challenge. Ethanol-inactivated vaccine was injected into the nasal submucosa and also had protective activity.

Among the herpesviruses, herpes simplex viruses 1 and 2 (human herpesviruses 1 and 2, HHV 1 and 2) have received the most extensive study relative to viral properties following inactivation. Inhibition of serum neutralization of HHV 1 by nonionic detergent-solubilized HHV 1-infected cell cultures was unchanged by formalin treatment, but was reduced by temperatures greater than 44°C for 1 hour (Kutinova et al., 1977). Lancz

(1980) demonstrated that HHV 1 inactivated by heating at 36°C retained gross physical integrity and mammalian cell adsorbtion activity. This relative stability of adsorptive activity also was demonstrated for HHV 1 by transformation assays of virus preparations inactivated by heat and UV (Rapp and Turner, 1978). Exposure of cell cultures to virus preparations inactivated by limited treatment resulted in transformation. The transformed cell cultures were virus-free, but expressed viral antigens. However, more rigorous treatment eliminated transformation activity. Equine herpesvirus 1 inactivated by combined BPL and UV treatment was reported by Campbell et al. (1982) to stimulate a secondary serological response in horses as measured by VN, but not a primary response in mice or rabbits.

Detergent-solubilized virus preparations have been used to enumerate and partially characterize the antigens of several herpesviruses. Sklyanskaya et al. (1977), using polyacrylamide gel electrophoresis (PAGE), detected 18 polypeptides (8 glycosylated) in BHV1. Using PAGE with sodium dodecyl sulfate (SDS-PAGE), Pastoret et al. (1980) reported 21 polypeptides (10 glycosylated) in BHV 1, Misra et al. (1981) reported 25 to 33 polypeptides (11 glycosylated) in BHV 1 and 15 nonvirion polypeptides in BHV 1-infected cells, and Bolton et al. (1983) reported 33 polypeptides in BHV 1. The reports concerning BHV 1 are comparable to the report of 33 polypeptides in HHV 1 (13 glycosylated) by Heine et al. (1974). Eleven precipitating antigens (4 glycosylated) were detected in HHV 1- and 2-infected cell cultures by two-dimensional immunoelectrophoresis (2DIE) (Vestergaard, 1973). Twenty precipitating antigens (8 glycosylated) were

detected in cell cultures infected with suid herpesvirus 1 (SHV 1) using this technique (Platt, 1982).

The development and testing of inactivated vaccines, as well as the evaluation of host animal immunologic responses, is dependent on the identification of viral antigens and the effect of common inactivation procedures. Identification and *in vitro* measurement of the protective antigen(s) would permit inactivation procedures to be optimized for vaccine production and would aid immunogenicity testing of the final product. If any antigen is selectively reduced or eliminated by inactivation, antigen-specific serologic testing might distinguish vaccinated from naturally-exposed host animals.

Some effects of BHV1 inactivation by BPL, formalin, heat, and UV were examined in this study. Effects on individual antigens were measured by 2DIE and fused rocket immunoelectrophoresis (FRIE) of treated and untreated BHV 1-infected cell cultures which had been solubilized by nonionic detergent. Effects on immunogenicity were measured by plaque reduction serum neutralization inhibition tests (PRSNIT) of these solubilized preparations.

MATERIALS AND METHODS

Virus

BHV 1 (Cooper strain) of low and high cell passage was used in this study. Low cell-passage viruses were standard NVSL BHV 1 challenge viruses and elicited signs of BHV 1 infection in calves exposed intranasally. High cell-passage BHV 1 was provided by Dr. G. L. Seawright, Los Alamos Scientific Laboratory (LASL), Los Alamos, New Mexico.

Cell Cultures

Cultures of third to fifth passage embryonic bovine kidney cells (EBK) were grown to confluency in 490 cm^2 roller bottles (Corning Glass Works, Corning, NY) using Eagle's minimum essential medium (EMEM) with Earle's salts (Grand Island Biological Company, Grand Island, NY), 10% bovine fetal serum, 1% L-glutamine, 0.1 mg/ml streptomycin sulfate, and 0.025 U/ml penicillin G potassium.

Virus Propagation and Inactivation

Cultures to be infected were inoculated using 5 to 12 ml/bottle of a suspension of BHV 1 (multiplicity of infection between 1 and 4) in EMEM, with 1% L-glutamine, 0.05 mg/ml gentamicin sulfate and 0.0025 mg/ml amphotericin B. Sham-inoculated (SI) cultures were inoculated with suspension medium only. After 60 to 90 minutes at 37°C, 0 to 7 ml/bottle of additional serum-free medium was added. When the cytopathic effect in the infected cultures reached 50 to 90% (16 to 32 hours), cells and supernatant fluids were harvested. Aliquots of the harvested suspensions were used immediately for treatment or stored at 4°C.

The BPL inactivation was effected by adding a 10% solution of BPL (Sigma Chemical Company, St. Louis, MO) to a final concentration of 0.2%, followed by the addition of 1 N NaOH to a final concentration of 0.02 N. Inactivation was allowed to proceed for 72 hours at 37°C in a container with a loose cap. Formalin inactivation was accomplished by adding formalin solution (37% formaldehyde, Mallinckrodt, St. Louis, MO) to a final concentration of 0.1% formaldehyde, stirring the suspension for 72 hours at room temperature, and adding 35% sodium bisulfite to a final concentration of 0.035%. Heat inactivation was effected by placement in a 56°C water bath for 60 minutes, with stirring at 15-minute intervals. The UV inactivation was accomplished by exposure to a Westinghouse Sterilamp 782L-30 bulb for 8 to 12 hours at an average dose of 10,000 to 15,000 ergs/sec/mm^2 as measured by a Blak-Ray ultraviolet intensity meter (Ultra-Violet Products, San Gabriel, CA) while constantly stirring in two 150 x 25 mm plastic tissue culture dishes (≈35 ml/dish).

Following treatment, samples of each aliquot were plaque titrated on EBK in 24-well cluster plates (Costar, Cambridge, MA) to confirm virus inactivation or absence of live virus in uninfected cultures. The infected-untreated (IU) aliquots contained 10^7 to 10^9 plaque forming units (PFU)/ml.

Antigen Preparation

The procedure followed was similar to that of Vestergaard (1973). Harvests were centrifuged for 4.5 hours at 36,000 X g. The pellets were resuspended in 3 to 4 volumes of either of two buffer solutions; glycine-Tris buffer, pH 9.0 (as utilized by Vestergaard and Bog-Hansen,

1975) containing 1% octylphenoxy polyethoxyethanol (Triton X-100, Sigma Chemical Company, and Fisher Scientific, Fairlawn, NJ), or Tris-Tricine buffer, pH 8.6 (buffer IV, Monthony, BioRad Laboratories, Richmond, CA), containing 1% (immunoelectrophoresis buffer, IEB) or 5% Triton X-100. These suspensions then were sonicated for four 15-second periods on ice with a Branson Sonifier, Model 3600, with microtip at a setting of 6. This was followed by 16 to 32 hours of agitation on a circular shaker at 100 to 250 rpm. The suspensions were centrifuged at 100,000 X g for 1 to 2 hours, and the supernatant fluids, comprising the antigen preparations, were harvested and stored at 4°C.

Four separate sets of antigen preparations were made and designated I, II, III, and IV. Set I was made with low cell passage virus, reconstituted from the pellet with glycine-Tris buffer, and consisted of an IU preparation only, which was used in 2DIE and PRSNIT tests. Set II also was made with low cell passage virus, reconstituted with glycine-Tris buffer, and consisted of IU, SI, and three treated preparations (formalin, heat, and UV), which were used in 2DIE, FRIE, and PRSNIT tests. Set III was made with low cell passage virus, reconstituted with Tris-Tricine buffer, 5% Triton X-100, and consisted of IU, SI, and three treated preparations (BPL, formalin, and heat), which were used in 2DIE, FRIE, and PRSNIT tests. Set IV was made with high cell passage virus and reconstituted with IEB. It consisted of IU and SI preparations, which were used as references in FRIE tests because of the similarity of IU-IV and IU-II patterns.

Antiserum Production and Globulin Preparation

The BHV 1 antiserum was produced by hyperimmunization of four cattle through intranasal exposure and intramuscular injection of low passage live BHV 1 (Cooper strain) without adjuvant. Serum obtained at intervals during the immunization schedule was fractionated by a modification of the anion-exchange chromatographic procedure of Stanworth (1960) using DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, NJ). The globulin preparations were concentrated to 25% of the original serum volume by pervaporation, filtered through a 0.45 micron filter, and pooled. A similar globulin preparation was made from anti-BHV 1 negative bovine fetal serum. Two antisera specific for a limited number of BHV 1 antigens were produced in calves by intramuscular injection with adjuvanted 2DIE gel sections containing specific antigen-antibody arcs (unpublished observations, D. E. Reed, Veterinary Medical Research Institute, Iowa State University). Globulin preparations were produced from these antisera by ammonium sulfate precipitation. One serum, "VN-", was specific for one antigen in this system, and lacked VN activity. The other serum, "VN+", was specific for four other antigens and had a VN titer of 64.

2DIE Procedure

The procedure followed was similar to that of Vestergaard (1973). Agarose gel was prepared from commercial immunoelectrophoresis tablets (BioRad Laboratories) by heating in water containing 1% Triton X-100. First dimension electrophoresis was performed on glass slides using IEB. Gel sections then were transferred to agarose support films (LKB Instruments, Rockville, MD) and 1% agarose gel, 48°C, containing 35%

anti-BHV 1 globulin, was pipetted onto the remaining area of the film. Following second dimension immunoelectrophoresis, films were stained with 0.2% Coomassie Brilliant Blue R-250 (BioRad Laboratories).

A modification of the 2DIE procedure described by Axelson and Bock (1972) was also used. The 2DIE with antigen addition (Ag-add) was performed by placing the preparation to be tested in a well centered below the well containing IU preparation prior to first dimension electrophoresis.

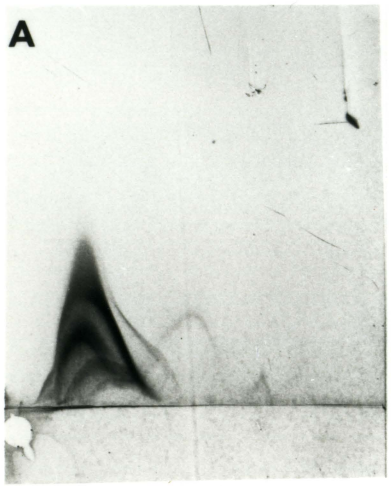
FRIE Procedure

Gels were cast on glass slides, cut, and transferred to agarose support films. Globulin-containing gel then was pipetted onto the remainder of the film, and wells were cut below the globulin-containing gel. Antigen preparations were pipetted into the wells, and immunoelectrophoresis was performed 1 to 3 hours later.

Evaluation of 2DIE and FRIE Results

Only arcs that were consistently reproduced and did not appear to be diffusion artifacts of other arcs (see Figure 1-B, antigens 5, 10, and 11) were used for analysis. Some unusual arcs were observed, complicating the evaluation. Examples of this are the connecting cathodal and anodal arcs of antigen 3. The anodal arc of antigen 3 and the anodal side of the arc of antigen 4 of Set I were used for evaluation. In some cases, a heavy arc was seen which was determined to represent two antigens, and was evaluated as such. In other cases, arcs were seen that could represent either or both of two antigens, and so were not used in evaluation. Correlation of

