Propagation of Infectious Bovine Rhinotracheitis Virus in Mouse Cell Cultures

by

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DEDICATION

To my parents, brother and sisters.

TABLE OF CONTENTS

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REVIEW OF LITERATURE

LITERATURE REVIEW

History.

Infectious Bovine Rhinotracheitis (IBR) was first recognized in California (McIntyre, 1954) and Colorado (Miller, 1955), in the early 1950's, as a severe respiratory disease affecting feedlot and dairy cattle. It was known under the synonyms "red nose," "dust pneumonia," or "necrotic rhinitis" (Miller, 1955) and the causative agent was designated Infectious Bovine Rhonotracheitis virus (McKercher et al, 1955). Madin et al (1956) isolated the virus and it was further characterized by Tousimis et al (1958). In 1961, Armstrong et al suggested that IBR virus belongs to the herpes virus group. The virus was soon recognized in dairy cattle associated with infectious pustular vulvovaginitis (IPV) (McKercher, 1963; Kendrick et al, 1967) and abortions (Owen et al, 1964; Wilson, 1974). McKercher and Theilen (1963) suggested that the virus, known as a cause of disease of the genital tract in Europe, was introduced into North America about 1930 and was exalted in virulence in the large concentration of feedlot animals acquiring the power to spread by the respiratory route. It is now known that IBR virus can produce a mild inapparent infection of a wide variety of clinical manifestations some of which have profound effects on feed efficiency, milk production, and reproduction (Kahrs, 1977). The virus has been diagnosed in many countries and is now reported to be world wide in distribution with the possible exception of South America (Mohanty, 1978; Blood et al, 1979).

Properties of the virus.

The etiological agent for IBR is a typical member of the herpes virus family. It has the physical, biochemical, immunological and epidemiological properties of this group (Kahrs, 1977). The IBR virus genome contains a double-stranded DNA with MW of 54 x 10^6 daltons and a buoyant density in cesium chloride of 1.730-1.732 g/cm^3 (Russel and Crawford, 1967; Plummer et al, 1969; Gibbs et al, 1970; Graham et al, 1972). The capsid is constructed of 162 capsomeres arranged in a icosahedral symmetry; an envelope surrounds the nucleocapsid (Watrach and Bahnemann, 1966; Bocciarelli et al, 1966). The nucleocapsid develops within the nucleus of the host cell and acquires the envelope on budding from the nuclear membrane (Jasty and Change, 1971; Zee and Talens, 1972). The envelope consists of a double membrane and has a diameter of approximately 200 nm (Watrach and Bahnemann, 1966). The virus has a buoyant density of 1.249-1.254 b/cm^3 in cesium chloride (Bagust, 1-72) and 1.22 g/cm^3 in potassium tartrate (Talens and Zee, 1976). It is ether sensitive, acid labile and relatively thermolabile (Snowdon, 1964). The IBR virus produces a cytopathic effect including rounded cells, frequently ballooned with small refractile snycitia, and large intranuclear, often eosinophilic (Cowdry type A), inclusion bodies in infected cells (Gibbs and Rweyemamu, 1977). The virus was named "Bovine Herpes Virus 1" (BHV1) according to the international committee for the toxonomy of viruses (Smith, 1976) and Gibbs and Rweyemamu (1977) stated that the term BHV1 refers to all virus isolates that are serologically related to IBR and IPV viruses.

Antigenic relationships.

A variety of techniques (neutralization kinetics, electron microscopy, growth yield in tissue cultures, buoyant density, plaque size in tissue cultures, resistance to trypsin, thermolability and zone electrophoresis) have been employed in search of antigenic differences among various BHV1 isolates and in an attempt to correlate viral characteristics to pathogenicity and clinical manifestations (Engels et al, 1981). Buening and Gratzek (1967) demonstrated minor differences between BHV1 (Enteritis-ISU-1 strain) and 3 different strains of IBR (Cooper, L. A., ISU-2) by neutralization kinetics and House (1972) stated that although minor strain differences were detected, they did not seem to be correlated with the affinity of the virus for particular tissue or organ. Pastoret et al (1980b) reported that it is difficult but not impossible to differentiate several IBR and IPV strains and the major distinguishing features are envelope proteins. The relative new technique of restriction endonuclease will help clarify differences among various isolates of BHV1 and may provide information on the source and epidemiological aspects of the various manifestation of this virus infection (Pastoret et al, 1980; Engels et al, 1981).

Antigenic relationships of BHV1 with other Herpes Viruses (HV) have been studied. Plummer (1964) could not detect any cross-neutralization between BHV1 and 8 different strains of Herpes Simplex, Equine HV 1 and 2, B-virus and Porcine HV1. No relationship was also found between BHV1 and Feline HV 1 (Johnson and Thomas, 1966); Canine HV (Poste, 1972), BHV2 (Martin et al, 1966) and BHV3 (Bartha et al, 1966). However, Carmichael and Barnes (1961) showed that there is antigenic

cross-reactivity between BHV1 and Equine HV1 by complement fixation and gel diffusion tests. Evans et al (1972) reported that BHV1, Marek's disease and Burkitt's lymphoma share at least one common antigenic component by gel diffusion immunoelectrophoresis and indirect fluorescent antibody techniques. Common viral antigen was detected by fluorescent antibody studies, in cultures infected with IBR virus (Los Angeles), between HSV1, Squirrel Monkey HV1 and Equine HV1 (Blue and Plummer, 1973); a common antigen was also detected between IBR virus and Caprine HV1 (Berrios and McKercher, 1975).

Cultivation and virus growth cycle.

Bovine Herpes Virus 1 infects a wide variety of tissues from the bovine and other species producing distinctive cytopathic changes that serve as a basis for virus isolation, virus titration, and neutralization tests for serum antibodies (Carbrey et al, 1972). Cell cultures derived from bovine tissue (fetal or neonatal kidney or testis cultures) are considered to be the most susceptible for virus isolation. Gross cytopathology induced by BHV1 shows rounded cells with small refractile syncitia being formed and as CPE progresses strands of cytoplasm are seen linking affected cells (Gibbs and Rweyemamu, 1977). Microscopic cytopathic effect shows large intranuclear inclusion bodies (Cowdry type A) in infected cells. The nuclear chromatin is displaced to the periphery of the nucleus and is separated from the inclusion by thin clear zone (Cheatham and Crandell, 1957).

The virus grows to high titer, produces intranuclear inclusions and abolishes ciliary activity in nasal and tracheal organ cultures

(Schroyer and Easterday, 1968; Chia and Savan, 1974a,b). The growth cycle is relatively short, with progeny completed in 12 hours (Mohanty, 1978; Luria et al, 1978). Bovine Herpes Virus 1 induces plaque formation under agar overlay in various cell cultures; only one infective particle of virus is necessary to produce one plaque (Stevens and Groman, 1963), and different BHV1 isolates cannot be differentiated by plaque morphology (Bagust, 1972; Buening and Gratzek, 1967).

The life cycle and sequential development of the IBR virus are similar to other herpes viruses (Schwartz and Roizman, 1969; Jasty and Chang, 1971; 1972; and Valicek and Smid, 1976). The eclipse period varies, depending upon the cell type, between 3 and 6 hours; the latent period is between 7 and 8 hours after which extracellular virus continues to rise for about 18 hours. Using acridine orange staining, Feulgen test reaction and fluorescent antibody techniques, the location of viral DNA and viral protein, during the growth cycle of BHV1 in cell monolayers, have been examined by several authors (Orsi and Cabasse, 1958; Theil et al, 1971).

Clinical diseases associated with IBR virus.

Infectious Bovine Rhinotracheitis virus has been reported to cause primarily respiratory and reproductive forms of disease (Schultz et al, 1977). The virus was also demonstrated to be associated with conjunctivitis (Timoney and O'Corner, 1964), central nervous system infection (Lomba et al, 1973), enteritis (Wellemans et al, 1974), fatal systemic IBR in neonates (Baker et al, 1960;) dermititis (Bwanganoi and Kaminjola, 1971) and mastitis (Gourley, 1974; Roberts et al, 1974; Greig and Bannister, 1965).

The respiratory form is usually described as an acute necrotic rhinotracheitis. The route of entry into animal is generally agreed to be by the respiratory tract and the incubation period varies from 2-7 days depending on exposure dose (Smith, 1976). The course of the disease is variable among individuals and apparently among outbreaks. The morbidity rate is high and mortality rate is low in most natural outbreaks (McKercker et al, 1957; Wiseman et al, 1980). Clinical symptoms include fever, increased respiratory rate, cough, anorexia and depression (Curtis et al, 1966; Smith, 1976). Frequently, in natural infection, the respiratory form is associated with conjunctivitis which may be prominent (Smith, 1976). Keratoconjunctivitis, usually without ulceration, is manifested by a copious serous discharge, extensive hyperemia and edema of the conjunctiva (Smith, 1976).

The genital form is recognized as infectious pustular vulvovaginitis (IPV) and balanoposthitis and characterized by hyperemia of genital mucous membrane leading to pustule formations and ulceration (Smith, 1976). The virus is widely known as a cause of abortion (Wilson, 1974; McKercher and Wada, 1964), stillbirth and infertility (Schultz et al, 1977; Elazhary et al, 1980). Abortion usually occurs between the 4th and 7th month of gestation and results from fetal death (Gibbs and Rweyemamu, 1977).

Several authors have reported the occurence of meningoencephalitis as sequel to IBR virus infection (Bartha et al, 1969; Bagdadi and Martin, 1974; Beck, 1975). Neurotropic characteristics appear to be associated with certain isolates of IBR virus and most cases of encephalitis occur in calves under 6 months of age (Smith, 1976).

Latent infection.

Like most herpes viruses, IBR virus has been shown to induce latency after a primary infection (Davies and Carmichael, 1973; Plowright, 1978). Snowdon (1965) was first to report long-term intermittent excretion of virus from experimentally infected cattle. Recrudescence of clinical disease with excretion of virus may occur as a reaction to stress (Gibbs and Rweyemamu, 1977) and the virus could be reactivated after corticosteroid administration in naturally infected cattle (Gibbs et al, 1975; Dennett et al, 1976). Studies on experimentally infected animals recovered from primary infection demonstrated that the virus could be reactivated after intravenous inoculation with corticosteroids (Davies and Carmichael, 1973; Sheffy and Rodman, 1973). Vaccination of cattle with modified live vaccine resulted in latent infection and the virus was reactivated after corticosteroid treatment (Sheffy and Rodman, 1973).

