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Development and evaluation of a subunit vaccine for infectious bovine rhinotracheitis

Harold Wayne Lupton
Iowa State University

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Development and evaluation of a subunit vaccine for infectious bovine rhinotracheitis

by

Harold Wayne Lupton

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

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Iowa State University
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1979
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INTRODUCTION

Infectious bovine rhinotracheitis (IBR) was first recognized as a distinct disease of cattle during the early 1950's (170, 238). However, McKercher (156) demonstrated that a disease which had been known in Europe since 1890 as Bläschenausschlag (coital exanthema) was caused by IBR virus (IBRV). Infection with IBRV results in diverse disease manifestations that include respiratory disease (146), conjunctivitis (1), vulvovaginitis (119), balanoposthitis (69), abortion (188), meningoencephalitis (57), alimentary tract disease (67) and fatal systemic infection (211). Kahrs (105) recently reviewed IBR.

Control of IBRV by management practices has been limited by the worldwide distribution of the virus in both active and latent forms. Maintenance of an IBR disease free herd requires that only IBR sero-negative animals can be added to the herd. Specialization of cattle production practices has eliminated most closed herds and has necessitated that disease control efforts be based on the use of vaccines.

Vaccines for IBR have been available since 1956 when Schwartz et al. (239) introduced an intramuscular modified live virus (MLV) vaccine. Subsequent research has resulted in development and production of formalin-killed vaccines (299) and intranasally administered MLV (272) and temperature-sensitive (TS) MLV vaccines (302). The IBR
vaccines have been widely accepted and millions of doses have been administered annually. Vaccination of pregnant cows with an intramuscular MLV vaccine was contraindicated because of vaccine induced abortions (116, 152, 172, 213). Furthermore, the use of intramuscular MLV vaccine was contraindicated in calves nursing seronegative cows because of the contact transmission of IBRV to the cows with the subsequent occurrence of IBRV abortions (116). Intranasal vaccination resulted in mild respiratory tract infection (106, 159) and shed of virus from the respiratory tract (60, 159, 272, 274, 303). Lateral transmission of intranasal vaccine virus was demonstrated (133) when 24% and 40% of contact controls seroconverted following intranasal vaccination of cattle in field trials. Formalin-killed vaccines have been criticized because of potential hypersensitivity reactions (104). Kucera et al. (133) reported that 83% of intranasal TS vaccinates seroconverted following vaccination, while Kelling et al. (115) reported less than 50% of intramuscular MLV vaccinates developed serum neutralizing (SN) antibody titers. Lack of immunity following vaccination was demonstrated by failure to prevent IBRV induced respiratory disease in cattle (42, 94, 99). The use of live virus vaccines must be questioned because of the possible establishment of latent or chronic IBRV infection in vaccinates. Davies and Duncan (45) determined that most cattle that developed IBRV specific humoral antibodies following natural infection or vaccination with live virus vaccines were latently infected. Latent
virus was recrudesced experimentally by injections of corticosteroid hormones (48, 242, 244); or naturally by suppression of cell-mediated immunity with other viral infections (169) and environmentally or physiologically induced stress. These facts have raised serious doubts about the efficacy and safety of available IBR vaccines. Furthermore, the safety of vaccines that contain live virus or nucleic acids has been questioned because of potential involvement in transfec- tion, oncogenesis and persistent or latent infections.

Subunit vaccines composed of only the necessary antigenic subunit required for evoking protective immunity are possible. The development of subunit vaccines has been aided by determination of the immunogenic characteristics of individual constituent viral proteins. A "split vaccine" produced by dissociation of influenza virus resulted in an effective vaccine which lacked the pyrogenicity that characterized whole virus vaccines (224). Experimental subunit vaccines composed of virus envelope glycoproteins have been developed from vesicular stomatitis virus (114), rabies virus (71) and Semliki Forest virus (177). Investigators have demonstrated humoral and cell-mediated immune responses after immunization with subunit vaccines containing herpesvirus glycoproteins (30, 136, 137, 150, 192, 282, 298).