Figure 1. 2DIE patterns of reactions between infected-untreated or sham antigen preparations and BHV1 antiserum or bovine fetal serum globulin preparations A - Infected-untreated preparation (II) with anti-BHV 1, B - infected-untreated preparation (I) with anti-BHV 1, C - sham preparation (II) with anti-BHV 1, D - infected-untreated preparation (II) with bovine fetal serum.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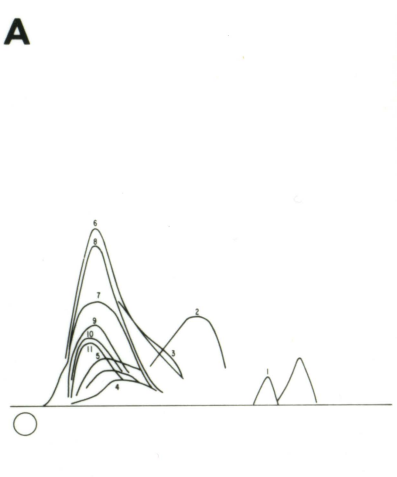
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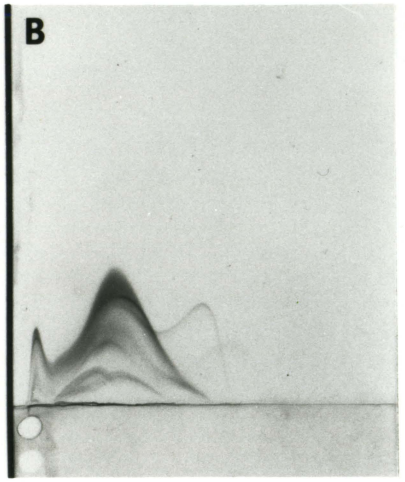
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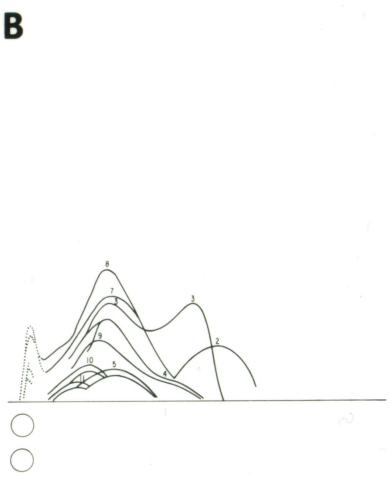
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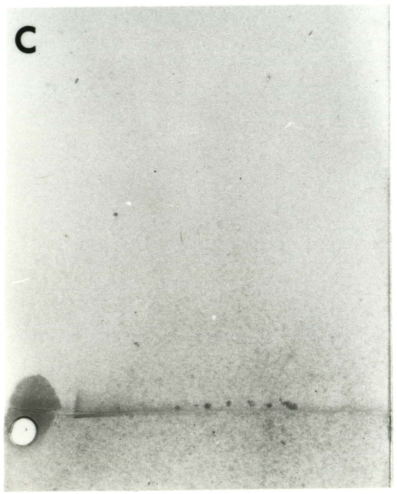
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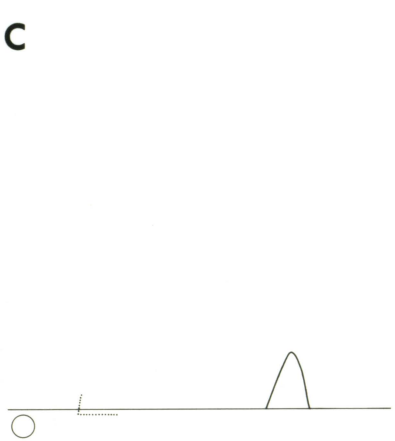
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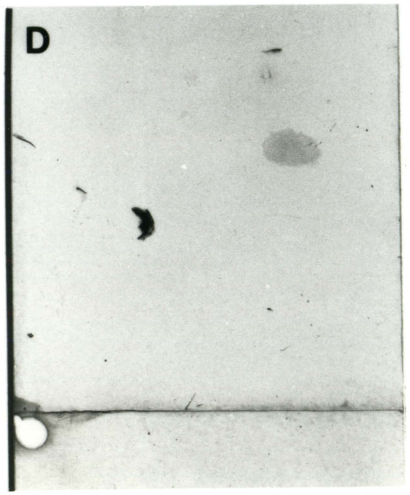
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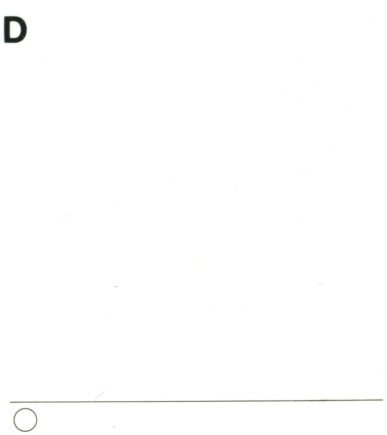

D

FRIE and 2DIE results was accomplished by performing 2DIE with first dimension electrophoresis times varying from 0 to 30 minutes.

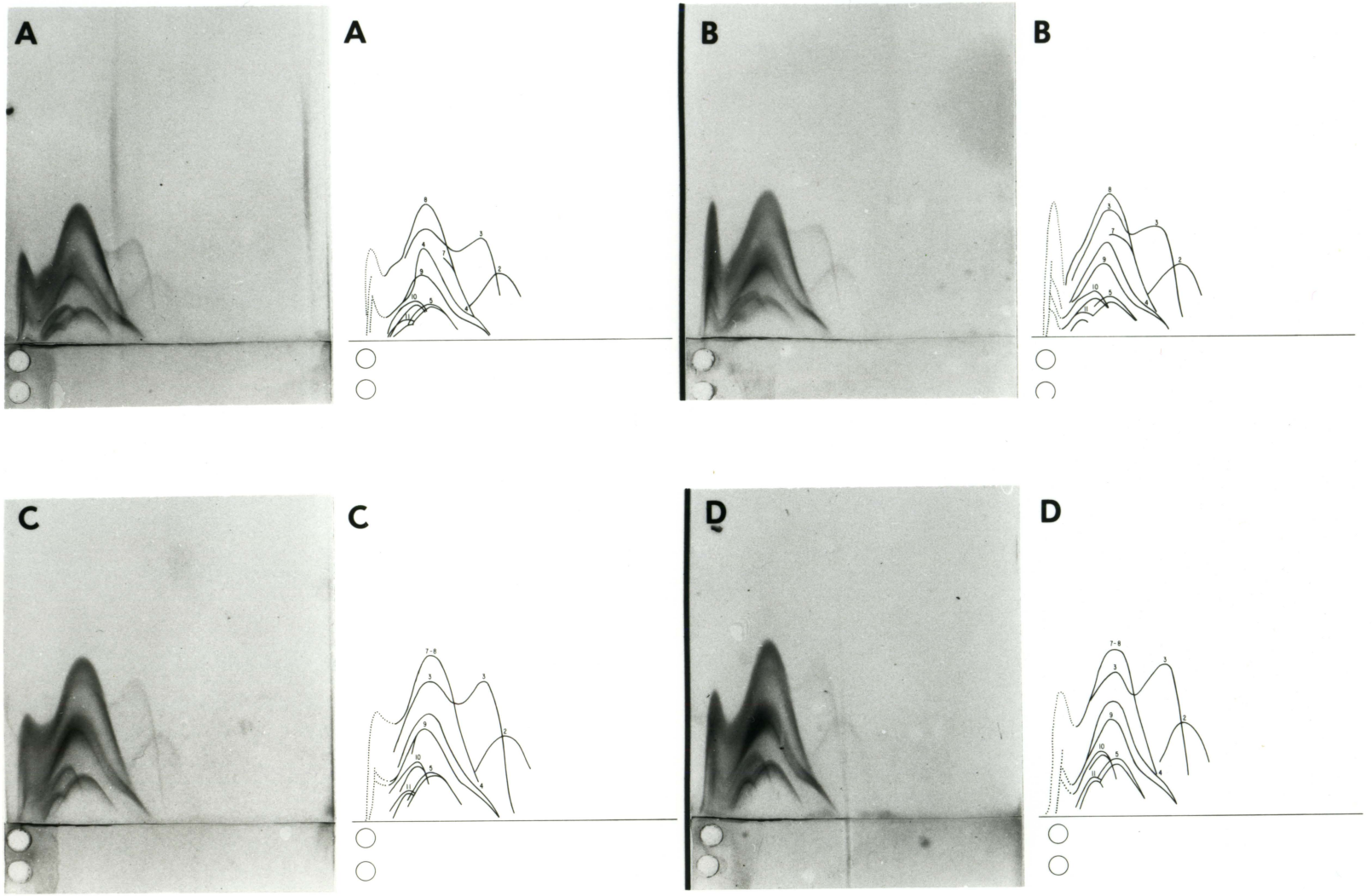
The viral antigens that survived were identified by increases in arc area when treated antigen preparations were added to IU 2DIE tests (Ag-add). This antigenic enhancement, as evaluated by superimposition of test and control films, was assigned relative values, "+" representing up to 25% of the enhancement produced by IU preparations (Figure 2-A), "++" representing 25 to 50% (Figures 2-A and 2-B), and "+++" representing greater than 50% (Figures 2-C and 2-D). For FRIE tests, the relative areas of fused rocket arcs demonstrating the reaction of identity were used. Quantities of antigen were again assigned relative values of "+", "++", and "+++". However, due to loss of definition in rocket patterns with dilution of control preparations, a calibration for these symbols was not possible.

PRSNIT Procedure

A modification of the technique described by Appleyard et al. (1964) and Cohen et al. (1972) was used. Dilutions of the preparation to be tested were incubated with equal volumes of a 1/30 dilution of BHV 1 antiserum for 1 hour at 37°C with agitation each 15 minutes. An equal volume of live BHV 1 suspension (Cooper strain) was then added, followed by another 1-hour incubation at 37°C with agitation each 15 minutes.

Following removal of the medium, 2- to 3-day old confluent EBK cell cultures in 24 well cluster cultures were inoculated with 0.1 ml/well. These were incubated at 37°C for 1 hour with agitation each 15 minutes, followed by addition of 0.5 ml of 1% gum tragacanth (Fisher Scientific, Fairlawn, NJ) in Earle's MEM. After 48 hours of incubation at 37°C, the

Figure 2. Enhancement of infected-untreated 2DIE arc areas by addition of homologous antigen (Ag-add 2DIE) Top well; A-D - infected-untreated preparation (I). Bottom well; A-D - infected-untreated preparation (I) diluted 1:4, 1:2, 3:4, and 1:1, respectively (see Figure 1-B).


A
A
B
B
C
C
D
D

plates were stained with crystal violet-formalin solution, washed, dried, and the microplaques counted using a dissection microscope.

The antiserum control consisted of equal volumes of diluent and 1/30 antiserum, and the virus control consisted of equal volumes of diluent and 1/30 bovine fetal serum. The virus suspension was standardized to yield 45 to 60 plaques/well, and the dilution of antiserum selected to neutralize 67% of the virus. For evaluation, the average virus control plaque count (e.g., 60, or 0% VN) was defined as 100% inhibition of VN activity of the antiserum. The average antiserum control plaque count (e.g., 20, or 67% VN) was defined as 0% inhibition of VN activity in the antiserum. The dilution of antigen preparation which yielded plaque counts halfway between these two points (e.g., 40, or 33% VN) would be responsible for inhibiting 50% of the VN activity in the antiserum. This dilution, calculated by arc-sine regression, was defined as the 50% PRSNIT titer. The SI plaque counts were used to correct for toxicity of the preparations.

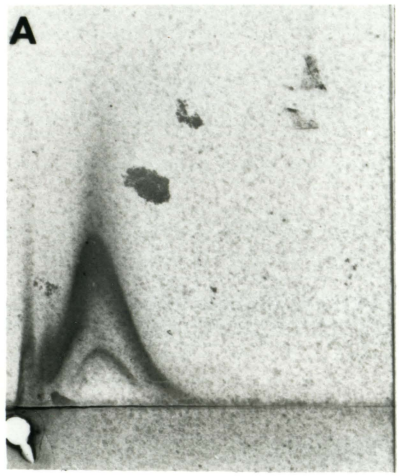
RESULTS

Twelve antigen-antibody arcs were repeatedly produced in IU 2DIE tests (Figure 1-A). One of these arcs is apparently cell-associated, as it was observed using SI, Set II (Figures 1-A and 1-C). However, the remaining 11 arcs were clearly virus-specific, as none of the arcs were due to precipitation by normal serum components (Figure 1-D). Other precipitation lines were observed infrequently, and were not used in this study. The IU 2DIE patterns showed some variation in the number of arcs detected, but those detected were very similar in position (Figures 1-A, 1-B, and 3-A).

When antigens corresponding to IU antigens were present in treated preparations, their presence in the Ag-add procedure resulted in an increase in area under the respective arc (Figure 2); this permitted the identification of reactive antigens following the various treatments. When BHV 1 globulin was used in 2DIE with the antigen preparations of Sets II and III, six arcs were observed with BPL-treated, five with formalin-treated, four with heat-treated, and three with UV-treated preparations (Figure 3). These same antigen preparations were added to IU 2DIE tests (Figure 4), with enhancement of arc areas as described in Table 1.

A FRIE test of Set II preparations was performed using Set IV as a control (Figure 5). This test demonstrated the large amounts of many IU antigens present in formalin-treated preparations, the small amounts of many IU antigens in heat-treated preparations, and the fact that none of the antigens in the UV preparations were identical to IU antigens.