Although the actual site of IBR virus latency and the mechanism involved in reactivating the virus are not fully understood, it is believed that the virus, like other herpes viruses (Luria et al, 1978), can remain latent in neurological cells (Stevens, 1978). Narita et al (1976) have shown that the virus can reside in the trigeminal ganglion of cattle, and Homan and Easterday (1980) have isolated IBR virus from trigeminal ganglion of normal animal. However, it is not known what form the latent virus takes and whether the mechanism of latency differs from other herpes viruses (Holland et al, 1980).

Host heterogeneity.

Cattle are believed to be the only significant natural host of IBR virus infection (McKercher, 1973). Serological and isolation techniques have been used to implicate several other species in natural infection, including goats (Mohanty et al, 1972), horse (Jubb and Kennedy, 1970), pigs (Saxegaard and Onstad, 1967; Derbyshire and Caplan, 1976), wild deer (Friend and Halterman, 1967), water buffalo (St. George and Philpott, 1972), several African species (Karstad et al, 1974; Hedger and Hamblin, 1978), mustelids (Porter et al, 1975) and man (Afshar and Tadjbakhsk, 1970).

Experimental infection, in addition to cattle, has been demonstrated in pigs (Nelson et al, 1972) goats (McKercher et al, 1958), mule deer (Chow and Davies, 1964), neonatal skunks (Lupton et al, 1980a), ferret (Smith, 1978b), adult and neonatal rabbit (Kelly, 1977; Lupton and Reed, 1979; Lupton et al, 1980b) and athymic nude mice (Geder et al, 1981).

Role in bovine respiratory disease complex.

Bovine respiratory disease complex is recognized as the most significant cause of economic loss in feedlot cattle. In addition to death of animals, losses are attributed to treatment, weight loss, prolonged feeding periods, prevention programs and research expenses (Adams et al, 1959; Jensen and MacKey, 1979). It has been suspected for many years that synergism between viruses and bacteria is an important factor in the etiology of bovine respiratory disease. Viruses known to be involved include BHV1, respiratory syncitial virus, bovine virus

diarrhea, adeno-, rhino-, and reoviruses (Straub, 1970). However, the importance of primary IBR within the respiratory disease complex is still poorly defined (Yates, 1982). Jansen (1980) stated that BHV1 contributes considerably to the causes of bovine respiratory disease, while Lupton and Reed (1980b) reported that respiratory IBR is a significant component of the disease complex. The virus has been implicated as a predisposing factor in the development of bacterial pneumonia in cattle (Carter, 1973; Joricho et al, 1978) and Bloom et al (1979) estimated that 1% of cattle infected with IBR die of secondary bacterial pneumonia. It is likely that IBR increases the susceptibility of the lung to bacterial infection by immunosuppression, indirectly resulting in impaired macrophage function, rather than by reducing the activity of a major proportion of macrophages as a direct result of infection (Forman et al, 1982a,b).

Immunity.

The immunological response of cattle to BHV1 can be divided into humoral and cell-mediated immunity (CMI). Though current evidence indicates that CMI is critical and the most important for recovery from herpes virus infections, humoral immunity may also play a significant role. Detectable neutralizing antibodies to IBR virus appear in blood between 8 and 14 days after infection (Davies and Carmichael, 1973) and persist for up to 5.5 years after infection (Chow, 1972). Passive antibodies are readily transferred to the calf in the colostrum and may be detected until the calf is 4-6 months old (Straub, 1969). Humoral immunity has been demonstrated to play an important role in

limiting virus spread between cells through the mechanism of antibody-complement lysis (Rawls and Tompkins, 1975), antibody-dependent cellular cytotoxicity (ADCC) (Bloom and Rager, 1975) and antibody-complement mediated inflammatory response (Osler, 1976). Though Herpes Viruses may not enter the extracellular environment, the virus being spread from infected to susceptible cells by intracellular route, infected cells express viral antigens on their cell surface (Roane and Roizman, 1964) and become susceptible to recognition and destruction by the immunological mechanisms. Rouse and Babiuk (1978) suggested that the humoral immunity plays a role in Herpes Virus infections by antibody-complement lysis and ADCC mechanisms, and it is more important in recovery from recrudescence than in primary infection.

In general, humoral immune response is used as an epidemiological tool. The techniques used to detect serum antibody include various serum neutralization tests (Dawson et al, 1962; Snowdon, 1964; McKercher and Saits, 1965; Mohanty and Lillie, 1965; Greig, 1969; House and Baker, 1971), passive hemagglutination test (Whitman and Hetrick, 1965; Kirby et al, 1974), gel diffusion techniques (Kaminjolo and Gicho, 1972), complement fixation test (Wellemans and Lenmen, 1973) and indirect fluorescent antibody procedure (Assaf et al, 1975). The constant virus varying serum, although widely used, is less sensitive than the constant serum varying virus test (House and Baker, 1971) and the passive hemagglutination test (Kirby et al, 1974). The sensitivity of the test can be improved either by reducing the concentration of challenge virus (Huck and Woods, 1972; Bitsch, 1970), increasing the virus-serum incubation period (Bitsch, 1978) or adding complement to the test

system (Rossi and Kiesel, 1974). Bommeli and Kihm (1980, 1982) reported that the ELISA may be a very important tool with many advantages within a disease control program. A great advantage of the ELISA is also its applicability to detect antibodies in milk samples (Stuker et al, 1980).

The importance of CMI in controlling herpes virus infection was demonstrated by several authors (Lodwell et al, 1973; Notkins, 1974; Russell et al, 1975). Herpes Virus infections are more severe in individuals with deficiency in CMI response (Allison, 1972; Merigan and Stevens, 1971). The appearance of antibody does not correlate with the time of recovery from infection (Rosenberg et al, 1972; Rouse et al, 1975) and Bottcher and Mahler (1970) reported the shedding of virus in naturally IBR infected cattle occurring in spite of the presence of circulating IBR antibody. The observations reported by Sheffy and Rodman (1973) and Schultz (1977) that cattle vaccinated with IBR virus vaccines cannot withstand the challenge of virulent strain of IBR virus despite the presence of high serum antibody supported the important role of CMI response in IBR infection. The measurement of CMI response to IBR virus are performed in vitro and have included lymphocyte blastogenic responses, direct cytotoxicity and lymphokine (interferon) production (Gibbs and Rweyemamu, 1977).

Nowell (1960) was first to report the measurement of thymic-derived lymphocytes (T-cells) function by evaluating lymphocyte blastogenesis in vitro in response to phytohaemagglutin (PHA), a specific T-cell nitrogen. Quantitation of lymphocyte stimulation by measuring the incorporation of ^{3}H-Thymidine (^{3}HTdR) into PHA-stimulated lymphocytes was developed by Sorensen et al (1969). Such assays have been shown

to be correlated with CMI status in animals (Cendrowski, 1970; Al-Sarraf, 1972) and to be useful in assessing the importance of CMI in virus recovery.

Lymphocyte stimulation assay for IBR virus has been described by Davies and Carmichael (1973) and Rouse and Babiuk (1974). Incorporation of ^{3}HTdR into sensitized lymphocytes (challenged with UV-inactivated IBR virus) from IBR infected cattle occurs at about 5 days after infection, peaks at 8-10 days and declines thereafter. The ability of anti-IBR serum to inhibit lymphocyte blastogenesis in response to UV-inactivated IBR antigen and the inability of other viruses to stimulate IBR-sensitized lymphocytes indicate specific antigen stimulation. Blastogenic assays, however, only give a measure of antigen recognition by immune cells, and do not give any information whether or not the lymphocytes can act as the effector cells. Rouse and Babiuk (1975) demonstrated that sensitized lymphocytes are able to prevent viral plaque formation and markedly decrease virus yield in IBR infected cell monolayer. The inhibition is shown to be immunologically specific and involves a suppression of viral replication rather than a destruction of free virus or virus infected cells. Subsequent observations by Babiuk and Rouse (1976) on the production of interferon by peripheral blood lymphocytes (PBL) of the IBR infected animals suggest that the inhibition is indeed produced by interferon. The immune interferon appears to be synthetized by immune T-lymphocytes with the cooperation of macrophages in response to IBR antigen. Immune T-lymphocytes are also involved in direct cytotoxicity against IBR infected cells as demonstrated by ^{51}CR release technique (Rouse and Babiuk, 1977).

Interferon.

The interferon system is an inducible genetic function of all somatic cell, which, when activated, can initiate several pathways which in turn can affect viral replication, the immune responses (Epstein, 1977), expression of cell membrane antigen (Lindahl et al, 1977) and proliferation of both normal and tumor cells (Ho and Armstrong, 1975). Interferons are a family of proteins produced in response to viral infection (Ho and Armstrong, 1973; Colby, 1977) and other stimuli such as bacteria (Youngner and Steinberg, 1964), endotoxin (Ho and Kono, 1965), protozoa (Remington and Merigan, 1968), rickettsiae (Hahon and Kozkowski, 1968), natural and synthetic nucleic acid (DeClercq, 1974), mitogen (Wheelock, 1965) and antibiotics (Merigan, 1973). A subclassification of interferon based on mode of induction and stability at pH2 was established by Youngner and Salvin (1973). Classical interferon or interferon type I is defined as acid-stable interferon produced by both lymphoid or nonlymphoid cells in response to virus or other stimuli and immune interferon or type II which is acid-labile and produced by lymphocytes in response to specific antigens or mitogens. These interferon types are different in immunological, biological and physiochemical properties (Epstein, 1977).