The objectives in this research were to develop a subunit IBR vaccine and to evaluate its immunogenicity, safety and efficacy. Preliminary studies were facilitated by development of a laboratory model of infection.
Explanation of Dissertation Format

This dissertation consists of an introduction, a literature review, four separate manuscripts, a general conclusion, references and acknowledgments. The Ph.D. candidate, Harold Wayne Lupton is the senior author and principal investigator for each of the manuscripts. He also performed all of the experimental procedures which are reported in the manuscripts with only limited assistance from the co-authors.
LITERATURE REVIEW

Infectious Bovine Rhinotracheitis Infection

The literature of IBRV infection has been reviewed extensively (25, 51, 98, 105, 154, 157, 160, 173, 215, 232, 252, 295). Consistent with the characteristics of other herpesviruses, IBRV replicates in a wide range of cell types and produces a variety of disease syndromes in cattle. The disease syndromes of IBRV infection are manifested either as distinct syndromes, as a complex of these syndromes or as clinically inapparent infections that are caused by antigenically homologous virus strains (1, 7, 58, 63, 65, 85, 142, 156, 164, 167, 168, 174, 203, 287).

Respiratory tract disease

The acute respiratory form of IBRV infection is restricted to the upper respiratory tract and is by far the most significant disease manifestation of IBRV infection (25, 35, 64, 98, 153, 160, 161, 170, 238, 289). The disease is characterized by hyperemia of the nasal and tracheal mucosae with herpetic pustules that coalesce and become ulcerated. Necrotic cellular debris adhere to the ulcerated lesions forming fibrinous pseudomembranes which slough during the latter stages of infection and result in a thick fibrinopurulent nasal discharge. Occasionally, inflammation, nasal secretions and sloughed fibrinous pseudomembranes cause a blockage of airways and result in open-mouthed breathing by infected cattle.
Vulvovaginitis

Infectious pustular vulvovaginitis (IPV) has been variously described as Bläschenausschlag, coital vesicular exanthema, vesicular venereal disease and vesicular vaginitis (63, 65, 69, 91, 109, 119, 130, 156, 164, 193, 254, 266). Originally described as a separate disease entity, IPV is now known to be a disease manifestation of IBRV infection. Vulvovaginitis has been reported in concurrent infections with conjunctivitis (95, 130), respiratory tract disease (38, 108) and abortions (108). Vaginal infection is characterized by hyperemia of the mucosa with sequential formation of herpetic nodules, vesicles and pustules which coalesce to form a fibrinous membrane. Detachment of the membrane results in formation of ulcers and a fibrinopurulent discharge. Infected cows have vulvar swelling and exhibit pain with frequent urination, back arching and tail switching.

Evidence that IBRV infection affects fertility adds increased significance to vulvovaginitis and balanoposthitis. Temporary infertility is caused by IBRV induced metritis (119, 120, 139) and oophoritis (194). There is controversy concerning the effect of IBRV infection on semen quality (3, 47, 107, 194, 232, 243, 290).

Balanoposthitis

In bulls, the genital form of IBRV infection, results in pustular balanoposthitis (16, 81, 90, 255, 265), orchitis (109, 148), epididymitis (27, 109, 148) or subclinical infection (90, 230, 231).
Infectious pustular balanoposthitis is characterized by inflammation of the mucosa of the penis and prepuce with pustule formation and development of ulcers. Secondary bacterial infection and edema occasionally result in tissue adhesions, excess scar tissue, distortion of the penis and paraphimosis.

Conjunctivitis

A mild to severe conjunctivitis or keratoconjunctivitis with or without accompanying respiratory signs is observed in cattle infected with IBRV (1, 14, 46, 55, 92, 95, 199, 207, 210, 262, 271). The conjunctival form of the disease results in hyperemia, inflammation and edema of the conjunctiva and is accompanied by increased lacrimation and mucous exudate. Frequently, multiple granular papillae form, coalesce into herpetic pustules and develop into ulcerated plaques covered by friable diphtheritic membranes. Keratitis occurs as an extension of edema from the conjunctiva that results in a clouding of the cornea. Investigators have associated IBRV with infectious bovine keratitis (206, 267) and squamous cell carcinoma (269). The significance of IBRV in these diseases is unknown.