Figure 3. 2DIE patterns of reactions between treated antigen preparations and BHV 1 antiserum globulin preparations A - BPL treated preparation (III), B - Formalin treated preparation (III), C - heat treated preparation (III), D - UV treated preparation (II) (see Figures 1-A and 1-B).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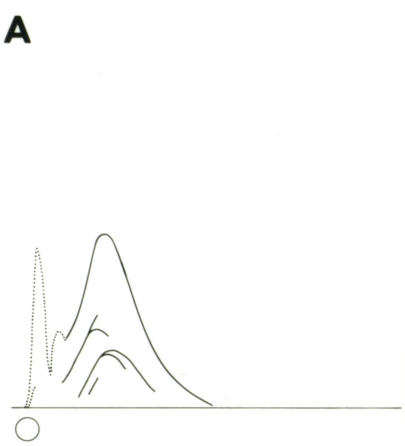
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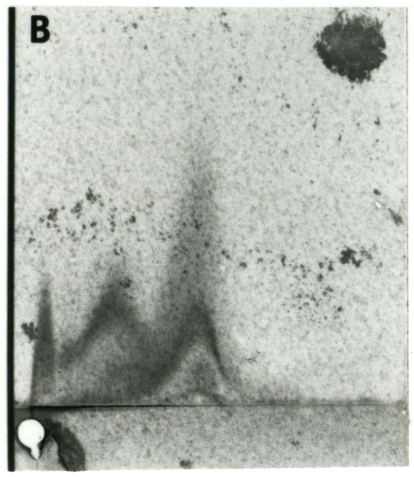
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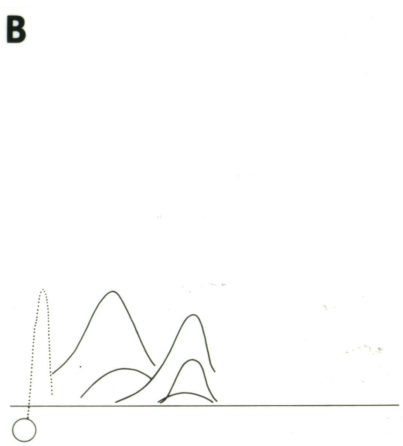
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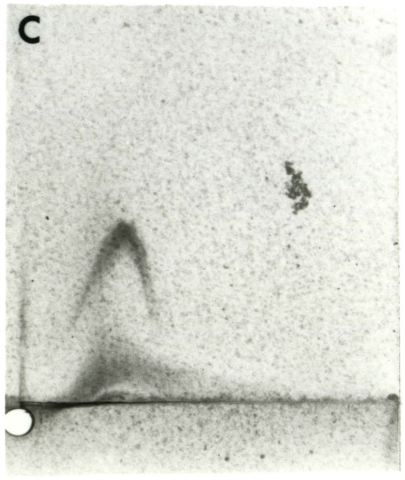
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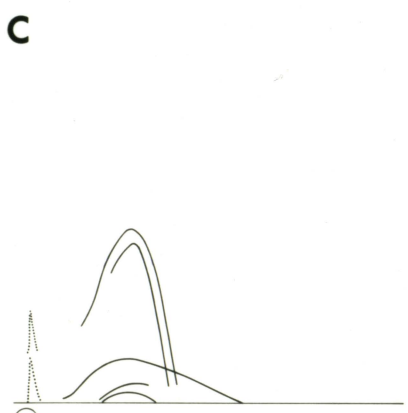
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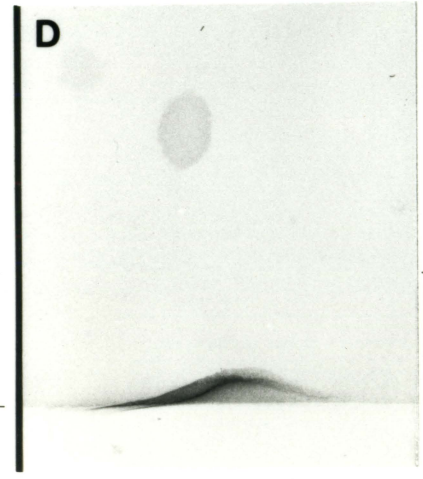
D

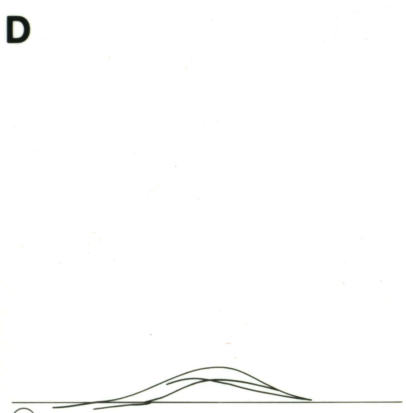
D

Figure 4. Identification of infected-untreated preparation antigens in treated preparations by enhancement of infected-untreated 2DIE arc areas (Ag-add 2DIE) Top well; A-D - infected-untreated preparation (I). Bottom well; A - BPL-treated preparation (III), B - formalin-treated preparation (III), C - heat-treated preparation (III), D - UV-treated preparation (II) (See Figure 1-A).

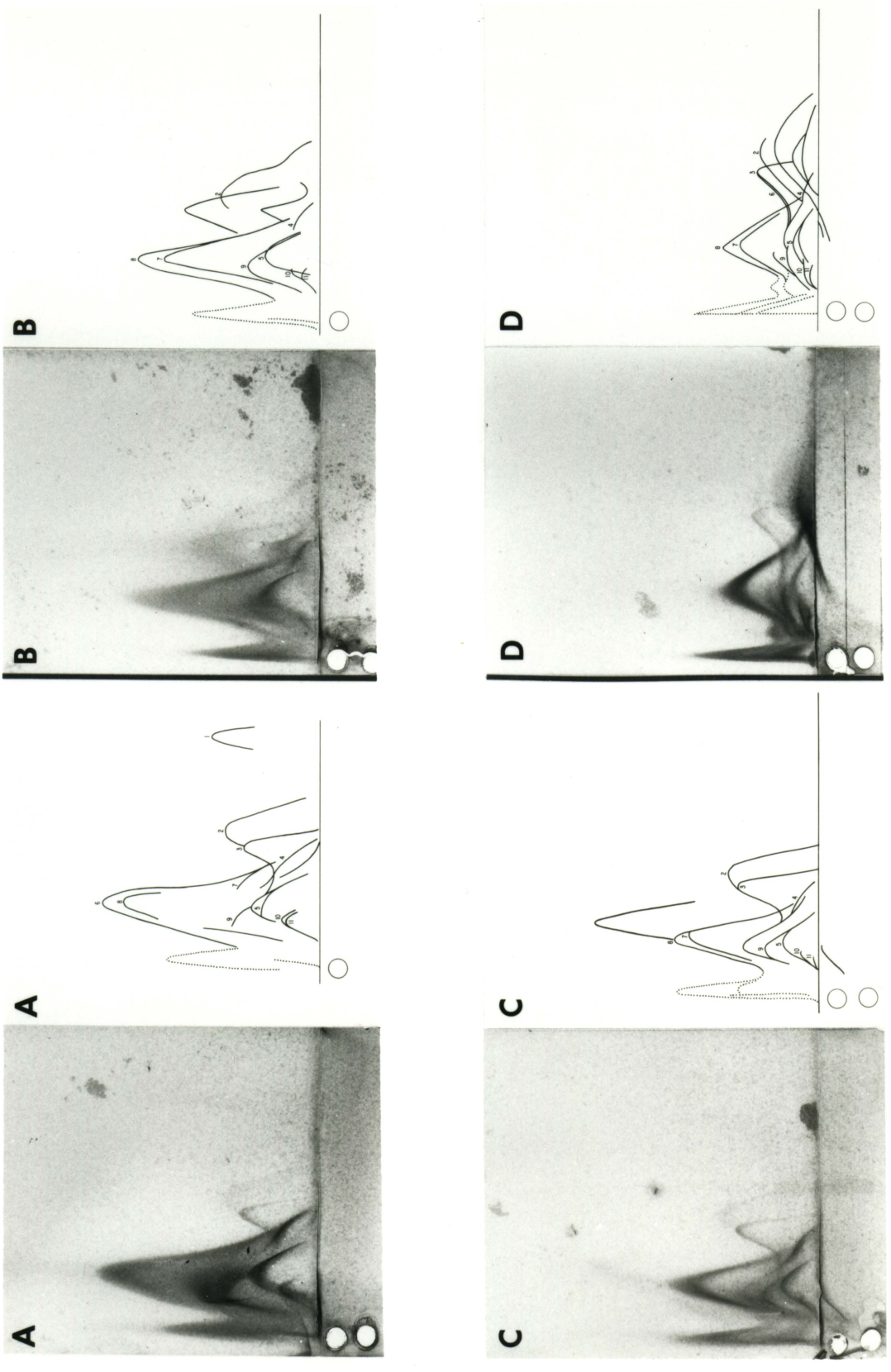
A
A
B
B
C
C
D
D

Figure 5. FRIE patterns of reactions between sham, infected-untreated, or infected-treated antigen preparations and BHV 1 antiserum globulin preparations A - sham preparation (II), B, C, E, G, - infected-untreated preparation (IV). D - formalin treated preparation (II), F - heat treated preparation (II), H - UV treated preparation (II).

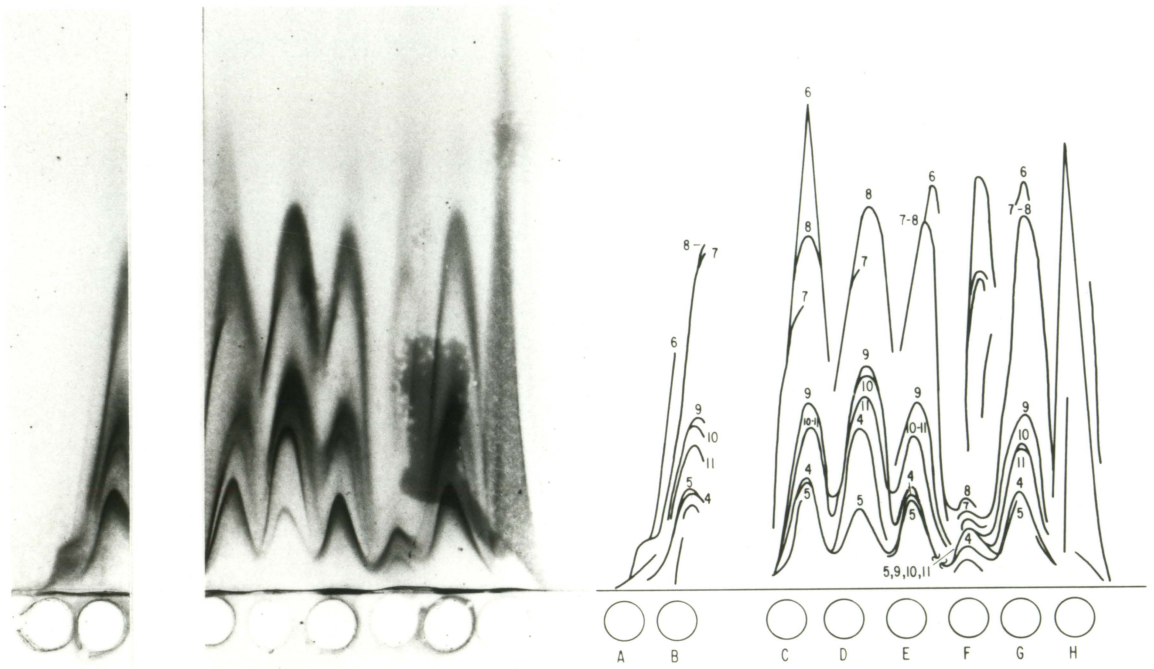
A
B
C
D
E
F
G
H
5,9,10,11

Table 1. 50% Plaque reduction serum neutralization inhibition test (PRSNIT) titers and relative antigen reactivities in two-dimensional immunoelectrophoresis with antigen addition of BHV 1-infected cell suspensions following inactivation of virus by various procedures

Treatment	PRSNIT Titer	% of titer (infected-untreated)	BHV 1 Antigen Specificities										
			1	2	3	4	5	6	7	8	9	10	11
Sham-inoculated	0	0	Neg[a]	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Infected-untreated	4300	100	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
BPL[b]-treated	1500	35	+++	++	Neg	+++	+++	NA[c]	+++	+++	+++	NA	+++
Formalin-treated	2500	58	+++	+++	+	++	++	NA	+++	+++	+++	+++	+++
Heat-treated	600	14	NA	+++	Neg	+	+	NA	+	+	+	+	+
UV[d]-treated	0	0	NA	Neg	Neg	Neg	Neg	NA	Neg	Neg	Neg	Neg	Neg

[a]Neg, +, ++, +++ = Antigen reactivities relative to infected-untreated preparation.

[b]BPL = Beta-propiolactone.

[c]NA = (Data) not available.

[d]UV = Ultraviolet radiation.

When treated antigen preparations were tested by FRIE with "VN+" (specific for IU antigens 2, 6, 7, and 8) two arcs were produced by BPL-treated, formalin-treated and heat-treated preparations, whereas only one arc was produced by the UV-treated preparation.

"Averaged" reactivities of individual antigens, as determined by 2DIE and FRIE tests illustrated in Figures 4 and 5, and averaged PRSNIT titers for the antigen preparations of Sets I, II, and III are presented in Table 1. The lability of antigen 3 and the relative stability of antigen 2 are evident in this table. The marked reduction in antigenicity resulting from UV- or heat-treatment is also notable.

DISCUSSION

Much of the work on inactivation of viruses has focused on the effect of treatment on the properties of intact virions, including infectivity (Griffin et al., 1958), absorption (Lancz, 1980), enzyme induction (Eglin et al., 1980), antigen induction (Ross et al., 1971), and immunogenicity (Matsuoka et al., 1972, Hristov and Karadjov, 1975, Schipper and Kelling, 1975, Cappel, 1976), without examining the effects on individual antigens. However, much of the work on individual proteins or antigens has focused on enumeration and characterization of these structures in virion or infected cell preparations (Vestergaard, 1973, Heine et al., 1974, Misra et al., 1981, Platt, 1982, Bolton et al., 1983), rarely examining their lability to common inactivation procedures or their roles except in regard to protection. The interface of these two emphases is important for the in vitro evaluation of inactivated vaccines, and for the differentiation of animals vaccinated with these products from those naturally exposed to the agent.