Herpes Viruses are reported to be both poor interferon inducers and to be insensitive to its effect (Trueblood and Manjara, 1972; Ho, 1973). Interferon production has been reported after inoculation of IBR virus into cattle (Rosenquist and Loan, 1969; Todd et al, 1971; 1972) and in IBR infected organs and cell cultures (Smorodintsev, 1968; Fulton, 1976). Rosenquist and Loan (1969) reported that interferon is detected in serum

as early as 6 hours post inoculation, reaches a peak at 1-2 days and declines thereafter; the later event being closely correlated with the presence of viremia. There appears to be a good correlation between the suppression of nasal viral titers and maximum levels of interferon (Todd et al, 1971; 1972). Vaccinated calves with high level of nasal interferon are refractory to challenge with virulent IBR virus (Todd et al, 1971; 1972) and calves given an interferon inducer (Poly I-Poly C) 3 hours before infection developed a milder clinical disease than untreated calves (Theil et al, 1971). These observations supported the protective role of interferon in IBR virus infection.

Prevention and control.

Hygiene, management and isolation procedures have been claimed to reduce morbidity and mortality from IBR; however, the virus is readily transmitted to susceptible cattle, and the success of these measures is limited by the wide geographic distribution of the infection in both active and latent forms (Kahrs, 1977). Most cattle with humoral antibody are latently infected (Davies and Duncan, 1974) and are potential sources of infection (Sheffy and Krinsky, 1973; Magwood, 1974; Dennett et al, 1976). Thus, control and prevention of the disease are based on vaccination. Vaccines for IBR consist of modified live virus (MLV) vaccines for intramuscular (IM) or intranasal (IN) use and inactivated vaccines.

Modified live virus vaccine for IM use.

The first IBR virus vaccine consisted of a parenterally administrated MLV vaccine that had been attenuated by rapid passage in bovine

cell cultures (Schwartz et al, 1957). Subsequently, MLV vaccines for IM inoculation were attenuated by adaption to porcine (Schwartz et al, 1958) or canine (Zuschek and Chow, 1961) cell cultures, by adaptation to cell cultures at 30°C (Inaba, 1975) or by selection of host stable mutant (56°C for 40 minutes) (Bartha, 1974). It was widely used and had the advantages of ease of administration and availability in combination with other vaccines (Kahrs, 1977). Intramuscular vaccination with MLV vaccine stimulated local CMI and antibody responses as well as systemic CMI and antibody responses to IBR virus (Gerber et al, 1978). The duration of humoral immunity persisted for 3-6 years after vaccination (Rosner, 1968; Chow, 1972) and early protection (40 to 96 hours) was demonstrated in calves given intramuscular MLV vaccine (Bordt et al, 1976). The efficacy of the vaccine was diminished by the presence of maternal antibodies in calves less than 6 months of age (Kahrs, 1977). Occasionally, it caused abortion in pregnant animals (McFeeley, 1968; Kelling et al, 1973; Mitchell, 1974; Wilson, 1974) and produced post vaccinal reactions (MacKey, 1971; Reed et al, 1973; Roberts et al, 1974; Frank et al, 1977). Many investigators have reported that vaccinated calves shed IBR virus into nasal secretions, urine, semen and milk which serve as sources of infection to susceptible cattle (Kelling et al, 1973; Straub, 1976).

Modified live virus vaccine for IN use.

Intranasally administrated MLV vaccine was introduced in 1969 (Todd et al, 1971) and has gained widespread acceptance since it may overcome the problem of causing abortion when administered into pregnant

cows (Kahrs et al, 1973; Smith, 1978a). Vaccine strains have been attenuated by several passages in rabbit cell cultures (Todd, 1974) or modified by treatment with HNO_2 followed by selection of temperature-sensitive mutants (Zygraich et al, 1974). Intranasal MLV vaccine induced local immunity involving interferon (Todd, 1972, 1974; Gerber et al, 1978; Cummins et al, 1982), secretory antibody (IgA and Ig G) (Zygraich et al, 1975; 1976; Gerber et al, 1978) and cell mediated immunity (Gerber et al, 1978). The intranasal vaccination of cattle with MLV provided protection within 72-96 hours post vaccination against virulent IBR challenge (Straub, 1976; Todd, 1974; 1972) and is shown to be efficaceous for vaccinating calves with colostral antibody (Todd, 1976). It was reported that the IBR vaccine is stable and does not infect the fetus (Gibson, 1973; Todd, 1976). However, vaccination with MLV vaccine by the intranasal route also resulted in the shed of virus from vaccinated animals (Todd et al, 1971; Zygraich et al, 1974) and subsequent transmission of IBR virus to nonvaccinated animals (McKercher and Crenshaw, 1971; Zygraich et al, 1974; 1974b). After vaccination of cattle with intranasal MLV vaccine, periodic shedding of IBR virus and development of mild clinical signs have been described by Hyland et al, (1975). Finally, administration of intranasal vaccine may be difficult and the vaccine may produce post vaccinal reactions (Kahrs, 1973). The duration of protection following vaccination was unknown and annual revaccination was recommended (Kahrs, 1977).

Comparing the effectiveness of IN and IM routes of injections, McKercher and Crenshaw (1971) reported no distinct advantage of IN over IM vaccine for systemic antibody response and Gerber et al (1978)

recorded no difference in the systemic CMI response to IBR virus following IM and IN vaccination. However, the local antibody response to IBR virus was greater in cattle vaccinated IN vaccine than in cattle vaccinated IM (Gerder et al, 1978).

The extensive use of live attenuated IBR vaccines for controlling IBR infections in cattle has raised some concern as to their safety, especially their potential to produce latency and later revert to virulence (Pastoret et al, 1980a). The discovery of temperature-sensitive (ts) mutants, which are able to replicate only in the cooler upper respiratory tract, is of special interest with regard to protection against IBR virus infections (Zygraich et al, 1974 a,c; 1976). The vaccine was reported to elicit neutralizing antibody production, protect the vaccinated cattle against challenge with virulent virus and to be safe for vaccination in pregnant cows (Kucera et al, 1978). In addition, both local and systemic humoral immune and CMI responses were elicited in vaccinated cattle (Gerder et al, 1978). However, Pastoret et al, (1980a) reported that ts-IBR vaccine can establish latency with the same frequency as a non-ts vaccine and the virus can be reactivated with dexamethasone.

Inactivated IBR vaccine.

Inactivated IBR vaccine available was an adjuvanted polyvalent vaccine, which included IBR virus, Pasteurella bacterin and inactivated PI-3 vaccine (Matsuska et al, 1972; Schipper and Kelling, 1975; Koonse and Overpeck, 1977; Frerichis et al, 1982). Inactivation has been obtained by formalin treatment (Zuscheck and Chow, 1961), ethanol

treatment (Haralambiev, 1976) and heat or ultraviolet (Hristov and Karadjov, 1975). Inactivated vaccines without adjuvant have poor efficacy (Schipper and Kelling, 1975). Efficacy has been improved, using Freund's adjuvant, oil, saponin, adsorbed aluminum hydroxide gel and sodium alginate (Lupton and Reed, 1980a). The vaccine has the advantage of overcoming concern about post vaccination abortion, unfavorable post vaccination reactions, latency and reactivation of IBR virus (McKercher, 1971; Kelling et al, 1973; Wilson, 1974; Roberts et al, 1974). The failure of the inactivated vaccine to induce detectable serum neutralizing antibody response, to confer protection against intranasal challenge or to moderate replication of the challenge virus was reported by several authors (Hamdy and Trapp, 1964; Schipper and Kelling, 1975; Msolla, 1979; Frerichis et al, 1982). However, some investigators have demonstrated that serum antibody titers increased after second injection of the vaccine (Kristov and Karadzhov, 1976; Karadjov, 1976; Inoue et al, 1977). The adverse effects of inactivated vaccines included concern for fatal hypersensitivity reaction (anaphylaxis) and nonfatal urticaria. The duration of protection is not known and a second and annual revaccination are recommended (Kahrs, 1977).

Finally, Lupton and Reed (1980a) reported that the utilization of subunit (Triton X-100 and NP-40 solubilized IBR virus) vaccine would provide many advantages. There were no live virus and therefore no transmission to other animals, no abortion or latency following administration of such vaccine. Pastoret et al (1980a) emphasized the importance of producing ts mutants which do not produce latency.

MANUSCRIPT

ABSTRACT

BALB/C mouse lung and kidney primary cultures were inoculated with Infectious Bovine Rhinotracheitis (IBR) virus. Virus infectivity titers were comparable in the two cell systems. Virion synthesis first occurred between 4 and 8 hours after virus inoculation and maximum virus titer ($5x10^5$ PFU/ml) was detected at 48 hours post infection (PI). Cytopathic effect (CPE) in cell cultures was initially observed at 8 hours and over 90% of the infected monolayers showed CPE in 48 to 72 hours PI. Approximately 60-80% of the newly replicated virus was cell associated as determined by plaque assay of extracellular and intracellular virus during virus growth cycle.

Pulse-chase experiments demonstrated the incorporation of radioactive precursors (^{3}H-thymidine and ^{3}H-valine) into viral macromolecules. Viral DNA synthesis was initiated at 2 to 4 hours, was maximum between 4 and 6 hours; viral protein synthesis began at 4 hours and peaked between 6 and 8 hours PI.

Enzyme-linked immunosorbant assay (ELISA) detected viral proteins and a gradual increase in specific viral antigen occurred in cultures during virus replication cycle.

INTRODUCTION

Infectious Bovine Rhinotracheitis Virus (IBRV) is a member of Herpesvirus group and is known to cause primarily respiratory, genital and sometimes nervous disease in cattle. The disease presents a major economic problem in feedlot animals and dairy herds and has been reported in most countries over the world. The virus may also be associated with ocular, enteric, neonatal and dermal infection in cattle (Gibbs and Rweyemamu, 1977). Natural infection has been described in goats (Karstad et al, 1974), swine (Derbyshire and Caplan, 1976), mink and ferrets (Porter, et al, 1975), and various species of wild life (Gibbs and Rweyemamu, 1977). Experimental infection has been reported in mule deer (Chow and Davis, 1964), goats (Mohanty et al, 1972),swine (Nelson et al, 1972),ferret (Smith, 1978), and rabbit (Kelly, 1977; Lupton et al 1980). The virus establishes persistent and latent infection in its natural host, replicates in a manner similar to herpes simplex virus and human cytomegalovirus in vitro cell systems (Geder et al, 1981). Snowdon (1965) reported long-term, intermittent excretions of IBRV, and several authors have reported excretions of latent IBRV activated by various stimuli including corticosteroids (Pastoret et al, 1979).