Abortion

Abortions occur following clinical or subclinical IBRV infection in pregnant cows (36, 41, 108, 124, 142, 227, 228) or following vaccination with intramuscular IBRV vaccines (see intramuscular MLV vaccines). Abortions associated with natural disease outbreaks have
been restricted until recently to the North American continent (56, 112, 245). This may be due in part to the fact that abortions are not common sequelae to vulvovaginitis (51) which is the predominant form of IBRV infection observed in other parts of the world.

Rapid diagnostic procedures (212, 236) have facilitated surveys which have demonstrated a high incidence of IBRV induced abortions (125). Abortions have been reported to occur during all 3 trimesters of pregnancy. However, most abortions occurred between the 5th and 8th month of gestation. Abortions usually occurred between 18 and 30 days (176, 190) following IBRV infection of the dam but longer incubation periods have been recorded (36, 165, 176). Virus was consistently isolated from placenta and cotyledons following IBRV induced abortion (118, 122, 191). Infected placentomes obtained by cesarean section (121) or less than 24 hours after fetal death (122) were normal on histological examination. Severe edema and necrosis of the placenta observed 24 hours after fetal death were nonspecific changes caused by fetal death (121, 122, 176, 191). Viremia in the fetus caused multifocal coagulative necrosis of liver and spleen (118, 122, 124, 191, 278) which resulted in fetal death. Abortion occurred several days following fetal death (118, 122).

**Systemic infection**

A fatal systemic IBRV infection occurs in calves infected in utero or shortly after birth (10, 171, 211). The clinical and pathologic manifestations of systemic IBRV infection are characteristic
of septicemia. Affected calves have ulcers throughout the alimentary tract, peritonitis, fibrinous pneumonia and multifocal coagulative necrosis of kidney and liver tissue.

**Encephalomyelitis**

Sporadic cases of meningoencephalitis are attributed to IBRV infection (11, 14, 57, 58, 100). Affected calves develop neurologic signs including ataxia, stumbling, generalized tremors and aimless circling followed by hyperexcitability, convulsions and death. Encephalitis has been reproduced experimentally in cattle inoculated by various routes: intracerebral (57, 72, 100, 101, 158); intranasal (8, 52, 58, 72, 178); epidural (8); oral (52); conjunctival (179); and intravaginal (180). Neural lesions included leptomeningitis, encephalomyelitis, perivascular cuffing, focal gliosis and sensory ganglionitis (8, 11, 72, 178, 179, 180).

**Enteritis**

Diarrhea is an inconsistent clinical finding in IBRV infections (35, 98, 149, 160, 163, 170, 214) and IBRV has been isolated from feces (40). Calves infected with IBRV in utero or as neonates frequently develop ulcers in the alimentary tract (10, 171, 211, 214). However, virus has been isolated infrequently from the gastrointestinal tract (166), esophagus (214) and Peyer's patches (67). Only two strains of IBRV consistently have induced enteritis (67, 68, 196, 214).
Distribution of IBRV virus in tissues

From the discussions above it is apparent that IBRV has a broad tissue tropism and a diverse pathogenicity. Rozkosny et al. (223) reported that infection of the mucosae (nasal, vaginal, tracheal and prepucial) was followed by a rapid one-peak pattern of virus replication. Following translocation of the virus via the bloodstream to secondary sites, virus replication occurred in atypical growth curve patterns. Clinical changes as a result of virus replication at secondary sites of infection were slight or absent, except in the newborn. Following inoculation by nasal, oral, vaginal and intravenous routes, Gratzek et al. (68) utilized fluorescent antibody tracings to demonstrate a widespread distribution of IBRV antigen throughout the body. Mc Kercher et al. (166) demonstrated a wide distribution of IBRV in tissues following intranasal inoculation. A transitory hematogenous phase of infection has been demonstrated (43, 45, 72, 165, 223, 244); however, most evidence for IBR viremia has been indirect. Rossi and Kiesel (216) added in vitro evidence by demonstrating that macrophage cultures were not susceptible to IBRV infection. Narita et al. (178, 179, 180) and Bagust and Clark (8) demonstrated ascending neural infections which suggested possible neural translocation of IBRV.