This study examined the effects of common inactivation procedures on individual antigens (as measured by 2DIE and FRIE) and antigen reactivity (as measured by the PRSNIT test) to identify similarities and differences between treatments and to identify any antigens selectively diminished by all treatments. Antigenic analysis following solubilization with one agent is clearly not exhaustive. Some antigens may have been destroyed and others not exposed by the solubilization procedure.

This information may be used to select the method of inactivation for the use of the viral preparation. The BPL and formalin treatments are

practical commercial techniques and formalin-inactivated vaccine is marketed in the United States. Most antigens were largely retained by either treatment. The PRSNIT titers were reduced, but retained significant activity compared to the control (averaging 30 to 60% activity). Of the treatments studied, BPL and formalin treatments are the most desirable for antigen survival and retention of immunogenicity. Heat treatment is a commonly used method for inactivation of viruses for experimentation, but this treatment markedly diminished PRSNIT titers (averaging 10 to 20% of control values). Heat treatment was relatively nonselective in its extensive diminution of BHV 1 antigens, sparing one.

The marked damage by UV was observed consistently and is probably due to the high dosages required for complete inactivation in this study. These extreme dosages may have been necessitated by the turbidity and high virus concentration of the suspensions treated. The turbidity of the suspensions, which were concentrated solutions of infected cell scrapings, reduced the penetration of the UV radiation. The high virus concentration in the suspensions resulted in the equivalent of high multiplicity of infection in tests for inactivation. The survival of UV-irradiated HHV 1 was reported to be increased at higher multiplicities of infection (Hall et al., 1980). This survival apparently was due to genetic exchanges between damaged HHV 1 genomes, allowing production of undamaged virus. Thus, higher doses of radiation may have been required for complete inactivation of these suspensions than would have been necessary for suspensions of lower turbidity or virus concentration.

The difficulties of inactivation and the resulting requirement of high doses (which in turn may have caused secondary chemical changes in the environment of the virions) may mean most virion proteins had undergone degradation before all the virions had been inactivated. This degradation may mean that the four antigens in the UV-treated preparation which did not cross-react with IU antigens are internal antigens of the complex IU antigens. Although not exposed by Triton X-100 treatment, these antigens would be exposed by in vivo antigen processing, so hyperimmune serum should be expected to contain antibodies against them, as was demonstrated. Treatment with UV radiation therefore eliminated IU antigens, while retaining other precipitating viral antigens.

The differences in stability between antigens relative to antigenicity as measured by PRSNIT titers may be important for the evaluation of multi-component vaccines containing inactivated BHV 1. Quantitation of a labile antigen (e.g., Antigen 3) in comparison to a stable antigen (e.g., Antigen 7) could provide a measure of inactivation of the BHV 1 fraction even in the presence of other fractions, which might prevent direct assays for live virus. The assay for the stable antigen could at the same time provide a measure of the BHV 1 antigenic mass of the product.

The results of this study also indicate that serologic testing relative to selected antigens might differentiate uninfected BHV 1 vaccinates from naturally-infected animals. If Antigen 3 is reduced and altered by virus inactivation procedures to the point that it is no longer immunogenic, then antibodies to this antigen would not be expected in the serum of vaccinates. In contrast, animals exposed naturally to infectious BHV 1

would be expected to have antibodies to this antigen in their serum. This potential differentiation assumes that inactivated vaccines are used, that the reduction of reactivity of Antigen 3 as measured by in vitro testing is a reflection of in vivo reactivity, and that naturally-exposed animals possess detectable antibodies to Antigen 3. It also assumes that Antigen 3 does not possess significant cross-reactivity with other infectious agents. The most likely agents to contain an antigen cross-reacting with Antigen 3 of BHV 1 are other bovine herpesviruses. In a study of three other herpesviruses of cattle, BHV 2, BHV 3, and SHV 1, only SHV 1 contained an antigen cross-reacting with Antigen 3 (Part II, Levings et al., 1984b). This should not interfere with diagnosis of natural exposure to BHV 1, because with rare exceptions, SHV 1 infection is fatal in cattle (Hagemoser et al., 1978, Toma and Gilet, 1978, Bironf et al., 1982). Testing for antibodies specific for Antigen 3 should therefore allow concurrent vaccination and eradication efforts, making eradication of the diseases caused by BHV 1 much less costly and more practical.

PART II. CROSS-REACTIONS OF BOVINE HERPESVIRUS 1 ANTIGENS WITH THOSE OF OTHER CATTLE HERPESVIRUSES

This manuscript has been submitted to Veterinary Microbiology.

CROSS-REACTIONS OF BOVINE HERPESVIRUS 1 ANTIGENS WITH THOSE OF OTHER CATTLE HERPESVIRUSES

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ABSTRACT

Bovine embryonic kidney cells were infected with bovine herpesviruses (BHV 1, 2, or 3), suid herpesvirus 1 (SHV 1), or were sham-inoculated. When cytopathic effect was apparent, the cells were solubilized using Triton X-100 detergent. Resulting antigen preparations were tested by two-dimensional immunoelectrophoresis using bovine fetal serum and antisera directed against BHV 1, BHV 2, BHV 3, SHV 1, or a restricted spectrum of BHV 1 antigens.

Interaction of BHV 1 antiserum with BHV 1 antigen preparations resulted in 11 precipitation arcs. The same antiserum produced three arcs with BHV 2, none with BHV 3, and five with SHV 1. The interaction of BHV 1 antigen preparations with BHV 2, BHV 3, or SHV 1 antisera failed to produce demonstrable arcs. However, when heterologous antigen or antibody preparations were added to BHV 1 homologous two-dimensional immunoelectrophoresis tests, all 11 BHV 1 arcs were modified by BHV 1, two by BHV 2, four by BHV 3, and four by SHV 1 preparations. Two antigens were common to the four herpesviruses.

Antigen preparations were evaluated for their ability to inhibit virus neutralization by BHV 1 antiserum; only the BHV 1 preparation was active. Sera were tested for BHV 1 neutralizing activity; only BHV 1 antiserum and a serum specific for a restricted spectrum of BHV 1 antigens were active. A glycoprotein antigen associated with BHV 1 neutralization was identified; this antigen may be important in protection of animals against disease.

INTRODUCTION

A variety of herpesviruses infects cattle, including infectious bovine rhinotracheitis virus (bovine herpesvirus 1, BHV 1), bovine mammillitis virus (bovine herpesvirus 2, BHV 2), Movar virus (bovine herpesvirus 3, BHV 3), pseudorabies virus (suid herpesvirus 1, SHV 1), and malignant catarrhal fever virus. The nomenclature applied to these viruses has been inconsistent; the system proposed by Roizman et al. (1981) is used here.

The immunologic relationships between these herpesviruses have been studied by a variety of experimental approaches. However, the relationships of bovine herpesviruses remain poorly defined (Killington et al., 1977), and it has been observed that "the number and kinds of shared components by any given herpesvirus depends on the methods of antigen preparation and the sensitivities of the tests for each particular system" (Evans et al., 1972).

The antigenic relationship of BHV 1 to other BHVs is subject to interpretation. Sterz et al. (1974) were unable to demonstrate cross-reactions between BHV 1 and BHV 2 by immunodiffusion (ID), immunofluorescence (IF), complement fixation (CF), or virus neutralization (VN) tests. Potgieter and Maré (1974b) were unable to demonstrate cross-reaction between BHV 1 and BHV 3 by IF or VN. Although BHV 1 and SHV 1 did not cross-neutralize when rabbit sera were used (Plummer, 1964), 33% of 90 cattle sera possessing neutralizing antibodies for BHV 1 as a result of infection or vaccination also possessed antibodies which neutralized SHV 1 (Aguilar-Setién et al., 1979c). The cattle sera had BHV 1 neutralizing antibody titers of 8 or greater. However, only 4% of 89 swine sera

possessing neutralizing antibodies for SHV 1 also possessed antibodies which neutralized BHV 1. Cattle sensitized to BHV 1 responded to injection of inactivated BHV 1 and SHV 1 with similar delayed hypersensitivity reactions (Aguilar-Setién et al., 1979a). Vaccination with temperature-sensitive mutants of BHV 1 did not protect cattle from SHV 1 challenge, but neither did vaccination with avirulent or killed SHV 1 (Bironi et al., 1982). Wellemans et al. (1980) observed that SHV 1 antibodies do not react in an indirect IF (IIF) test for BHV 1 antibodies. However, BHV 1 and SHV 1 have been reported to share two antigens detectable by immunoelectrophoresis (IE) (Evans et al., 1973).

Smith (1976) reported work by Bartha indicating that BHV 2 is not serologically related to BHV 3. Although BHV 2 does not cross-react with SHV 1 by IF, CF, or VN (Sterz et al., 1974), ID studies revealed an antigen common to BHV 2, SHV 1, and two other herpesviruses (Killington et al., 1977). This polypeptide antigen was identified by polyacrylamide gel electrophoresis (PAGE) of antigens precipitated by heterologous antisera (Yeo et al., 1981), and shown to be a major DNA-binding protein for each virus (Littler et al., 1981), with a highly conserved amino acid sequence.

Elucidation of relationships between herpesviruses of cattle has application beyond the delineation of taxa. Identification of unique antigens in each bovine herpesvirus would aid specific diagnosis, e. g. by the production and use of monospecific antisera or monoclonal antibodies. Identification of antigens cross-reacting between bovine herpesviruses, especially those common to all these viruses, would be of value in detection of extraneous herpesviruses in bovine biologics and in group-

specific diagnosis. The role of shared antigens in infection and immunity, including cross-protection, is of theoretical and practical (vaccination) importance.

The serological relationships of BHV 1 to three other herpesviruses that infect cattle were examined in this study by the use of two-dimensional immunoelectrophoresis (2DIE), plaque reduction serum neutralization (PRSN), and plaque reduction serum neutralization inhibition tests (PRSNIT). Cell cultures infected with each of the viruses were solubilized using a nonionic detergent, and tested against globulin preparations derived from homologous and heterologous antisera.

MATERIALS AND METHODS

Viruses

The bovine herpesviruses and their sources were as follows: BHV 1 (Cooper strain, NVSL), BHV 2 (New York-1 strain, NVSL), BHV 3 (3373 strain, D. E. Reed), and SHV 1 (S62-26 strain, NVSL). All viruses were type viruses of limited cell passage.

Antigen Preparation

The procedure followed has been described previously (Part I, Levings et al., 1984a). Briefly, cell cultures (embryonic bovine kidney, EBK) were grown to confluency in roller bottles (Corning Glass Works, Corning, NY) using Eagle's minimum essential medium (EMEM, Grand Island Biological Company, Grand Island, NY) containing 10% bovine fetal serum. These cultures were infected with BHV 1 at a multiplicity of infection (MOI) of 0.5 to 3.0, BHV 2 at MOI 0.02 to 0.1, BHV 3 at MOI 0.0002 to 0.01, or SHV 1 at MOI 0.5 to 3.0 in EMEM without serum. Sham-inoculated (SI) cultures were inoculated with EMEM only. When the cytopathic effect reached 50 to 90%, the cultures were harvested and centrifuged for 4.5 hours at 36,000 X g. The pellets were resuspended in glycine-Tris (Sigma Chemical Company, St. Louis, MO) buffer (pH 9.0) containing 1.0% Triton X-100 (Sigma Chemical Company, and Fisher Scientific, Fairlawn, NJ) or Tris-Tricine (BioRad Laboratories, Richmond, CA) buffer (pH 8.6) containing 1.0% Triton X-100 (immunoelectrophoresis buffer, IEB). Following sonication and agitation, the suspensions were centrifuged for 1 to 2 hours at 100,000 X g and the

supernatant fluids, comprising the antigen preparations, were harvested and stored at 4°C.

Four separate sets of antigen preparations were made and designated I, II, III, and IV. Set I was made using glycine-Tris buffer and consisted of a BHV 1-infected preparation only. Set II was made using glycine-Tris buffer and consisted of BHV 1-infected and SI preparations. Sets III and IV were made using IEB and consisted of SI and BHV 1-, BHV 2-, BHV 3-, and SHV 1-infected preparations.

BHV 1 antigen preparations were subjected to affinity chromatography using Sepharose-linked Ricines communes agglutinin (RCA, E-Y Laboratories, San Mateo, CA), a lectin specific for beta-linked D-galactose and structurally similar residues. Following a 16-hour incubation at 4°C, unbound materials were washed from the column with four volumes of IEB. One hour later, bound antigen was eluted with three volumes of IEB containing 0.2 M galactose. This glycoprotein preparation was concentrated to the original preparation volume by pervaporation and dialyzed against IEB.