Studies of IBRV pathogenesis and vaccine evaluation currently are limited to cattle. However, neonatal rabbits are susceptible to the virus (Kelly, 1977), and IBR virus has been demonstrated to transform mouse macrophages and mouse embryofibroblasts and to cause a persistent infection in athymic nude mice (Geder et al, 1980; 1981).

Although the virus has been propagated in wide variety of cell cultures (Madin et al, 1965; McKercher, 1959; Plummer et al, 1969 and House, 1972), its growth in mouse primary cultures has not been reported. The purpose of the present study was to investigate replication of IBRV in mouse primary cell cultures.

MATERIALS AND METHODS

Stock virus preparation:

Cooper strain of IBRV was obtained from Dr. David Reed (Iowa State University, Ames, Iowa). Stock virus was prepared by infecting confluent cultures of Madin-Darby bovine kidney (MDBK) cells at a multiplicity of 0.1 PFU/ml. The cultures were harvested when extensive cytopathic degeneration appeared (48 to 72 Hours). Cultures were frozen, thawed and the cellular debris was removed by centrifugation at 5,000 x g in a Sorvall GSA rotor for 20 minutes. The supernatants were saved and stored at -70°C.

Plaque assay:

The virus infectivity was determined by plaque assay. MDBK monolayers were inoculated with serial tenfold dilutions of virus suspension in Minimal Essential Medium (MEM) Eagle's supplemented with 5% heat inactivated fetal calf serum (FCS) free of IBR antibodies. The virus was adsorbed for 1 hour at 37°C and monolayers were overlaid with a mixture of equal parts of 2% agar in deionized distilled water and 2x MEM containing 5% FCS. Cultures were incubated at 37°C for 72 hours in a 5-8% CO_2 atmosphere. Cells were strained with 0.01% neutral red in agar MEM mixture and plaque forming units (PFU) titer was determined by enumerating plaques.

Growth cycle of the virus in mouse cell cultures:

Mouse primary cell cultures were prepared from 6 to 9 day old mice. Kidneys and lungs were aseptically removed, finely cut, washed two times with Phosphate-Buffered Saline (PBS) and trypsinized with 0.25% trypsin. After centrifugation, cell pellets were resuspended in MEM Eagle's containing 10% FCS; cells were counted, seeded in 60 x 15 mm dishes, and incubated at 37°C in a CO_2 incubator. Monolayers were formed in 2-3 days in kidney cultures and 3-4 days in lung cells; media was aspirated, cultures were washed twice with PBS and inoculated with IBR virus at a multiplicity of 1. The virus was adsorbed for 1 hour, cultures were washed twice with Hanks Balanced Salt Solution (HBSS) to remove unadsorbed virus and reincubated in MEM with 5% FCS. At various times post inoculation, the infected monolayers were observed microscopically for CPE. Both cells and fluids were harvested together and frozen at -70°C until they were assayed for virus propagation by plaque assay on bovine kidney (BK) cells.

Cultures were also prepared as described above to study the intracellular and extracellular virus yields. At various times after infection, cultures were harvested by scraping the cells gently with a rubber policeman. Cells and fluids were collected, centrifuged at 1000 x g for 15 minutes to pellet the cells. Supernatants were stored frozen at -70°C until assayed for extracellular virus yield. The cells were washed twice with HBSS, resuspended in MEM with 5% FCS, frozen-thawed, sonicated and plaque assayed for intracellular virus infectivity titer.

Synthesis of viral macromolecules:

Pulse-chase experiments were performed to detect IBR virus de novo DNA and protein synthesis for progeny virus. Mouse primary cell monolayers were inoculated with IBR virus, incubated for 1 hour, washed twice and medium containg 5% FCS was added. At various times, medium was aspirated from a set of cultures and radioactive medium containing 5uci/ml ^{3}H-thymidine (^{3}HdTR) was added for 2 hours. Simultaneously, uninfected control cultures received the same amount of radioactive medium. Following two hours incubation period, labelled medium was removed and the cells were washed 3 times with HBSS. MEM containing 10 ug/ml cold thymidine was added and the radioactivity was chased into the progeny viral DNA. Cultures were incubated then harvested 24 hours PI. Samples were frozen, thawed, sonicated and centrifuged at 2000 x g for 20 minutes to remove cell debris. The virus was concentrated and purified in an ultracentrifuge at 10^{5} x g, washed once with HBSS and resuspended in PBS. After a brief sonification, the radioactivity in partially purified was counted. The virus was also titrated by plaque assay and the protein content was determined by the method of Lowry et al (1951) using bovine serum albumine (BSA) as standard.

In order to examine viral protein synthesis, cultures were prepared as described in the experiment for DNA synthesis. Cultures were inoculated with IBR virus and radioactive medium containing 5uci/ml ^{3}H-valine was added to follow the incorporation of radioactivity into viral protein in a pulse-chase experiment.

Determination of specific viral antigen synthesis by ELISA test:

Rabbit anti-IBR serum was prepared from New Zealand white rabbits (8-10 weeks of age) injected with purified IBR virus (in Freund's incomplete adjuvant) grown in primary rabbit kidney cell cultures. Gamma globulin was isolated by repeated precipitation with ammonium sulfate at a final concentration of one-third saturation (Garvey et al, 1977).

Horseradish peroxidase type VI (Sigma Chem. Co.) was conjugated to anti-IBR immunoglobulin essentially by the method of Nakane and Kawaoi (1974). The substrate for the experiment was 2-2' Azino-Di-(3-Ethylbenz-thiazoline Sulfonic Acid) diammonium salt (ABTS) and the antigen was prepared from IBR virus infected mouse cultures which were harvested at various times PI. Cultures were centrifuged and the cell pellets were washed twice with HBSS, resuspended in a minimum volume of PBS, and sonicated.

Protocol for the ELISA technique has been used to detect the presence of specific viral antigens (Mills et al, 1978; Edwards et al, 1983). The present study used the sandwich assay in which antigen was incubated with an excess of antibody coupled to a solid phase. After incubation and washing, the solid phase was incubated with enzyme-labeled antibody. The amount of enzymatic activity bound to the solid phase was a measure of the amount of antigen found in the first incubation. Checkerboard titration demonstrated that 10ug of anti-IBR immunoglobulin /ml per well and 1 in 100 dilution of stock conjugate were the best working dilutions for the ELISA assay under consideration.

The ELISA was performed in microtiter plates (Dynatech) which were coated with 200 ul per well of anti-IBR immunoglobulin prepared in carbonate buffer (0.01 M, pH9.6) for 90 minutes at 37°C. The plates were washed three times with PBS containing 0.1% tween 80 and 0.5% Horse serum (PBS-T-HS), and 100 ul aliquots of IBR antigen was added in duplicate wells and incubated for 1 hour at 37°C. After three more washes, 200 ul of anti-IBR-peroxidase conjugate was added at a dilution from stock of 1 to 100 in PBS-T-HS. The plates were further incubated 1 hour at 37°C, washed three times and 100 ul of freshly prepared substrate in 0.045M of citrate buffer (pH4.0) with 0.5M of hydrogen peroxide was added to each well. The reaction was stopped after 30 minutes by the addition of 50 ul per well of 0.1% EDTA (38%) in 0.2M of hydrofluoric acid and 1N NAOH and the optical density was measured at 405 nm on a titerteck photometer.

RESULTS

Growth of IBR virus in mouse cultures:

Comparison of growth curves of IBR virus, in mouse primary lung and kidney cells is presented in Fig. 1. The virus titer, as determined by plaque assay, demonstrated a slight decrease in virus infectivity during the first 4 hours post virus inoculation and indicated virus eclipse period. Virion synthesis began thereafter, increased exponentially and reached a peak titer of $3x10^5$PFU/ml in lung cultures and $2.2x10^5$ PFU/ml in kidney cultures, by 48 hours PI. Adsorption of the virus, one hour PI, was relatively less in lung cultures (1/3 of kidney cultures) in which the virus growth started at 4 hours PI with a maximum increase

of approximately 2 log PFU in 24-48 hours. In kidney cultures, virus maturation began at 6 hours PI with a maximum increase of 1 log at 48 hours PI.

Comparing cytopathic effect (CPE) in IBR inoculated cultures (Fig. 1), complete cell destruction occurred in 48 hours in lung cultures whereas maximum CPE was observed at 72 hours in kidney cells. The development of CPE started earlier in lung cultures (8 hours PI) than in kidney cultures (12 hours PI).

Extracellular and intracellular virus:

Greater percentage of virus was cell associated throughout the virus replication cycle (Fig. 2); approximately 60-80% of the newly replicated virus was demonstrated in the sedimented cells. After a latent period of about 4 hours, both intracellular and extracellular virus titers started increasing between 6-10 hours PI, thereafter increased rapidly to a maximum titer of $8x10^3$PFU/ml (20%) as cell free virions and $5x10^4$PFU/ml (80%) as cell associated virions by 24 hours PI.

DNA synthesis:

Since the pulse-chase experiment determined the amount of labeled DNA in purified virions, the increase in radioactivity was directly related to an increase in IBR virus DNA synthesis. Figures 3 and 4 showed the amount of newly replicated virus DNA which was incorporated into the virus progeny and was expressed as specific activity of DNA synthesis. Increased viral DNA synthesis occurred in infected mouse kidney cells beginning at 2 hours with a maximum synthesis at 6

hours PI (Fig. 3). Synthesis of viral DNA in lung cultures started at 2 hours, increased at 4 hours and peaked at 6 hours PI (Fig. 4). Another peak of DNA synthesis occurred at 14 hours PI in mouse kidney and at 12 hours PI in mouse lung cells indicating a second cycle of viral DNA synthesis (Figs. 3 and 4). Since all samples were harvested 24 hours after infection, plaque assay demonstrated an identical amount of virus progeny made during the growth cycle.

Protein synthesis:

Synthesis of IBRV proteins is presented in Table 1. Comparing uninfected and infected cultures, viral proteins increased between 6 and 8 hours in kidney cultures as measured by the incorporation of radioactive valine in purified virus. In lung cultures the increase was first observed at 2-4 hours and was maximum at 6 hours PI. There were also second peaks of protein synthesis at 12 hours PI in both cultures indicating another replication cycle of the virus.