Persistence of IBRV infection

Persistent or chronic IBRV infections with periodic virus excretion and recurrent disease episodes have been demonstrated (17, 69,
Consistent recrudescence of IBRV from latently infected cattle has been demonstrated following corticosteroid injections \((43, 44, 45, 242, 244)\). Injections of corticosteroids have been utilized to detect IBRV venereal carrier status in breeding animals \((16, 48, 49, 61, 131)\).

**Immunity to IBRV infection**

The primary purpose of vaccination programs is to elicit specific humoral and cell-mediated immune responses to prevent infection. There is lack of agreement on the duration and degree of immunity to IBRV afforded by vaccination or natural infection and on the relationship of a particular level of antibody to the degree of immunity or resistance. It has been reported that any SN antibody induced by a primary IBRV infection provided a long-term or lifetime immunity to subsequent challenge with virulent virus \((33)\). Intranasal vaccines were developed and advocated because they evoked local secretory antibody and interferon secretion which provided "a more complete immunity" at the natural site of infection \((272, 273, 274, 275)\). Unfortunately, persistent or latent IBRV infections have occurred in the presence of SN antibodies and virus was recrudesced with corticosteroid injections \((43, 242, 244)\).

Rouse and Babiuk \((220)\) recently reviewed the immune mechanisms of recovery from herpesvirus infections and emphasized cellular immunity. A recent series of papers provided *in vitro* evidence that cell-mediated immunity is important in recovery from IBRV infection.
However, it is not presently possible to evaluate the *in vivo* role of these mechanisms.

**Host range for IBRV infection**

Natural infections with IBRV have been described in goats (175), swine (50, 187, 233), wildebeest (276), water buffalo (263), mink and ferrets (201). Experimental infections have been established in goats (162), swine (181, 293), ferrets (253), mule deer (34) and rabbits (4, 15, 28, 117, 195, 254). Serologic studies indicated that IBRV infections may exist in many additional species (2, 12, 59, 76, 110, 135, 208, 225, 226, 260, 270).

Experimental IBRV infection in rabbits has resulted in diverse disease manifestations. Bindrich (15) produced vulvovaginitis and conjunctivitis in rabbits with IBRV but was unable to serially transfer infection. Armstrong et al. (4) produced dermal inflammation and testicular necrosis with IBRV but concluded that the reactions were a toxic response and not due to virus replication. Bwangamoi and Kaminjolo (28) isolated IBRV from dermal and testicular lesions in rabbits. Kelly (117) provided electron microscopic evidence of herpesvirus replication in the liver and the adrenal glands of IBRV infected rabbits. Kelly (117) suggested that the neonatal rabbit may be useful as an experimental host for studies on the pathogenesis of IBRV infection.

Smith (253) studied the pathogenesis of IBR in English ferrets and stated that ferrets are a potential laboratory model of infection.
Van Houweling (277) determined that the goat was unsatisfactory as a laboratory model of infection. Studies of IBR pathogenesis and vaccine evaluation currently are limited to cattle.

Infectious Bovine Rhinotracheitis Vaccines

The propagation of IBRV in cell culture (146) made development of an IBR vaccine feasible. York et al. (296, 297) experimentally reproduced IBRV infection in cattle with cell culture virus and determined by cross-protection and serum neutralization tests that known IBRV isolants were closely related to one another.