Antiserum Production

BHV 1 antiserum production and pooling has been previously described (Part I, Levings et al., 1984a). Briefly, two calves were injected intramuscularly (IM) with live virus at intervals and bled five times. The harvested sera had BHV 1 VN titers greater than 32 and SHV 1 VN titers greater than 8. Two other calves were exposed, once intranasally and twice IM, and were bled once. The harvested pooled serum had a BHV 1 VN titer greater than 256 and a negligible SHV 1 VN titer. All calves were BHV 1

and SHV 1 seronegative prior to immunization. The sera, negative by VN for BHV 2 and negative by IIF for BHV 3, were pooled for use in this study.

BHV 2 antiserum was provided by M. L. Snyder (NVSL). One calf was injected IM at intervals and ultimately intravenously (IV) with BHV 2. The serum had an anti-BHV 2 titer of 90 by VN and 20,480 by IIF. This serum was negative by these tests for BHV 1 and SHV 1 and was negative by IIF for BHV 3.

BHV 3 antiserum was provided by F. A. Osorio (Iowa State University). One calf was injected IV with BHV 3, 3374 strain (Osorio and Reed, 1983). A persistent infection ensued during which virus was repeatedly isolated from peripheral blood leukocyte cultures. Harvested serum had an anti-BHV 3 titer of 160 by IIF, and a negligible titer by VN. This serum was negative by these tests for BHV 1, and was negative for SHV 1 by VN. This calf was seropositive by IIF for BHV 2 pre- and post-injection.

SHV 1 antiserum was provided by P. D. Beard (NVSL). One calf was injected IM twice with a Triton X-100 solubilized antigen preparation of SHV 1, incorporated in incomplete Freund's adjuvant (Grand Island Biological Company). Harvested serum had an SHV 1 VN titer of 13, was negative by VN for BHV 1 and BHV 2, and was negative by IIF for BHV 3.

These sera were fractionated by a modification of the anion-exchange chromatography method of Stanworth (1960), using DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, NY). The globulin preparations were concentrated by pervaporation to 25% of the original serum volume and filtered through a 0.45 micron filter. A similar globulin preparation was made from bovine fetal serum.

Two antisera specific for a limited number of BHV 1 antigens were produced in calves by intramuscular injection with adjuvanted 2DIE gel sections containing specific antigen-antibody arcs (unpublished observations, D. E. Reed, Veterinary Medical Research Institute, Iowa State University). Globulin preparations were produced from these antisera by ammonium sulfate precipitation. One serum, "VN-", was specific for one antigen in this system, and lacked VN activity. The other serum, "VN+", was specific for four other antigens and had a VN titer of 64.

2DIE Procedure

The procedure followed was similar to that of Vestergaard (1973). Agarose gel was prepared from commercial immunoelectrophoresis tablets (BioRad Laboratories) by heating in water containing 1.0% Triton X-100. First dimension electrophoresis was performed on glass slides using IEB. Gel sections were then transferred to agarose support films (LKB Instruments, Rockville, MD) and 1% agarose gel, 48°C, containing globulin, was pipetted onto the remaining area of the film. Following second dimension immunoelectrophosesis, films were stained with 0.2% Coomassie Brilliant Blue R-250 (BioRad Laboratories).

Modifications of 2DIE procedure described by Axelson and Bock (1972) were also used. The 2DIE with antigen addition, (Ag-add), was performed by placing the preparation to be tested in a well centered below the well containing BHV 1 preparation prior to first dimension electrophoresis. The 2DIE with antibody addition, (Ab-add), was performed by incorporating the globulin preparation to be tested into 1% agarose gel containing BHV 1

globulin and pipetted onto the film prior to second dimension immuno-electrophoresis.

Evaluation of 2DIE Results

Only arcs that were consistently reproduced and did not appear to be diffusion artifacts of other arcs (see Figure 1-B, antigens 5, 10, and 11) were used for analysis. Some unusual arcs were observed complicating the evaluation. Examples of this are the connecting cathodal and anodal arcs of Antigen 3. The anodal arc of Antigen 3 and the anodal side of the arc of Antigen 4 of set I were used for evaluation. In some cases, a heavy arc was seen which was determined to represent two antigens and was evaluated as such. In other cases, arcs were seen that could represent either or both of two antigens, and were not used in evaluation.

Common viral antigens were identified by increases in arc area when heterologous antigen preparations were added to homologous BHV 1 2DIE tests (Ag-add). This antigenic enhancement, evaluated by superimposition of test and control films, was assigned relative values, "+" representing up to 25% of the enhancement produced by BHV 1 antigen preparations (Figure 2-A), "++" representing 25 to 50% (Figures 2-A and 2-B), and "+++" representing greater than 50% (Figures 2-C and 2-D).

Common antibodies in the antisera studied were identified by the decrease in arc area observed when heterologous globulin preparations were added to homologous BHV 1 2DIE tests (Ab-add). This antibody-mediated depression was assigned relative values of "+", "++", and "+++", representing the percentage of effect BHV 1 globulin had on arcs.

Figure 1. 2DIE patterns of reactions between BHV 1-infected or sham antigen preparations and BHV 1 antiserum or bovine fetal serum globulin preparations A - BHV 1-infected preparation (II) with anti-BHV 1, B - BHV 1-infected preparation (I) with anti-BHV 1, C - sham preparation (II) with anti-BHV 1, D - BHV 1-infected preparation (II) with bovine fetal serum.

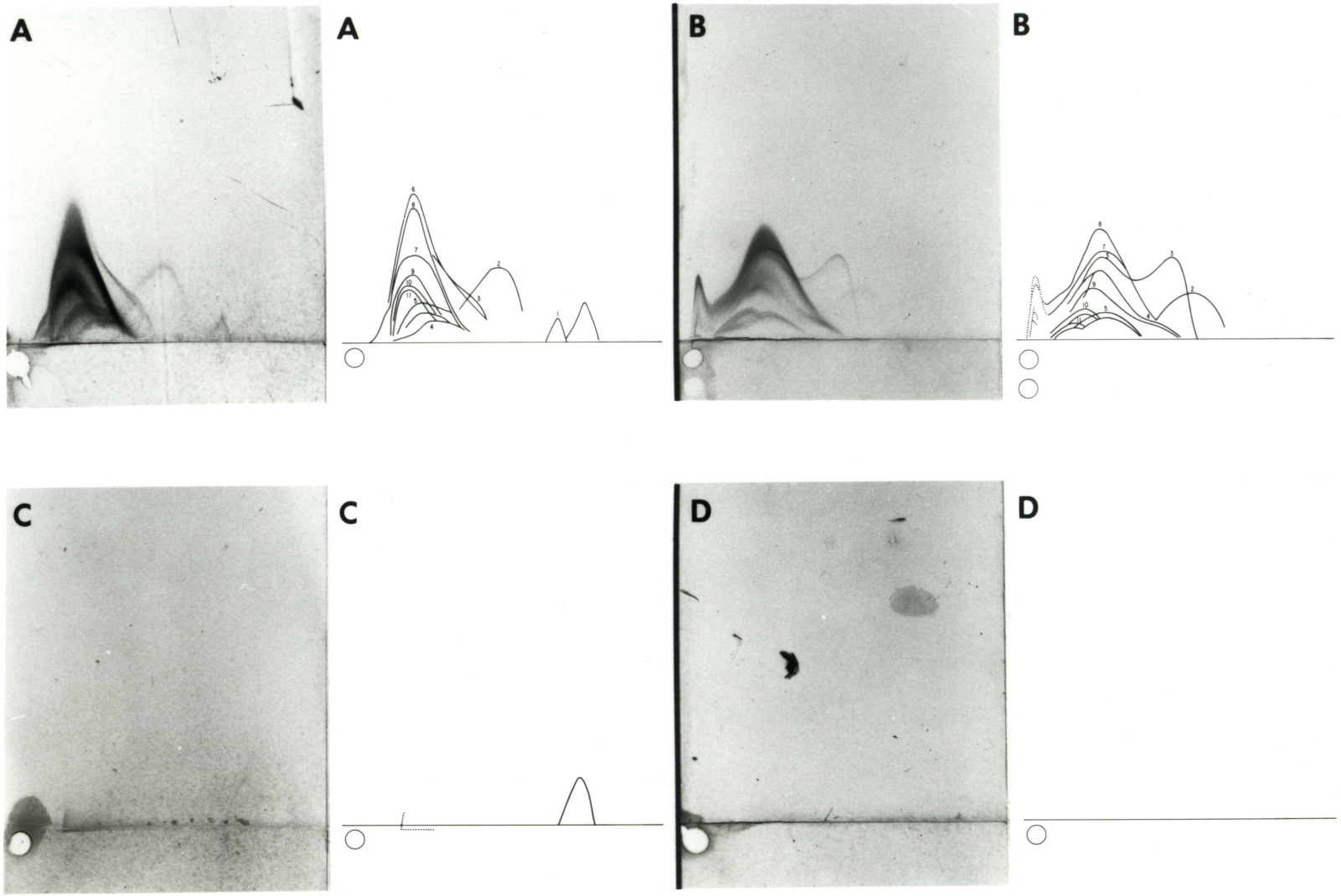
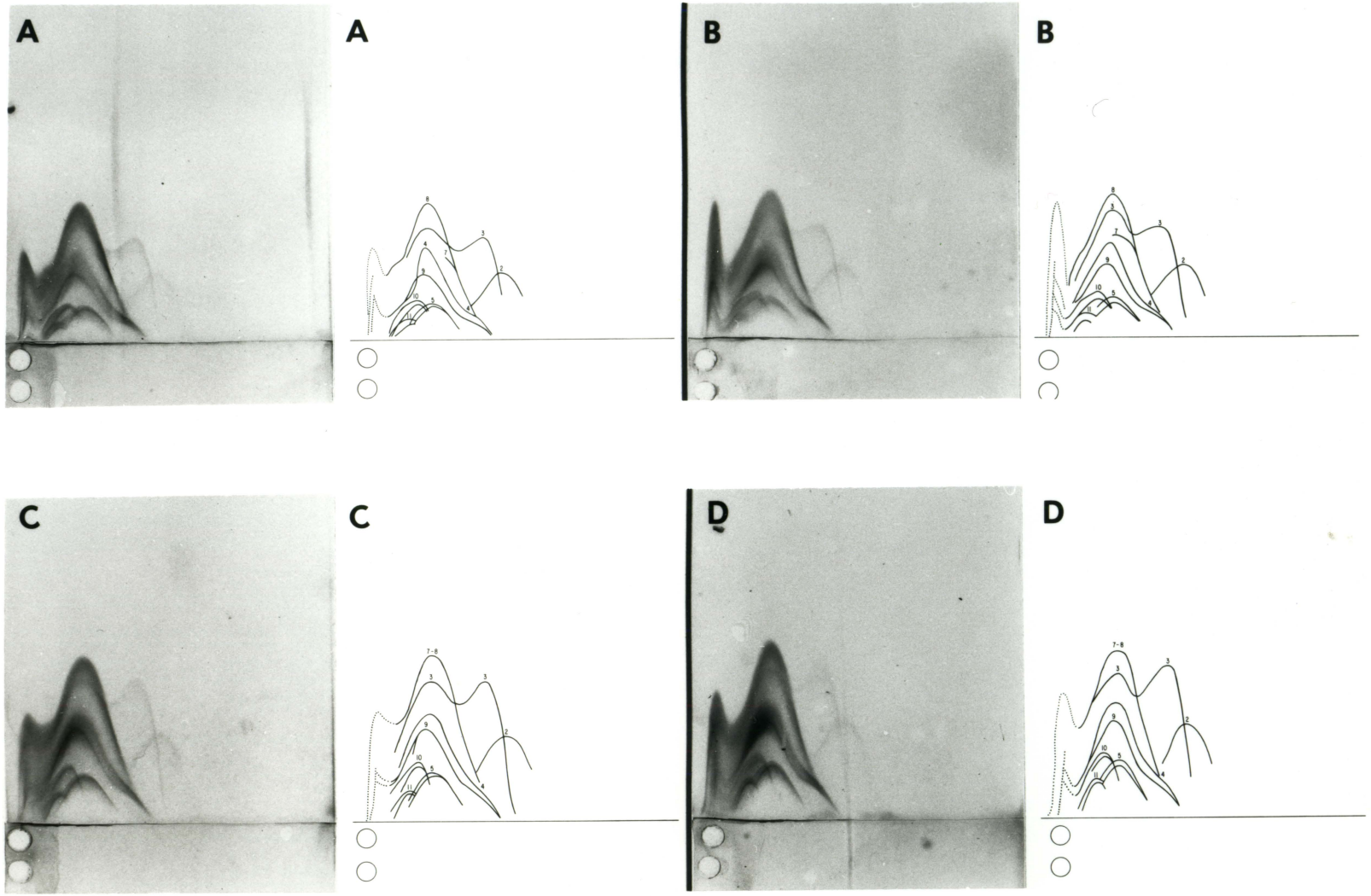

A
A
B
B
C
C
D
D

Figure 2. Enhancement of BHV 1 homologous 2DIE arc areas by addition of homologous antigen (Ag-add 2DIE) Top well: A-D - BHV 1-infected preparation (I). Bottom well: A-D - BHV 1-infected preparation (I) diluted 1:4, 1:2, 3:4, and 1:1 respectively (See Figure 1-B).