ELISA assay:

The optical density measurement of samples prepared from uninfected and infected cultures at various times PI were presented in Fig. 7 and 8. There was a gradual increase in optical density (0.16 to 0.4) of samples, prepared from infected lung cultures during 0-48 hours PI, indicating an increase in the specific viral protein synthesis during the virus replication cycle (Fig. 8).

Infected kidney cultures demonstrated an increase of optical density from 0.16 at 0 hour to 0.43 at 48 hours PI (Fig. 7). Samples prepared from uninfected lung and kidney cultures did not show any significant

increase in the optical density (Fig. 7 and 8). These experiments demonstrated that IBR specific viral protein were made both in mouse kidney and lung cultures and were detectable by ELISA technique.

DISCUSSION

Infectious Bovine Rhinotracheitis (IBR) virus multiplies in a wide variety of cell cultures such as bovine (Madin et al, 1965), ovine, caprine, rabbit, (McKercher, 1959), canine (House, 1972), monkey (Plummer, et al, 1969) and human lung WI-38 (Michalski et al, 1976). The available data suggests that the synthesis of IBR virus in infected cells is similar to that known for Herpes Simplex Virus and other herpes viruses (Gibbs and Rweyemamu, 1977). Jasty and Chang (1971) reported the growth of IBR virus in bovine kidney cells and demonstrated that after a latent period of 6 hours, the cell-associated virus increased exponentially and reached a maximum titer at 16 hours after inoculation. Viral yield remained more or less stationary at this concentration for about 8 hours and then declined. The cell-free virus started to increase from the 10th hour and steadily increased to reach a maximum titer at 32 hours post inoculation (PI).

Our findings that IBR virus is capable of replicating in mouse primary kidney (MKC) and lung cell cultures (MLC) bring a new evidence of the susceptibility of the mouse cultures to IBR virus infection. Propagation of IBR virus in mouse kidney and lung cell cultures demonstrates similar pattern of growth. At input of multiplicity of 1, total virus infectivity titers are comparable in the two cell systems and the highest virus titer is reached in 2-3 days PI (Fig. 1). After 4 hours exposure to virus in MLC and 4 to 6 hours in MKC, the total virus begins to increase exponentially

until 48 hours PI in MLC and 24 hours PI in MKC; maximum virus titer is obtained about 48 hours PI in both cultures (Fig. 1).

Mouse cell cultures undergo degenerative alterations in their general morphology. The cells round up and detach from the dish starting at 8 to 10 hours in MLC and at 12 hours in MKC indicating a characteristic cytopathic effect (CPE) of Herpes Virus infection. Cytopathic degeneration of the host cells continues and 85-100% of the cell cultures demonstrate CPE by 72 hours PI (Fig. 1).

There are some differences observed in the growth of IBR virus and the CPE in MLC and MKC cultures. Virus adsorption appears to be relatively greater (3 fold) in MKC at the beginning of infection and shows a longer latent period (6 hours) than in MLC (4 hours). Total virus progeny yield starts earlier in MLC and increases at a higher rate to reach a maximum titer at 48 hours PI. These observations can also be correlated to CPE in cell cultures. Cell degeneration starts earlier (8-10 hours PI) in MLC and almost 100% cells round-up and detach at 72 hours PI. In contrast, CPE is observed at a later time (12 hours PI) in MKC and only 85-90% of the cells are degenerated (Fig. 1). These differences may suggest that primary kidney cell cultures originate from clones of cells thus a greater number of cells are present as compared to MLC cultures and may cause greater virus adsorption. However, it may also be attributed to the presence of a high number of receptor sites for virus adsorption in MKC cultures. Relatively lower virus yield in MKC during the virus replication cycle may be due to low degree of virus maturation.

The eclipse phase in MLC lasts six hours followed by rapid increase of intracellular virus until 24 hours PI. Extracellular virus is first detected between 6 and 8 hours and reaches a peak at 24 hours (Fig. 2). Approximately 60-80% of the newly replicated virus is cell-associated indicating a slow release after maturation of the virus. In addition, at 24 hours, the concentration of intracellular virus surpasses that of extracellular virus. This finding illustrates that the release of virus is not concommitant with maturation of IBR virus in mouse cells and it is in accord with the growth of IBR virus in other cell types.

Synthesis of viral DNA is first observed at 2 hours PI in both types of mouse cultures; the bulk of viral DNA is synthesized between 4 to 8 hours PI, and then declines (Fig. 3 and 4). The results are in accord with these reported for Herpes Virus DNA synthesis (Roizman, 1969). During the latent period (0-4 hours) of IBR virus infected bovine kidney cells, viral DNA and viral multiplication are not detected; however between 4 and 6 hours, viral antigens make their appearance. In addition, during the logarithmic phase (7 to 15 hours) maximum viral DNA synthesis occurs (Jasty and Chang, 1969).

The incorporation of ^{3}H-valine into the virus in the pulse-chase experiment shows that viral protein synthesis occurs in both mouse kidney and lung cultures starting at 4 hours and a maximum synthesis between 4 and 8 hours in MLC and 6 and 10 hours in MKC (Table 1).

To further investigate the susceptibility of mouse cells to IBR virus, Enzyme-Linked Immunosorbant Assay (ELISA) was performed on samples from both cell cultures. The ELISA, described by Engval and Perlmann (1972), has been used in several studies for quantitative

determination of antibodies to viral antigens. Voller et al, (1976) have noted the potential of ELISA method for the detection and quantitative assay of viruses. The ELISA technique is sensitive, easily performed and requires inexpensive equipment. The rapidity with which the test results can be obtained suggests that the technique can be a good alternative to present methods for serotyping viruses (Mills et al, 1978). Edwards et al, (1983) demonstrated that ELISA results for the detection of IBR antigen, in nasal swab samples from experimentally infected calves, were very encouraging.

The ELISA assay was used to follow the growth of IBR virus in mouse primary cultures and showed a gradual increase in the optical density measurement indicating an increase in the specific IBR virus proteins (Fig. 7 and 8). The fact that almost identical amounts of IBR virus specific proteins were detected by ELISA assay in both MKC and MLC may suggest that an abortive infection was associated with IBR virus infected MKC cultures.

Finally, IBR virus has been reported to establish persistent infection in its natural host (Gibbs and Rweyemamu, 1977), and has a replication cycle similar to herpes simplex virus and human cytomegalovirus in in vitro cell systems (Roizman, 1969). Several authors have made efforts to find a suitable laboratory animal for the study of pathogenesis of IBR virus, viral latency or vaccine evaluation. Ferrets (Smith, 1978), neonatal rabbit (Kelly, 1977; Lupton et al, 1980) have been reported as potential laboratory model for IBR viral infection. IBR virus was also capable of establishing a persistent infection in athymic nude mice and transforming mouse macrophages and embryofibroblast

(Geder et al, 1980; 1981). Both humoral and cell mediated immune responses were elicited in mice immunized with IBR virus and interferon was demonstrated in lymphocyte cultures from immune mice after exposure to IBR viral antigens (unpublished data). Our findings that IBR virus replicates in mouse primary kidney and lung cell cultures would aid in (1) conducting biochemical studies on the virus replication including latent infection of cells and cellular transformation and (2) investigating immune mechanisms by preparing polyclonal and monoclonal antibodies using mouse cell culture grown stock virus for hyperimmunization.

Figure 1: Growth curves of IBR virus in mouse primary kidney and lung cultures. Monolayers were inoculated with IBR virus and incubated. At various times post inoculation, cultures were observed for CPE, fluids and cells were harvested, frozen, thawed, and assayed for virus infectivity titers by plaque assay. Symbols: infected kidney cultures (●); infected lung cultures (▲); CPE in kidney cultures (■); CPE in lung cultures (□).

Replication of IBR Virus in Cell Cultures

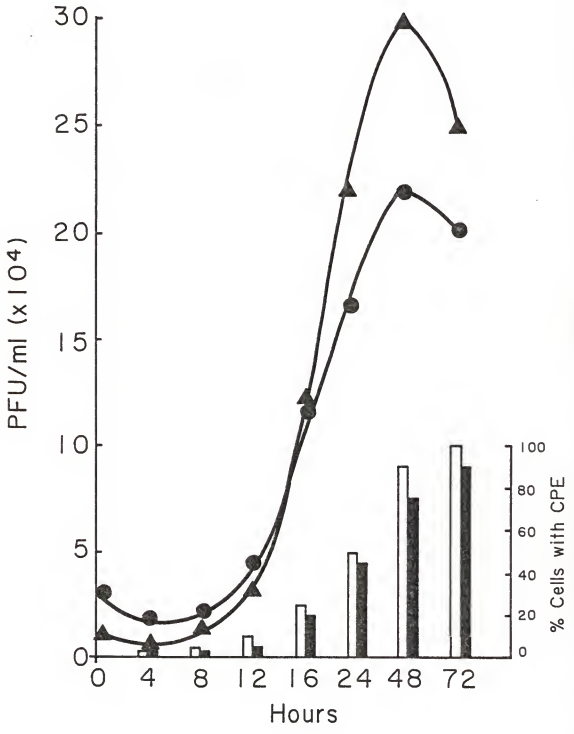
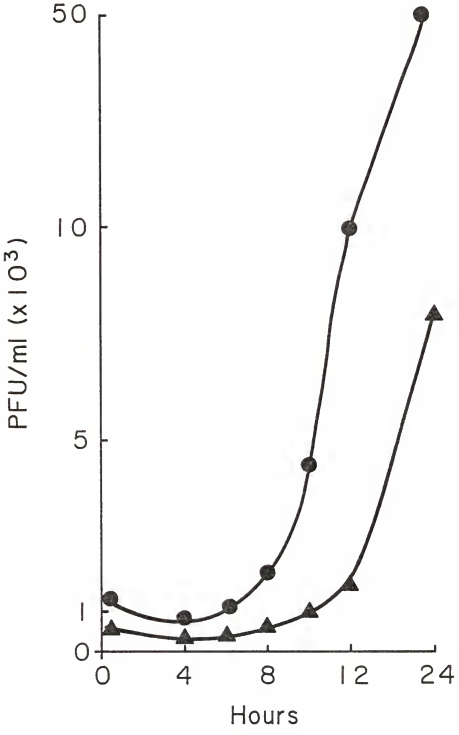


Figure 2: Cell-associated and cell-free virus production during growth cycle. Monolayer cultures were inoculated with IBR virus and incubated. At various times post inoculation, fluids and cells were harvested and centrifuged. Cells were washed 3 times with HBSS, resuspended in MEM, and both fluids and cells were stored. Intracellular and extracellular virus yields were determined by plaque assay of these samples. Symbols: intracellular virus yield (●); extracellular virus yield (▲).