A
A
B
B
C
C
D
D

PRSN Procedure

Dilutions of antisera to be tested were incubated with equal volumes of a fixed dilution of BHV 1 (Cooper strain) for 1 hour at 37°C with agitation every 15 minutes. Following removal of the medium, 2- to 3-day-old confluent EBK cell cultures in 24 well cluster cultures (Costar, Cambridge, MA) were inoculated with 0.1 ml/well. These were incubated at 37°C for 1 hour with agitation every 15 minutes, followed by addition of 0.5 ml of 1% gum tragacanth (Fisher Scientific, Fairlawn, NJ) in EMEM. After 48 hours incubation at 37°C, the plates were stained with crystal violet-formalin solution, washed, dried, and the microplaques counted using a dissection microscope. Virus control consisted of equivalent dilutions of bovine fetal serum and virus. The dilution used in the virus suspension was standardized to yield 45 to 60 plaques/well. Fifty percent neutralization was calculated by arc sine regression.

PRSNIT Procedure

A modification of the technique described by Appleyard et al. (1964) and Cohen et al. (1972) was used. Dilutions of the preparation to be tested were incubated with equal volumes of a 1:30 dilution of BHV 1 antiserum for 1 hour at 37°C with agitation each 15 minutes. An equal volume of live BHV 1 suspension (Cooper strain) was then added, followed by another 1 hour incubation at 37°C with agitation each 15 minutes. Inoculation, incubation, staining, etc., was performed as described in "PRSN procedure".

The antiserum control consisted of equal volumes of diluent and 1:30 antiserum, and the virus control consisted of equal volumes of diluent and

1:30 bovine fetal serum. The virus suspension was standardized to yield 45 to 60 plaques/well, and the dilution of antiserum selected to neutralize 67% of the virus. For evaluation, the average virus control plaque count (e.g., 60, or 0% VN) was defined as 100% inhibition of VN activity of the antiserum. The average antiserum control plaque count (e.g., 20, or 67% VN) was defined as 0% inhibition of VN activity in the antiserum. The dilution of antigen preparation which yielded plaque counts halfway between these two points (e.g., 40, or 33% VN) would be responsible for inhibiting 50% of the VN activity in the antiserum. This dilution, calculated by arc-sine regression, was defined as the 50% PRSNIT titer. The SI plaque counts were used to correct for toxicity of the preparations.

RESULTS

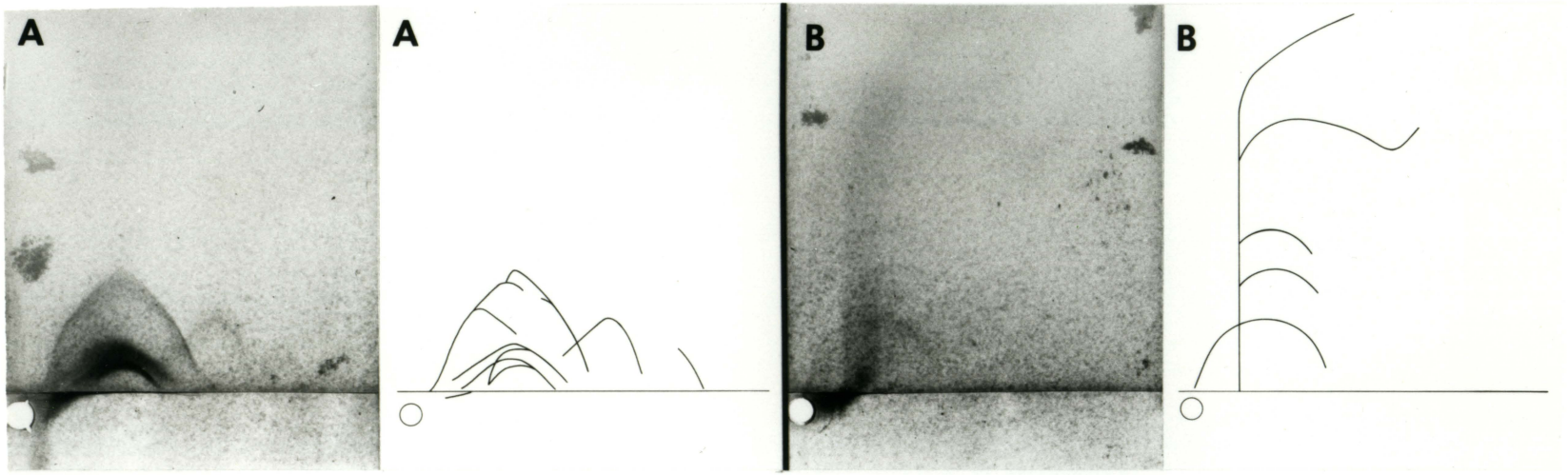
Twelve antigen-antibody arcs were repeatedly produced in homologous BHV 1 2DIE tests (Figure 1-A). One of these arcs is apparently cell-associated, as it was observed using SI set II (Figures 1-A and 1-C). However, the remaining 11 arcs were clearly virus specific, as none of the arcs were due to precipitation by normal serum components (Figure 1-D). Other precipitation lines were observed infrequently and were not used in this study. The BHV 1 homologous 2DIE patterns showed some variation in the numbers of arcs detected, but those detected were very similar in position (Figures 1-A, 1-B, and 3-A).

When BHV 1 globulin was used in 2DIE with the antigen preparations of sets III and IV, 10 arcs were produced by BHV 1, three by BHV 2, none by BHV 3, and five by SHV 1 (Figure 3). These same antigen preparations were added to BHV 1 homologous 2DIE tests and enhanced arc areas as follows: BHV 1, Antigens 1 to 11; BHV 2, Antigen 1; BHV 3, Antigens 1 and 2; and SHV 1, Antigens 1, 3, 6, and 8 (Figure 4 and Table 1).

When heterologous globulin preparations were used in 2DIE with BHV 1 antigen preparations, no arcs were produced. These same globulin preparations were added to BHV 1 homologous 2DIE tests and decreased arc areas as follows: BHV 1 globulin, Antigens 1 to 11; BHV 2 globulin, Antigens 1 and 8; BHV 3 globulin, Antigens 1, 6, and 8; and SHV 1 globulin, none of the antigens (Figure 5 and Table 1).

Antisera produced against 2DIE gel sections containing antigen-antibody arcs had very limited specificity as determined by 2DIE (Figure 6). The

Figure 3. 2DIE patterns of reactions between herpesvirus-infected antigen preparations (III) and BHV 1 antiserum globulin preparations A - BHV 1, B - SHV 1, C - BHV 2, D - BHV 3 (See Figures 1-A and 1-B).


A
A
B
B

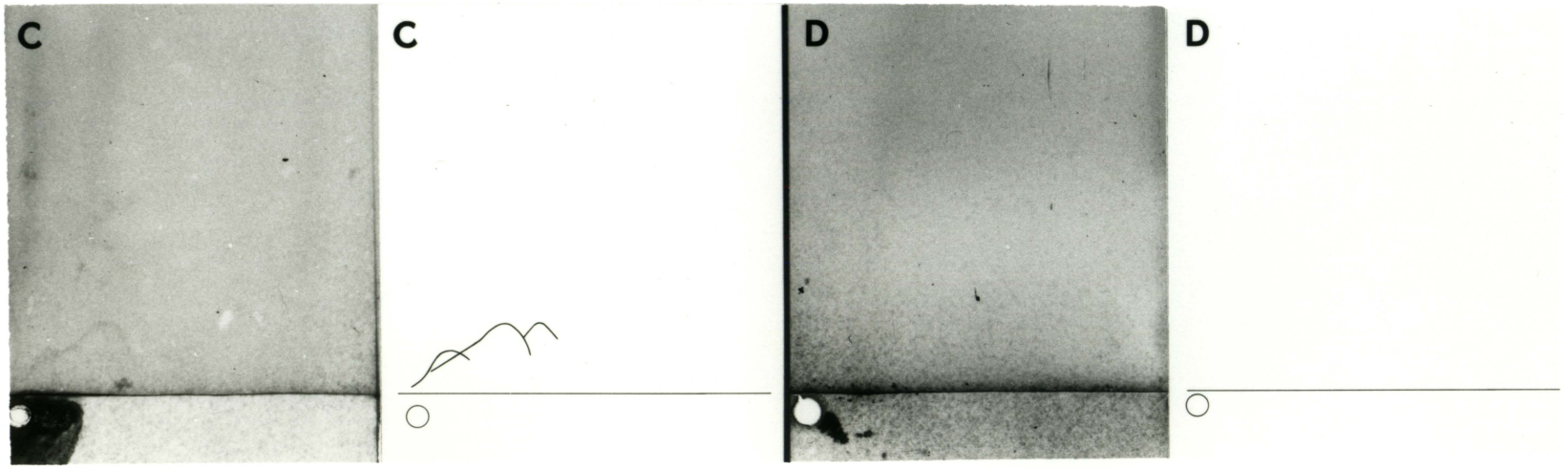

C
C
D
D

Figure 4. Identification of BHV 1-infected preparation antigens in herpesvirus-infected antigen preparations (III) by enhancement of BHV 1 homologous 2DIE arc areas (Ag-add 2DIE) Top well: A-D - BHV 1-infected preparation (II). Bottom well: A - BHV 1, B - SHV 1, C - BHV 2, D - BHV 3 (See Figure 1-A).

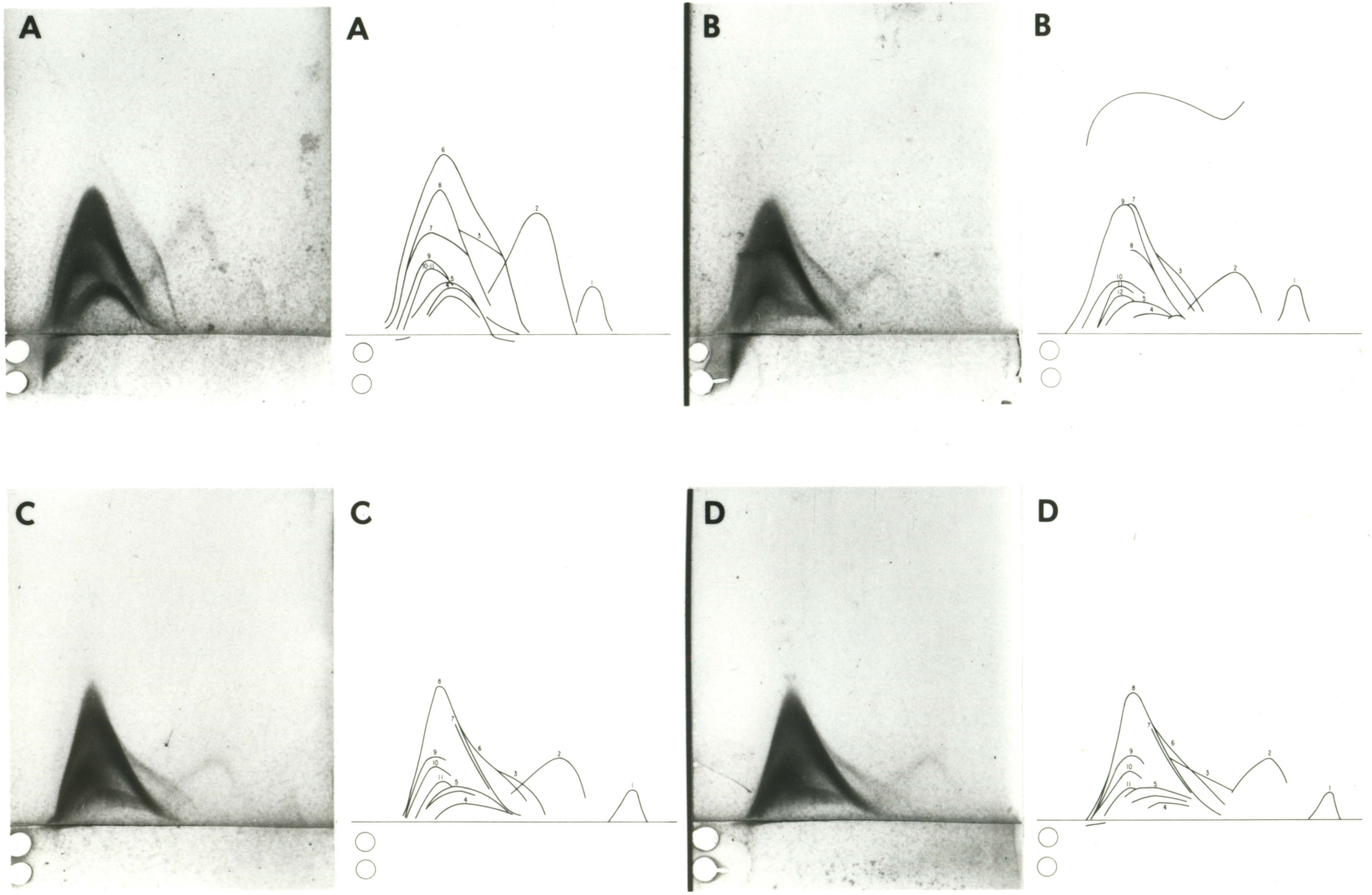
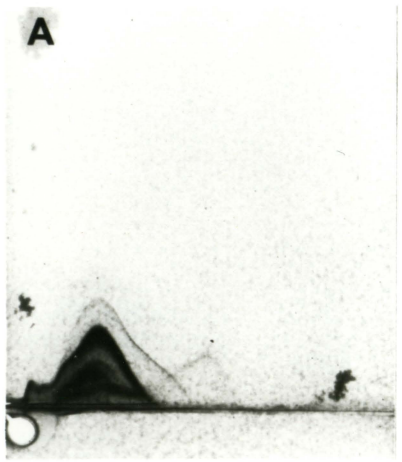
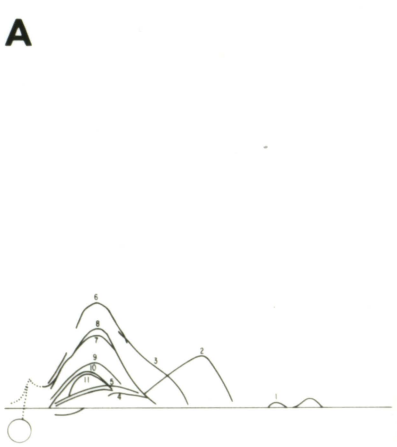
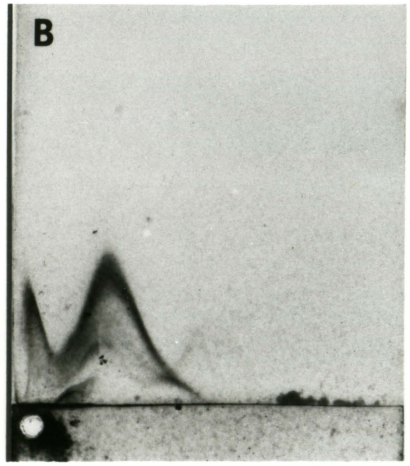

A
A
B
B
C
C
D
D

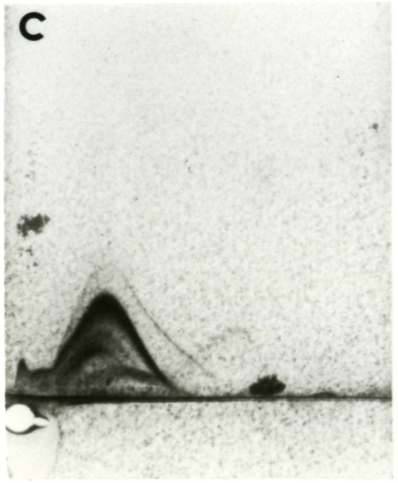
Figure 5. Identification of BHV 1 antibodies in herpesvirus antisera globulin preparations by depression of BHV 1 homologous 2DIE arc areas (Ab-add 2DIE) A - BHV 1 antiserum; B - SHV 1 antiserum; C - BHV 2 antiserum; D - BHV 3 antiserum (see Figures 1-A and 1-B).

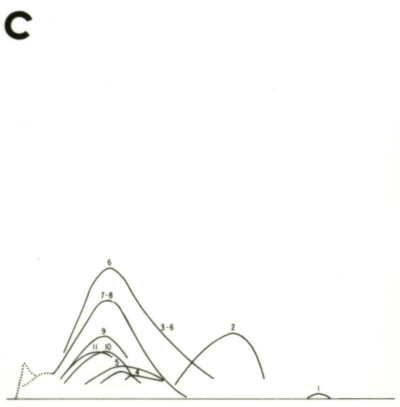

A


A


B


B


C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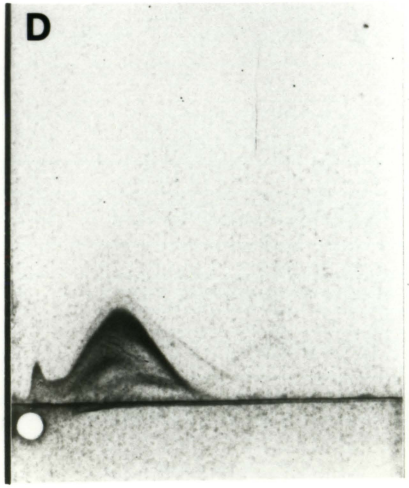
D

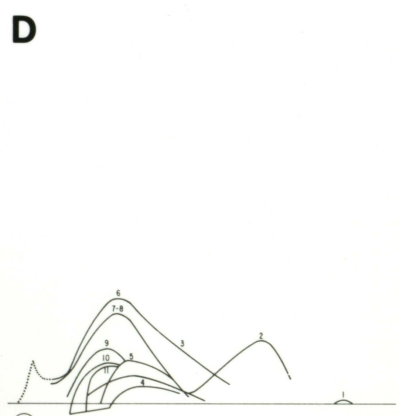
D

Figure 6. Detection and identification of BHV 1 antibodies in "VN+" and "VN-" by 2DIE with BHV 1-infected preparation (II) and by depression of BHV 1 homologous 2DIE arc areas (Ab-add 2DIE) A - "VN-", B - "VN+", C - "VN-", D - "VN+" (See Figure 1-A).

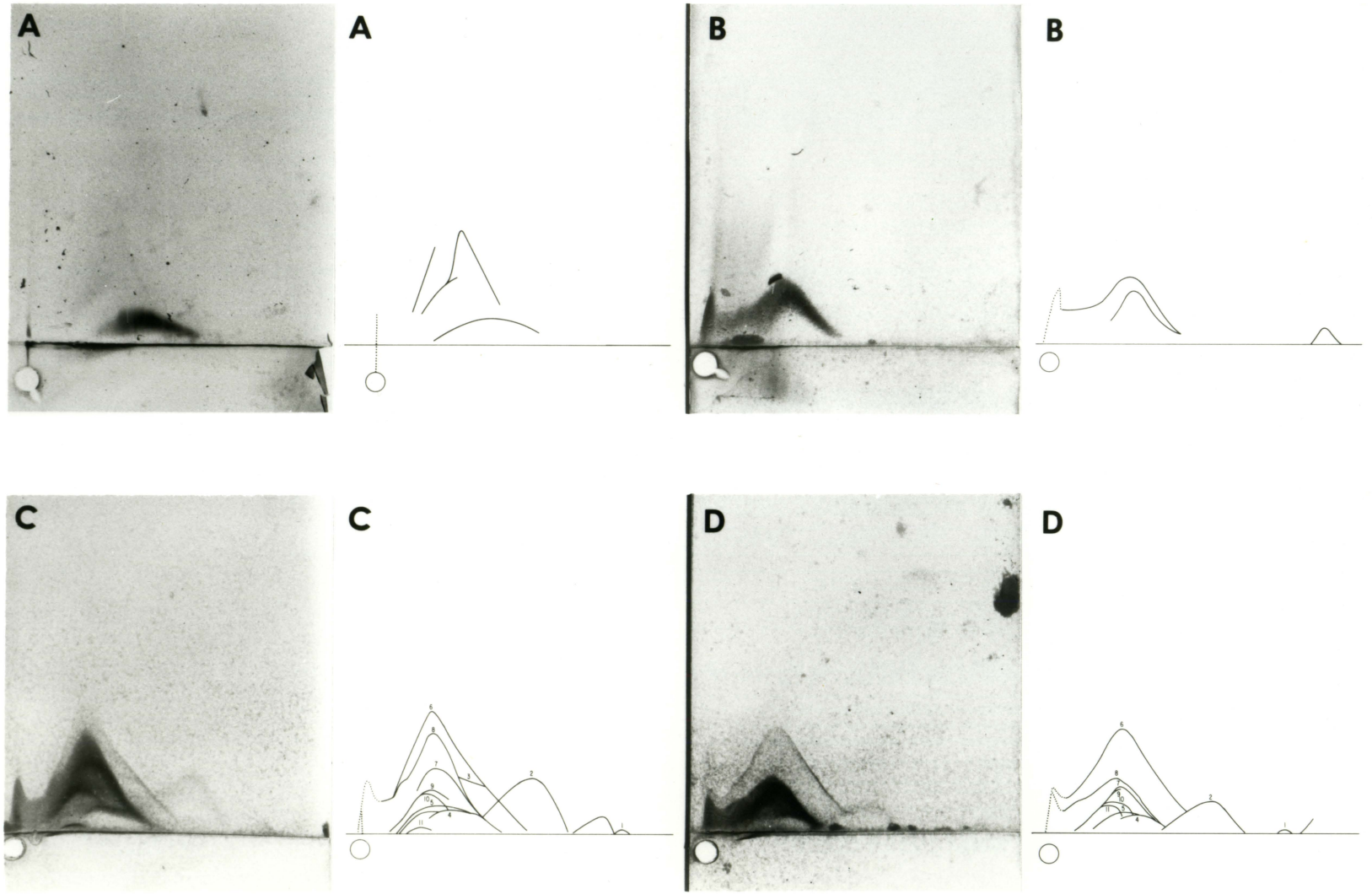
A
A
B
B
C
C
D
D

Table 1. Distribution and relative reactivity in two-dimensional immunoelectrophoresis with antigen or antibody addition of BHV 1 antigen specificities associated with various herpesviruses

Antigen or Antibody Preparation Added	Association with BHV1[a] Specificities				
	1	2	3	6	8
BHV 2[b]	+[c]	Neg.	Neg.	Neg.	Neg.
Anti-BHV 2	++	Neg.	Neg.	Neg.	++
BHV 3[d]	+	+	Neg.	Neg.	Neg.
Anti-BHV 3	++	Neg.	Neg.	+	++
SHV 1[e]	++	Neg.	+	+	++
Anti-SHV 1	Neg.	Neg.	Neg.	Neg.	Neg.

[a]BHV 1 = Infectious bovine rhinotracheitis virus.

[b]BHV 2 = Bovine mammillitis virus.

[c]+,++ = Antigen reactivities relative to BHV 1 preparations.

[d]BHV 3 = Movar virus.

[e]SHV 1 = Pseudorabies virus.

"VN-" serum was specific for antigen 10 by antibody-addition 2DIE. The "VN+" serum was specific for Antigens 2, 6, 7, and 8 by the same technique.

Antigen preparations of each of the four herpesviruses studied were evaluated for ability to inhibit PRSN of BHV 1; only the BHV 1 preparation was positive. Sera were tested for PRSN of BHV 1; only anti-BHV 1 and "VN+" were postitive.

The BHV 1 Antigens 7, 8, and 11 were identified as glycoproteins by antigen-addition 2DIE using RCA-fractionated preparations.

DISCUSSION

The serological cross-reactions between BHV 1 and three other herpesviruses that infect cattle were examined to identify antigens common to two or more of these viruses. The experimental procedure, antigenic analysis by 2DIE of virus-infected cell preparations solubilized by Triton X-100, was selected because: (1) Triton X-100 treatment was reported by Vestergaard et al. (1977) to free 90% of the protein of cells infected with human herpesvirus 1 (HHV 1); (2) when controlled as in this study, only virus-determined antigen specificities should have been detected; (3) use of hyperimmune anti-BHV 1 globulin together with modification of the 2DIE with Ag-add and Ab-add techniques should have detected most BHV 1 precipitating and nonprecipitating antigen-antibody reactions (Axelson and Bock, 1972). Although the procedure only measured antigens resulting from solubilization of virus-infected cells, and did not define those components on the basis of size or complexity, the system did have the potential for relating antigenic composition of the viruses by serologic reactivity.

This study delineated certain antigenic relationships between the viruses including the demonstration of certain virus-restricted (unique) and shared (cross-reacting) antigens. Cross-reacting antigens may have value in construction of "serons," defined by Honess and Watson (1977) as "a cluster of viruses defined by significant serological relationships." This study has demonstrated five BHV 1 antigens common to other cattle herpesviruses. Two of the antigens were common to the four viruses. The shared antigens may correspond to structures or functions common to herpesviruses in general, e.g., the DNA-binding protein described by Killington

et al. (1977), or they may represent a cattle herpesvirus-common structure, e.g., a protein necessary for infection of bovine cells similar to that described by Sarmiento et al. (1979) for HHV 1 penetration. Identification of specific antigens common to all cattle herpesviruses, or even all herpesviruses, could allow development of a serological test to detect the extraneous viruses in bovine constitutions. This is a problem which cannot be addressed practically by testing individually for each possible contaminant.

In addition to the two antigens common to the four viruses, one BHV 1 antigen was shared with BHV 2, two were detected in BHV 3, and two were found in SHV 1. One BHV 1 antigen (6) was observed in BHV 3 and SHV 1, but not BHV 2. This variation in the number and identity of common structures may be related to molecular variation, e.g., the thymidine kinase variation observed in HHVs by Honess and Watson (1977). An alternative explanation is that some of these antigens are common to all the cattle herpesviruses, or all the herpesviruses, but because the antigens are present in limited concentration, they are not detected with some antigen or antibody preparations.