Association of IBR Virus with Lung Cultures



Figures 3: Viral DNA synthesis in IBR virus infected mouse kidney cultures. Monolayers were inoculated with IBR virus and incubated. Uninfected and IBR virus infected cultures were pulse-labeled for 2 hours with 5uci of ^{3}H-Thymidine (^{3}HTdR)/ml at different times post-inoculation (PI). Cultures were harvested at 24 hours PI, ultracentrifuged and the amount of protein and the incorporation of radioactive labeled DNA into partially purified progeny virus were determined. The specific activities were calculated on the basis of DPM/ug protein. Symbols: uninfected cultures (▲); infected cultures (●); PFU/ml (o).

Synthesis of Viral DNA
in IBR-Infected Kidney Cultures

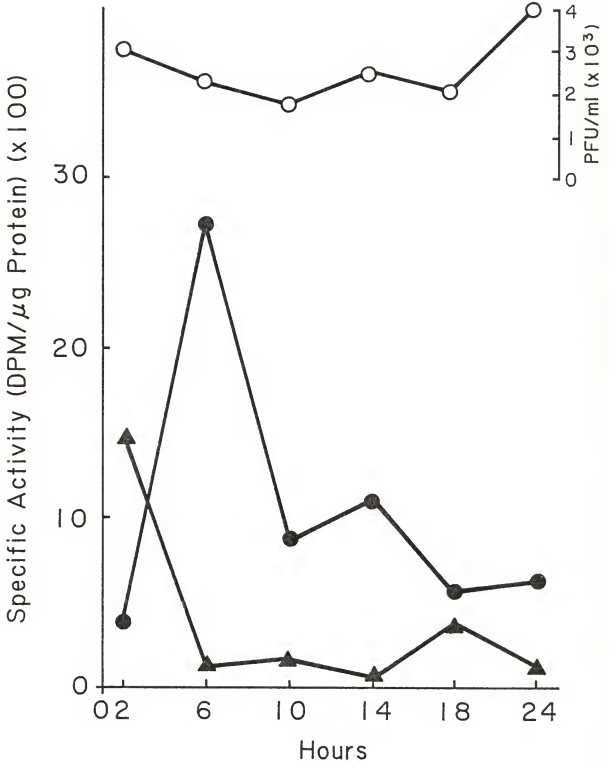
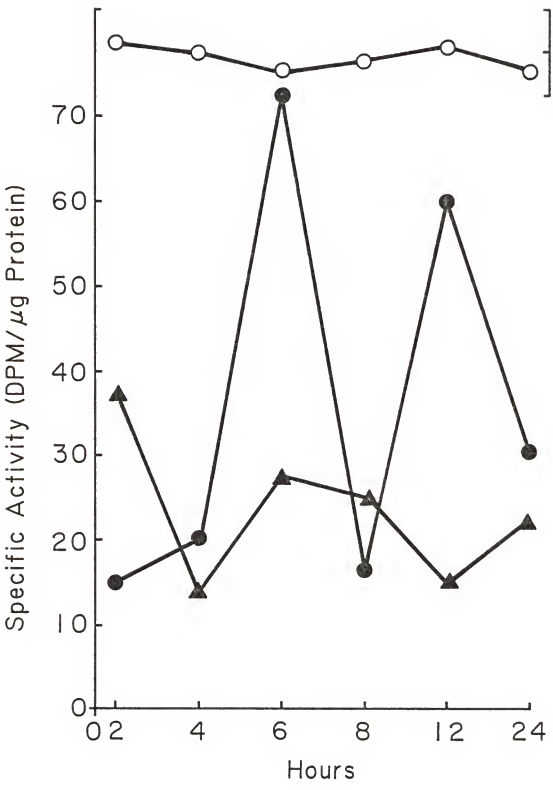


Figure 4: Viral DNA synthesis in IBR virus infected lung cultures. Monolayers were inoculated with IBR virus and incubated. Uninfected and virus infected cultures were pulse-labeled for 2 hours with 5uci of ^{3}HTdR/ml at different times post inoculation. Cultures were harvested at 24 hours PI, ultracentrifuged and protein content and ^{3}HTdR incorporation into DNA of partially purified virus were determined. The specific activities were calculated on the basis of DPM/ug protein. Symbols: uninfected cultures (▲); infected cultures (●); PFU/ml (○).

Synthesis of Viral DNA
in IBR-Infected Lung Cultures



Synthesis of Viral Protein in Mouse Cell Cultures

Time	Kidney Cell Cultures*		Lung Cell Cultures*	
(Hours PI)	Uninfected	Infected	Uninfected	Infected
2	18	15	4	7
4	15	14	4.6	7.6
6	12	14.5	5.4	10.4
8	13	21.5	6.6	7.8
12	7	12	5.4	8
24	11.5	11.5	8.6	7

*Disintegration per minute (DPM)/ug Protein.

Cell layers were inoculated with IBR virus and incubated. Uninfected and IBR virus infected cultures were pulse-labeled for 2 hours with 5 uci of ^{3}H-valine/ml at different times PI. Cell cultures were harvested at 24 hours PI, ultracentrifuged and protein content and the radioactivity were determined. The specific activities were calculated on the basis of DPM/ug protein.

Figure 7: Replication of IBR virus in mouse primary kidney cultures as determined by the specific viral protein antigen in ELISA test. IBR virus antigens, prepared from IBR virus infected cultures at different times PI, were added to microtiter wells coated with anti-IBR immunoglobulin; incubated and washed. Horseradish-peroxidase conjugate was added, the plate was incubated, washed, followed by the addition of the substrate (ABTS). The reaction was stopped and the optical density of each well was read at 405nm. Symbols: uninfected cultures (▲); infected cultures (●).

Determination of IBR-Antigen by ELISA

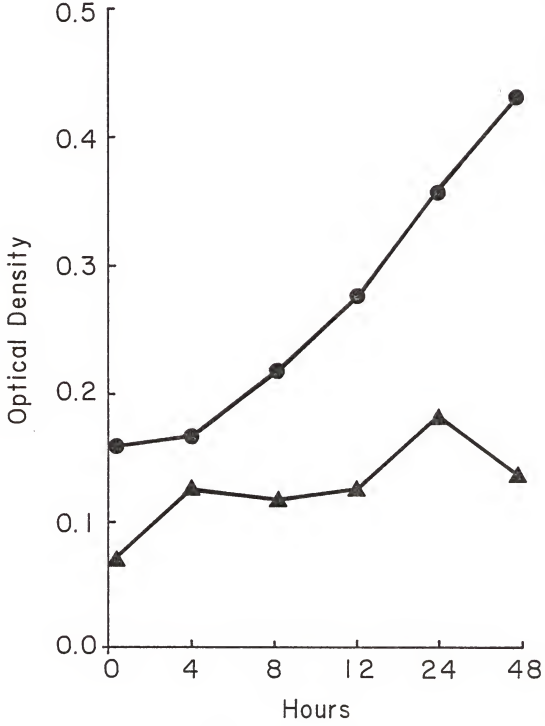
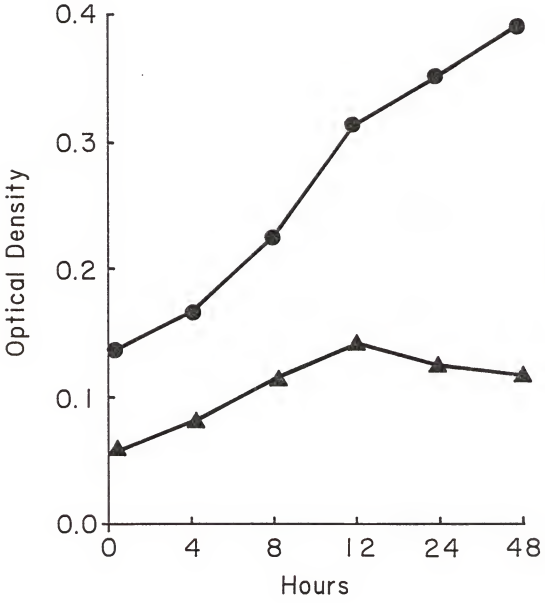


Figure 8: Replication of IBR virus in mouse primary lung cultures as determined by the specific viral protein antigen in ELISA test. IBR virus antigens, prepared from IBR virus infected cultures at different times PI, were added to microtiter wells coated with anti-IBR immunoglobulin, incubated and washed. Horseradish-peroxidase conjugate was added, the plates were incubated, washed, followed by the addition of substrate (ABTS). The reaction was then stopped and the optical density of each well was read at 405nm. Symbols: uninfected cultures (▲); infected cultures (●).

Determination of IBR-Antigen by ELISA



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APPENDIX

DETAILED METHODOLOGY

Determination of TCID50:

Tissue culture infectious dose 50 (TCID50) was performed in 96 wells microtiter plates. Monolayers of MDBK cell cultures were infected with 0.2ml of serial log dilutions of stock virus (4 wells per dilution). The cultures were observed for CPE under microscope 48-72 hours post inoculation. One unit of TCID 50 was determined as the dilution that showed CPE in 50% of cultures.

Plaque assay: Calculation of virus titer = number of (PFU)/ml of original suspension = $\frac{\text{Average number of plaques}}{\text{Volume of inoculum x dilution}}$

Preparation of UV-inactivated IBR virus:

Rabbit primary kidney cell cultures were prepared from 1-2 week old New Zealand white rabbits, essentially in the same manner as described for mouse primary cell cultures in materials and methods. Cells layers were inoculated with IBR virus at a multiplicity of 0.1 PFU/ml and incubated at 37^{o}C. The cultures were harvested when they showed extensive cytopathic degeneration (48 hours later), frozen, thawed, centrifuged at 5000 x g for 20 minutes to remove cell debris and the supernatant was saved and stored at -70^{o}C. Stock virus was further purified by ultracentrifugation over a 3 ml cushion of 25% sucrose and the pellet was resuspended in PBS. The infectivity titer was determined by plaque assay or TCID50 methods on MDBK cell.