The norm in this study was for antigen and antibody preparations to evidence greater activity when added to BHV 1 homologous 2DIE tests than was demonstrated when tested alone. These observations may be due to the superior sensitivity of the addition techniques. However, interesting exceptions were noted with BHV 2 and SHV 1 antigen preparations which produced more arcs with BHV 1 globulin than were affected by these preparations in BHV 1 homologous 2DIE tests. This result may be due to the

molecular characteristics of the cross-reacting antigens. The dissociated viral antigens are probably complexes of antigenic molecules, or molecules with multiple determinants, with heterogeneity among the herpesviruses studied. This would account for the differing expression of antigenic preparations in the test procedures. Thus, the three antigens of BHV 2 which reacted with BHV 1 globulin may represent separate molecules, while those specificities may be distributed on only two BHV 1 molecules (Antigens 1 and 8, see Table 1). The SHV 1 component which did not influence a BHV 1 homologous 2DIE arc, but precipitated separately (Figure 4-B), may be due to an SHV 1 antigen which shares only one of many determinants with a BHV 1 antigen, with an indetectable line of partial identity (Axelson and Bock, 1972). Alternatively, this SHV 1 arc may be due to an SHV 1 surface determinant which is hidden in a BHV 1 antigen complex because it was not exposed by the solubilization procedure. However, this antigen would be exposed by in vivo antigen processing, so antibodies against it should be present in hyperimmune BHV 1 antiserum, as was shown (Figures 3-B and 4-B). This antigen is of particular interest, because this same mechanism (surface versus hidden) may explain why BHV 1 antiserum is capable of neutralizing SHV 1, while SHV 1 antiserum has been reported not to neutralize BHV 1 (Aguilar-Setién et al., 1979c).

The BHV 1 unique antigens may be useful for species-specific diagnosis. Those BHV 1 antigens (4, 5, 7, 9, 10, and 11) that did not cross-react with other cattle herpesviruses in this study are not likely to cross-react with other, less closely related, viruses infecting cattle. If these antigens

are not found in other cattle herpesviruses, they could be used to refine BHV 1 diagnosis.

The experimentation revealed that an association with BHV 1 neutralization could be made for one antigen, while this association was ruled out for several others. A role for cross-reacting antigens in BHV 1 neutralization was eliminated by the failure of the other cattle herpesviruses to inhibit neutralization of BHV 1 or induce antibodies capable of neutralizing BHV1. For this reason, Antigens 1, 2, 3, 6, and 8 are probably not BHV 1 neutralization epitopes. The VN+ serum reacted with BHV 1 Antigens 2, 6, 7, and 8. Therefore, by elimination, Antigen 7 is associated with neutralization of BHV 1. Similarly, Antigen 10 probably is not associated with BHV 1 neutralization because VN- is specific for it.

The association of Antigen 7 with neutralization of BHV 1 confirms and extends the observations of one of the authors (D. E. Reed), who demonstrated neutralizing activity in a serum directed against a limited mixture of BHV 1 antigens, including Antigen 7. Therefore, a subunit or genetically engineered vaccine containing this antigen may have potential for immunoprophylaxis. Such an antigen could be protective without the safety or latency problems that may be encountered using other herpesvirus vaccines containing DNA (Reed, 1980). Such a vaccine would allow concurrent vaccination and serological testing for naturally exposed animals by testing for antigens not included in the vaccine. This would make eradication of the diseases caused by BHV 1 much less costly and more practical.

GENERAL SUMMARY

This study involved the identification of those bovine herpesvirus 1 (BHV 1) antigens affected quantitatively by virus inactivation and those cross-reacting serologically with antigens of other cattle herpesviruses. Two-dimensional and fused rocket immunoelectrophoresis were used to detect 11 viral antigens in detergent-solubilized BHV 1-infected cells.

The effects of four widely used methods of virus inactivation on BHV 1 antigens were examined. Antigen preparations treated with beta-propiolactone (BPL) or formalin retained a large measure of most of the individual antigens, as well as substantial total antigen reactivity. Antigen reactivity was measured by a plaque reduction serum neutralization inhibition test (PRSNIT). Heat treatment (56°C x 1 hr) was relatively nonselective in its extensive reduction of BHV 1 antigens, and PRSNIT activity of these treated preparations was low. Treatment with ultraviolet (UV) radiation eliminated all of the antigens studied, and PRSNIT activity of these preparations was not detectable. One BHV 1 antigen (3) was found to be labile to all treatments (negative or <25% of control levels). Another (2) was found to be relatively stable (>50% of control levels in BPL-, formalin-, and heat-treated preparations).

The cross-reactions of the BHV 1 antigens with those of other cattle herpesviruses (BHV 2, BHV 3, and suid herpesvirus 1, SHV 1) were also investigated. Five BHV 1 antigens (1, 2, 3, 6, and 8) were found to cross-react with the antigens of at least one of these other herpesviruses. Two of the BHV 1 antigen specificities (1 and 8) were common to all of the

viruses studied. One (6) was shared with two other cattle herpesviruses; two (2 and 3) were shared with one other virus.

Variation in the effect of the inactivation treatments may be valuable for selection of inactivation methods as they relate to antigenicity of the final product, cost, ease of preparation, and toxicity. The antigen preparations used in this study were very turbid and had high preinactivation titers. Therefore, excessive UV irradiation may have been necessary for complete inactivation, which may account for the destruction of all 11 antigens studied. The use of tissue culture assays for inactivation may have contributed to this necessity for excessive treatment, as repair of UV-inactivated virion infectivity is known to occur under conditions of high multiplicity of infection.

A one-way neutralization of SHV 1 with BHV 1 antisera has been reported and was confirmed in this study. A SHV 1 antigen was detected which was precipitated by BHV 1 antiserum but did not coprecipitate with BHV 1 antigens. This antigen may represent an internal or "hidden" BHV 1 determinant (unavailable to antibody, neutralization not possible) which is a surface SHV 1 determinant (available to antibody, neutralization possible). Although hidden in whole (and gently solubilized) BHV 1 virus preparations, such a determinant would be exposed by in vivo antigen processing. Thus, antibodies against it would be expected to occur in hyperimmune BHV 1 antisera, which were used in this study to precipitate the antigen. A similar one-way neutralization of HHV 1 with BHV 2 antiserum has been reported (Pauli et al., 1979). Ludwig (1983) noted that a variety of evidence suggests the common antigen of HHV 1 and BHV 2 is an "integral

part of the membrane" in HHV 1, but on the "internal side" of the envelope of BHV 2.

Those antigens found only in BHV 1 (BHV 1 unique) are important for the development of BHV 1-specific serodiagnostic tests. Six BHV 1 antigens were not observed to cross-react with the three other cattle herpesviruses used in this study. Serologic cross-reactions would be most likely in such closely related viruses, so cross-reactions with other cattle viruses are improbable. Should further experimentation fail to demonstrate cross-reactions with another virus, these antigens could be considered BHV 1-specific and used to refine BHV 1 diagnosis.

Six BHV 1 antigens identified in this experimentation warrant special attention. Antigens 1 and 8 are common to all four cattle herpesviruses studied. The shared antigens may be responsible for structures or functions common to all herpesviruses or to all cattle herpesviruses. If either of these antigens could be shown by further experimentation to be common to all cattle herpesviruses or all herpesviruses, a test based on that antigen might be used to detect extraneous viruses of that group in bovine biologics. Contamination of products with these viruses is more practically addressed by group-specific testing than by virus-specific testing. Antigen 6 cross-reacted with antigens of BHV 3 and SHV 1, but not BHV 2. This may be due to limited concentrations of reagents (allowing cross-reactions to go undetected) or degrees of variation of similar structures in bovine herpesviruses. BHV 1 Antigen 2 was shown to be stable to treatment with BPL, formalin and heat. As the only heat-stable antigen detected, it is enriched relative to the other BHV 1 antigens by heat

treatment of virus or antigen preparations. It is also an antigen of cross-reaction with BHV 3. In view of the low titers in which BHV 3 is produced in tissue culture, a heat-treated BHV 1 preparation might have potential as a diagnostic reagent for BHV 3 screening, when used in conjunction with BHV 1 diagnosis.

Of interest is the fact that only one BHV 1 antigen (3) was found to be labile to all inactivation techniques tested. Its unique lability to three divergent inactivation treatments (BPL, Formalin, and Heat) suggests a common site and perhaps a common mechanism for inactivation. It might further be suggested that (being the only antigen eliminated by heat) Antigen 3 is the site of temperature sensitive mutant modification. It might be supposed that BHV 1 Antigen 3 represents a vital structure or function in the infectivity of bovine herpesviruses, which could be expected to be highly conserved, but Antigen 3 is not a cattle herpesvirus-common antigen as detected in this study. Because BHV 1 Antigen 3 was reduced in quantity by all treatments, it may be that the humoral immune response to Antigen 3 in those animals exposed to treated preparations is also reduced. Thus, an assay for antibody against Antigen 3 relative to those directed against another antigen might distinguish noninfected animals vaccinated with inactivated product from naturally-infected animals. This assumes antibodies against Antigen 3 would not be present from infection with another agent. Cattle herpesviruses would be the most likely agents to contain antigens cross-reacting with BHV 1 Antigen 3, but of those studied only SHV 1 (almost uniformly fatal in cattle) contains such an antigen. If other cross-reacting antigens are not found, a BHV 1-

infection specific serodiagnostic test based on Antigen 3 might be practical. It would aid epidemiologic investigation, allow more widespread vaccination of animals destined for export, and make eradication of the diseases caused by BHV 1 possible with existing vaccines.

Of those BHV 1 antigens reacting with a BHV 1-neutralizing antiserum, Antigen 7 would appear to be of singular importance. BHV 1 Antigens 2, 6, 7, and 8 reacted with a BHV 1-neutralizing serum raised against an antigen-antibody precipitate in agarose. Those BHV 1 antigens which cross-reacted with other cattle herpesviruses in this study are not likely to play a role in BHV 1 neutralization, because antisera directed against these viruses did not neutralize BHV 1, and antigen preparations of these viruses did not inhibit neutralization of BHV 1. Thus, Antigen 7 is singled out as an important BHV 1 neutralization epitope. The use of such an antigen in immunoprophylaxis might be protective without the safety and latency problems inherent in the use of current products. The use of a defined vaccine would also allow more certain differentiation between uninfected vaccinates and infected animals than that allowed by the present vaccines.

This study identifies several areas of additional research that should be pursued. These include correlation of 2DIE and PAGE results, investigation of cross-reactions between BHV 1 antigens and those of additional herpesviruses, and isolation and use of particular BHV 1 antigens.

There is a need to correlate information obtained from two dimensional immunoelectrophoresis (2DIE) and polyacrylamide gel electrophoresis (PAGE) because of the unique information which may be attained from modifications of agarose immunoprecipitation and modifications of PAGE. Correlation may

be accomplished using radiolabeled specific antisera or monoclonal antibodies following electrophoretic protein transfer to nitrocellulose from each pattern. Antigens 1, 3, 7, 8, and that antigen which may be on the surface of SHV 1 but hidden in BHV 1 merit particular attention. The extension of this correlation to 2DIE and PAGE of SHV 1 and other herpesviruses would also be useful.

The relationship of BHV 1 antigens to those of a broad spectrum of herpesviruses needs to be investigated. Cross-reactions with BHV 1 antigens 1, 3, 7, and 8 could be especially meaningful. BHV 1 Antigens 1 and 8 cross-reacted with each of the cattle herpesviruses examined in this study. Further investigation is required to characterize them as common to herpesviruses, bovine herpesviruses, or some other restricted group of herpesviruses. BHV 1 Antigen 7 was not observed to cross-react with any of the cattle herpesviruses studied. It may be shown to not cross-react with other herpesviruses that do not cross-neutralize, due to its association with BHV 1 neutralization. If cross-reactions are not found, Antigen 7 could be used in a BHV 1-specific serodiagnostic test. The use of BHV 1 Antigen 3 in a BHV 1-infection specific serodiagnostic test requires that it not cross-react with other herpesviruses which infect cattle nonlethally. Also of interest would be the occurrence of this antigen in temperature-sensitive mutants of BHV 1 and SHV 1 at non-permissive temperatures.

Isolation and characterization of many BHV 1 antigens (e.g. 1, 2, 3, 7, 8 and the "hidden" antigen) would be worthwhile, but Antigens 3 and 7 warrant special attention. Antigen 3 could be used to test sera from

animals vaccinated with inactivated virus (uninfected) and sera from infected animals. Observation of a difference between these groups in antibody activity against Antigen 3 relative to total activity against BHV 1 or against Antigen 7 might then lead to a BHV 1-infection specific serodiagnostic test. Antigen 7 could be tested for protective activity, contributing to BHV 1 subunit vaccine development. The use of a defined, DNA-free BHV 1 protective vaccine might eliminate many of the safety and latency problems inherent in the use of less restricted products. If Antigen 7 is also shown to be BHV 1-specific, the development of serodiagnostic tests based on Antigen 7 and a "complementary antigen" (e.g. Antigen 3) would allow more certain differentiation between vaccinated and infected animals than that allowed by present vaccines. A BHV 1-infection specific serodiagnostic test would be extremely valuable in simultaneous vaccination-eradication efforts, epidemiologic investigations, and certification of animals as free of BHV 1 infection.

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