Partially purified virus, in 1ml amount, was UV-irradiated for 5 minutes from a UV germicide sterile lamp ($630T_8$, 30 watt, Westinghouse Electric Co., Bloomfield, N. J.). Inactivation of virus was confirmed by the absence of plaques in the plaque assay.

Inoculation of rabbits and collection of serum:

Two New Zealand white rabbits (8-10 weeks old) were used to prepare anti-IBR serum. They were injected with UV-inactivated IBR virus in Freund's adjuvant according to the following protocol.

-First injection: UV-inactivated virus was suspended in Freund's complete adjuvant and divided into four doses given in foot pads.

-Second injection: two weeks later, the virus dose was doubled; the antigen being suspended in incomplete Freund's adjuvant and given intramuscularly in several sites.

-Third injection: two weeks later, the dose of second injection was doubled and given intramuscularly in divided doses.

The serum titer was checked one week later and the rabbits were bled through ear artery once a week. Freshly drawn blood was allowed to stand for clot formation and stored at $4^{o}C$ for 12-24 hours to permit clot contraction. The serum was decanted, centrifuged at 1000 x g for 30 minutes at $4^{o}C$, and stored at $-20^{o}C$ until assayed for anti-IBR antibody titer by the serum neutralization test.

Serum neutralization test:

Rabbit anti-IBR serum was heat inactivated at $56^{o}C$ for 30 minutes. Two-fold dilutions of inactivated serum were prepared in MEM with 5%

FCS and incubated with 100 TCID50 of IBR virus for 1 hour. Serum-virus mixture (0.2ml) were added on monolayers of MDBK cell cultures in a microtiter plates (4 wells per dilution). The titers of serum antibody were expressed as the reciprocal of the serum dilution that protects 50% of cell cultures after 48 hours incubation. Serum titers were determined by the method of Kaerber (1931). The serum neutralization titer was 64.

Protein determination:

Crystalline bovine albumin was used as a standard protein (1mg/ml stock). Standard curves of 10, 20, 30, 40, 50, 75 and 100 ug of bovine albumin were determined. Standard and experimental samples were made to 1.0ml with distilled water, then 5ml of solution B (2% of $NaCO_3$ in 0.1N NaOH) was added and mixtures were incubated for 10 minutes at room temperature. As soon as 0.5ml of folin solution was added, each tube was rapidly mixed, reincubated for 30 minutes at room temperature and read in Klett (#166 filter). The amount of protein in each sample was extrapolated from standard curve.

Preparation of immunoglobulin:

The isolation of rabbit anti-IBR immunoglobulin from the serum involved repeated precipitation with ammonium sulfate at a final concentration of one-third saturation, (Garvey et al, 1977). Five milliliters of saturated ammonium sulfate solution (pH7.8) were added slowly, with a constant stirring, to a 10ml serum sample. Upon completion of the addition of the salt solution, the stirring of the suspension was continued for an additional 2-3 hours in order to avoid mechanical

trapping of serum components other than gamma globulin in the precipitate. The suspension was then centrifuged at room temperature for 30 minutes at 1400 x g. The precipitate was dissolved in distilled water and purified by a second and a third precipitation and finally dissolved in 5ml of 0.01M of carbonate buffer (pH9.5). The ammonium sulfate was then removed from the precipitate by dialysing against 3 times changes of 0.01M of carbonate buffer at $4^{o}C$. The final solution was centrifuged at $4^{o}C$ for 30 minutes at 1400 x g to get rid of insoluble material formed during dialysis and stored at $4^{o}C$.

Preparation of conjugate:

Conjugate was prepared essentially by the method of Nakane and Kawaoi (1972). Horseradish peroxidase type VI (5 mg) was dissolved in 1.0ml of freshly made sodium bicarbonate (0.3M, pH8.1). To the mixture was added 0.1ml of 1% dinitrofluorobenzene (FDNB) in absolute ethanol. The above solution was gently mixed for 1 hour at room temperature, followed by the addition of 1.0ml $NaIO_4$ (0.01M) in distilled water and the mixture was gently mixed for 30 minutes at room temperature. Ethylene glycol, 0.16M (1ml), in distilled water was added and the solution was gently mixed for 1 hour at room temperature. The solution was then dialysed against 3 one liter changes of 0.01M of sodium carbonate buffer, pH9.5 at $4^{o}C$. After dialysis, 1.0ml of carbonate buffer containing 5 mg of immunoglobulin was added to the 3 ml of horseradish-peroxidase-aldehyde solution and mixed for 3 hours at room temperature. The solution was dialysed against 3 changes of 1 liter volume of PBS (0.01M) at $4^{o}C$. The small amount of precipitate that formed was removed by centrifugation at 1800 x g for 15 minutes. The supernatant fluid was stored at $4^{o}C$.

Substrate:

A stock solution of (2-2'-Azino-Di-(3-Ethylbenzthiazoline Sulfonic Acid) diammonium salt (ABTS) was prepared by dissolving 0.5487g of ABTS in 25ml distilled water. Immediately before use, 0.125ml of stock ABTS was mixed with 25ml citrate diluent (0.045M) containing 0.1ml hydrogen peroxide (0.5M).

ELISA protocol:

A checkerboard titration to find the approximate working dilutions of the reagents was set up. Ten-fold dilutions of anti-IBR immunoglobulin (100 ug to 0.0001 ug) were placed into wells of a polyvinyl microtiter plate and tested against two-fold dilutions of conjugate (1/100 to 1/3200) in PBS containing 0.1% Tween 80 and 0.5% horse serum (PBS-T-HS). Infected and uninfected samples were used as antigen. Two hundred microliters from each dilution of immunoglobulin in carbonate buffer was placed into wells of the horizontal rows of the microtiter plate (12 wells per dilution). These were incubated in a humid chamber for 90 minutes at 37°C. The fluid was removed by vacuum, and the wells were filled with PBS-T-HS which was removed after 3 minutes. The washing procedure was repeated three times, then 200 ul of antigens was placed into each well. The plate was incubated for 1 hour at 37°C followed by another wash procedure. Two hundred microliters of each dilution of conjugate, in PBS-T-HS, was placed into wells of the vertical rows; the first six vertical rows contained viral antigen, the last six vertical rows contained negative sample. The plate was incubated for 1h at 37°C and the wash procedure was repeated. This was followed by the

addition of 100 ul of freshly prepared substrate. The reaction was stopped after 30 minutes by the addition of 50 ul of 0.1% EDTA (38%) in hydrofluoric acid (0.2M). A clear difference in color was detected by viewing the microtray against a white background. The test result was then read in a titerteck photometer at 405 nm and the optical density of each well was recorded. Repeated assays demonstrated that 10 ug of anti-IBR immunoglobulin per well and 1 to 100 dilution of the conjugate were the best working dilutions for the ELISA test under consideration.

REAGENTS

1. ABTS - (2,2'-Azino-Di(3-Ethylbenzthiazoline Sulfonic Acid)
   diammonium salt----------0.5487 g
   distilled water----------25 ml
   Wrap bottle in foil and keep covered. Store at $4^{o}C$.

2. Agar - 2% purified agar is dissolved in deionized distilled water and autoclaved for use. A mixture of 50% agar - 50% 2 x MEM is used for plaquing.

3. Ammonium Sulfate - 50.42g are dissolved in 100 ml distilled water at $100^{o}C$.

4. Antibiotics - Stock solution (100 ml) contains:
   Penicillin G (1,000,000 units)
   Streptomycin (1 g)
   Kanamycin (50 mg)
   One ml of PSK stock solution is added to give a final concentration of: 100 units penicillin, 10 mg streptomycin and 5 mg kanamycin per ml media.

5. Carbonate Buffer - 10X, 0.15MpH9.6. Adjust pH with pellets of sodium hydroxide.
   Na_2CO_3----------15.9 g
   $NaHCO_3$----------29.3 g
   Q.S. 1 titer with distilled water
   Use this diluted to 1 x to coat antigen or antibody to the tray.

6. Citrate diluent for ABTS - 0.045 M

   Citric Acid----------9.6g

   Add 900 ml distilled water and adjust pH to 4.0 with IN NaOH (about 60 ml). Q.S. 1 liter with distilled water - Autoclave.

7. 2.4-Dinitrofluorobenzene - (FDNB), 1% solution, ethanol (vol/vol). Prepare on day of use.

8. EDTA tetrasodium salt 38% in distilled water.

9. Ethylene glycol 0.16M (0.45 ml/50 ml) in distilled water. Prepare on day of use.

10. Hanks Balanced Salt Solutions (HBSS):

    10 x HBSS containts:

Solution #1		
1) NaCl	100 g	Dissolve in 500 ml
2) KCL	5 g	deionized water
3) $MgSO_4 \cdot 7H_2O$	2.5 g	
Solution #2		
1) $Na_2HPO_4 \cdot 7H_2O$	1.4 g	Dissolve in 500 ml
2) (or $12H_2O$)	1.9 g	deionized water
3) KH_2PO_4	0.8 g	
Solution #3		
$CaCl_2 \cdot 2H_2O$	2.3 g	Dissolve in 125 ml deionized water.

Combine solution #1 and #2, add 250 ml deionized water, shake well and add solution #3. Add 0.25 g of pheonol red.

Complete Hanks (1 X):

10X Hanks	100 ml
Antibiotics	10 ml
Fungizone	0.2 ml
Dextrose (10%)	10 ml
Deionized water	
$NaHCO_3$ (7.5%) to pH 7.4 or 7.6	
Filter to sterilize for use.	

11. Hydrogen peroxide - H_2O_2: 0.5 M

H_2O_2----------0.5 ml

H_2O----------2.5 ml

Store in covered bottle at 4°C.

12. Hydrofluoric Acid, 0.2 M (Use a Hood):

Hydrofluoric acid----------3.47 ml

1 N NaOH----------6 ml

Q.S. to 1 liter with distilled water.

13. Folin and Ciocalteu's Phenol Reagent:

Dilution of 1 part of folin to 2 parts of distilled water is used.

14. For 25 ml of substrate (ABTS):

    Citrate diluent (solution #6)----------25 ml

    H_2O_2 (solution #11)----------0.1 ml

    ABTS (solution #1----------0.125 ml

    Prepare when ready to use.

15. Four liters 1X sodium carbonate buffer (solution #5 diluted 1:10). Adjust pH to 9.5. Chill to 4^oC.

16. Mix 0.1 ml of #8 and 100 ml of #12. This solution chelates any heavy metal in the water and stops the action of the enzyme. Prepare on day of use.

17. $NaIO_4$ - 0.08M (0.428 g/25 ml in distilled water). Prepare on day of use.

18. Neutral Red:

    A solution (0.01%) for plaquing id prepared from powder with deionized water and dissolved in 37^oC water bath. Whatman #2 filter is used for filtration. Sterilized by autoclaving and stored at 4^oC, protected from light. Add 1 ml of stock/ 100 ml of agar and media.

19. Phosphate buffered saline - 0.01M, pH 7.4. Use this solution for dialysis (preparation of the conjugate).

NaCl----------8.0 g

Kh_2PO_4----------0.2 g

KCl----------0.2 g

Plus one of the following 3:

Na_2HPO_4 (anhydrous)----------1.15 g

or $Na_2HPO_4 \cdot 7\ H_2O$----------2.17 g

or $Na_2HPO_4 \cdot 12\ H_2C$----------2.9 g

Q.S. to 1 liter with distilled water.

20. Phosphate-Buffered Saline (PBS): 0.25 M . pH; 7.2

Solution #1

$Na_2HPO_4.7H_2O$	134 g/l deionized H_2O

Solution #2

$NaH_2PO_4.H_2O$	69.1 g/l deionized H_2O

Buffer Stock:

$Na_2HPO_4.7H_2O$	386 ml - solution #1
$NaH_2PO_4.H_2O$	114 ml - solution #2
Deionized water	500 ml

PBS:

80 ml	buffer stock
17 g	NaCl
1920 ml	deionized H_2O

pH 7.2

Autoclave at 15 lbs. for 15 minutes.

21. Solution for diluting test antigen and conjugate, and for washing the coated plate:

    Solution #19 plus 0.1% tween 80 and 0.5% horse serum.

22. Solvent for Horseradish peroxidase. MAKE FRESH.

    0.3 M sodium bicarbonate = 2.529 g/100 ml distilled water

    pH 8.1.

23. Tris Buffer (Hydroxymethyl aminomethane, 0.1 N, pH 7.0):

    12.1 g of Tris is dissolved in 1,000 ml of deionized distilled water,

    HCl is used to adjust pH.

    Autoclaved for use.

24. Trypsin: 0.25%.

    25 g Trypsin are dissolved in 10 liters of PBS (solution #20), filtered through 0.45 u filter and stored at -20°C.

25. Trypsin: 1 g Trypsin in 400 ml PBS. Stored frozen.

    Working solution:

    0.25% trypsin----------20 ml

    PBS (solution #20)----------30 ml

    10% EDTA----------0.5 ml

REAGENTS AND ADDRESSES

ABTS (2,2'-Azino-Di-(3-Ethylbenzthiazoline Sulfonic acid) diammonium salt	Sigma, Chemical Co., St. Louis, MO.
Agar purified	Difco, Detroit, MI
Ammonium Sulfate	J. T. Baker Chemical Co., Phillipsburg, NJ
Calcium Chloride	Mallinckrodt Chemical Co., St. Louis, MO.
Citric Acid	Allied Chemical, Morristown, NJ.
Cupric Sulfate ($CuSO_4.5H_2O$)	Mallinckrodt Chemical Co., St. Louis, MO.
EDTA (Ethylene Diamine Tetraacetic Acid) tetrasodium salt	Sigma Chemical Co., St. Louis, MO.
Ethylene Glycol	Fisher Scientific Co., Fair Lawn, NJ.
FDNB (2,4-Dinitrofluorobenzene)	Sigma Chemical Co., St. Louis, MO.
Fetal Calf Serum	Kansas City Biological Co., Kansas City, KS.
Folin and Ciocalteus Phenol reagents	Sigma Chemical Co., St. Louis, MO.
Horseradish-peroxidase type VI	Sigma Chemical Co., St. Louis, MO.
Horse Serum	Kansas City Biological Co., Kansas City, KS.
Hydrochloric Acid (HCl)	Mallinckrodt Chemical Co., St. Louis, MO.
Hydrofluoric Acid	Fisher Scientific Co., Fair Lawn, NJ.
Hydrogen peroxide	Fisher Scientific Co., Fair Lawn, NJ

Kanamycin	Bristol Laboratories, Syracuse, NY.
Magnesium Sulfate	Sigma Chemical Co., St. Louis, MO.
Minimum Essential Media (MEM)	Grand Island Biological Co., Grand Island, NY.
Neutral Red	Fisher Scientific Co., Fair Lawn, NJ.
Penicillin G	F. R. Squibb and Sons, New York, NY.
Potassium Phosphate Monobasic (KH_2PO_4)	Fisher Scientific Co., Fair Lawn, NJ.
Potassium Chloride (KCL)	J. T. Baker Chemical Co., Phillipsburg, NJ.
Scientillation Fluid	Research Products International, Corp., IL.
Sodium Chloride (NaC1)	Fisher Scientific Co., Fair Lawn, NJ.
Sodium Bicarbonate ($NaHCO_3$)	MC & B, Norwood, OH.
Sodium Carbonate (Na_2CO_3)	Fisher Scientific Co., Fair Lawn, NJ
Sodium Hydroxide (NaOH)	Allied Chemical, Morristown, NJ.
Sodium M-periodate ($NaIO_4$)	Sigma Chemical Co., St. Louis, MO.
Sodium Potassium Tartrate (NaK tartrate)	Mallinckrodt Chemical Co., St. Louis, MO.
Sodium phosphate dibasic ($Na_2HPO_4.7H_2O$)	Fisher Scientific Co., Fair Lawn, NJ.
Streptomycin	Eli Lily, Indianapolis, IN.
Tris (base) ultra pure	Schwarz/Mann Orangeburg, NY.

Tritiated Thymidine	Schwarz/Mann Spring Valley, NY.
Tritiated Valine	ICN, Pharmaceutical, Inc., Irvine, CA.
Trypsin	Difco, Detroit, MI.
Tween 80	Fisher Scientific Co., Fair Lawn, NJ.

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PROPAGATION OF INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS IN MOUSE CELL CULTURES

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AN ABSTRACT OF A MASTER'S THESIS

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ABSTRACT

The growth of Infectious Bovine Rhinotracheitis (IBR) progeny virus and the synthesis of viral macromolecules were investigated in mouse primary cell cultures. BALB/C mice (6 to 9 days old) were asphyxiated with carbon dioxide; lungs and kidneys were aseptically removed, finely cut and trypsinized with 0.25% trypsin. After centrifugation, cell pellets were resuspended in Minimal Essential Medium (MEM) Eagle's with 10% Fetal Calf Serum (FCS), counted, seeded in dishes and incubated until monolayers were formed (2-3 days for kidney and 3-5 days for lung cultures). Cultures were inoculated with IBR virus and at various times post infection (PI), they were observed microscopically for cytopathic effect (CPE). Cells and fluids were harvested together, frozen, thawed and assayed for progeny virus by plaque assay.

Virus infectivity titers were comparable in both cultures. Virion synthesis first occurred between 4 and 8 hours after virus inoculation and maximum virus titer was detected 48 hours PI. Cytopathic effect was observed at 8 hours in lung cultures and 12 hours PI in kidney cultures, and over 90% of the infected monolayers showed CPE in 48 to 72 hours in both cultures. Intracellular and extracellular virus yields were determined in lung cultures. Infected lung cells were pelleted by centrifugation and progeny virus was assayed in both the supernatants and sedimented cells. After an eclipse period of 4 hours, both, cell free and cell associated virus increased gradually from 6 to 10 hours PI, peaked thereafter to a maximum titer at 24 hours PI. Majority of the newly replicated virus (60% to 80%) was cell associated throughout the virus replication cycle.

Viral macromolecular synthesis (DNA and protein) was determined by the pulse-chase experiments. Infected cultures were pulsed for 2 hours with radioactive precursors (^{3}H-thymidine or ^{3}H-valine) and the labeled viral macromolecules were chased, in the presence of non-radioactive medium, into the progeny virus. Viral DNA synthesis started at 2 to 4 hours and was maximum at 4 to 6 hours PI in lung and kidney cultures. Plaque assay of purified virus obtained in pulse-chase experiments showed identical amounts of virus progeny made in all samples pulsed at various times and harvested 24 hours after infection. Viral protein synthesis, in infected lung cultures, started increasing at 4 hours PI, peaked between 6 and 8 hours PI, then decreased. In kidney cultures, viral protein syntehsis increased at 6 hours, was maximum at 8 hours PI, then sharply declined by 12 hours.

In order to identify synthesis of specific viral proteins, enzyme-linked immunosorbant assay (ELISA) was performed in microtiter plates coated with ammonium sulfate-precipitated anti-IBR immunoglobulin (prepared in rabbit). Concentrated samples of IBR infected cells, harvested at different times PI, were added in duplicate to the wells, incubated, and then washed. Horseradish-peroxidase conjugated to anti-IBR immunoglobulin was added, the plates were incubated, washed, followed by the addition of freshly prepared substrate (2-2'-Azino-Di (3-Ethylbenzthiazoline Sulfonic Acid)) (ABTS). The reaction was stopped by adding EDTA in hydrofluoric acid and the optical density of each well was read at 405 nm in a titerteck photometer. Comparing samples prepared from uninfected and IBR virus infected cells, a gradual increase in the optical density measurements was demonstrated in infected samples at various

times PI. This indicated that a gradual increase in specific IBR virus proteins occurred at various times PI in both lung and kidney mouse cultures infected with the virus.