Intramuscular MLV vaccines

Schwartz et al. (239) produced the first IBR vaccine with a virus strain modified by rapid passage in cell culture. The modified virus caused clinical signs when administered intranasally and produced a transient temperature rise when injected intramuscularly. Retention of intranasal pathogenicity was required as further attenuation destroyed immunogenicity (239). Schwartz et al. (240) reported that passage of IBRV in porcine kidney cells resulted in loss of infectivity for the natural host. Intranasal inoculation of calves with modified virus did not produce disease or elicit a SN antibody response while intramuscular inoculation resulted in production of SN antibodies. Fastier and Smith (54) selected a field strain of IBRV with reduced pathogenicity and further modified it by cell culture passage to produce a vaccine strain. Zuschek and Chow (299)
evaluated a vaccine strain modified by passage in canine kidney cells. Bartha (13) developed an IBR vaccine which contained a heat stable mutant (56°C for 40 minutes) with reduced virulence. Inaba (96) obtained an attenuated IBR vaccine strain by repeated cell culture passage at 30°C. This strain remained nonpathogenic after five serial passages in cattle and did not evoke SN antibodies in rabbits following intravenous, subcutaneous or intradermal inoculations. Animals vaccinated with all the modified IBR vaccine strains discussed above reportedly produced specific SN antibodies and remained clinically normal following challenge with virulent virus (13, 54, 96, 239, 240, 299). Multivalent vaccines that combine IBR with bovine viral diarrhea (BVD) (18, 102); parainfluenza-3 (PI-3) (235); BVD and PI-3 (66); PI-3 and pasteurella bacterin (235); BVD, PI-3 and pasteurella bacterin (235); or BVD and leptospirosis bacterin (9) have proven effective as determined by production of specific SN antibodies. Smith et al. (250) advocated the simultaneous administration of IBR vaccine and strain 19 brucellosis vaccine.

Kendrick et al. (123) described a transient temperature rise following intramuscular vaccination of cattle. Brown and Chow (24) reported a temperature rise in contact controls as well as in intramuscular vaccinates. McKercher and Crenshaw (159) reported the isolation of virus from the nasal cavity of intramuscularly vaccinated animals and a contact control but attributed the presence of virus to accidental intranasal contamination with the vaccine. Challenge of intramuscularly vaccinated cattle with virulent IBRV resulted in a
transient temperature rise (24, 123, 159, 229), intranasal lesions (123, 159, 229) and shed of virus (60, 96, 123, 159, 246).

Brown and Cabasso (23) reported that a transient temperature rise was the only clinical sign associated with the intramuscular injection of virulent IBRV. Infection of animals with IBRV by an unnatural route of innoculation accounted for the lack of clinical response (239). Cabasso and Brown (29) reported that the intramuscular inoculation of virulent IBRV met the criteria for an acceptable live virus vaccine.

Prior to 1971, research on intramuscular IBR vaccines had not demonstrated shed of virus from vaccinates or transmission of IBRV infection to contact controls (9, 24, 29, 53, 54, 66, 123, 159, 239, 296, 299). However, serologic studies and field observations implicated intramuscular vaccines as a cause of abortion (152, 155, 165, 172, 213). Although the initial descriptions of natural outbreaks of IBRV infection included reference to abortions, IBRV was not recognized as an abortifacient agent until experimental reproduction of abortion (36, 124, 165) verified field observations (41, 142, 183, 188). Following publication of this evidence, vaccination of pregnant cows was curtailed to circumvent abortions. Saunders et al. (229) demonstrated that intramuscular vaccination of cows prior to breeding prevented abortion when the cows were challenged with virulent virus during any trimester of pregnancy. In 1971 Kelling et al. (116) provided epizootiological evidence that IBRV was transmitted from vaccinated calves to pregnant cows and resulted in abortion.
Demonstration that IBRV was shed from the respiratory tract of cattle following intramuscular vaccination (32) resulted in the promulgation of warnings to avoid vaccination of calves nursing nonvaccinated cows.

**Adjuvanted, inactivated vaccines**

Formalin inactivated IBR vaccines have been produced to overcome the disadvantages inherent in live virus vaccines. Hristov and Karadjov (86) found that formalin and saponin inactivation decreased immunogenic activity of the vaccine, whereas, heat inactivation and ultraviolet light inactivation preserved immunogenic activity. Schipper and Kelling (237) observed that inactivated vaccines without adjuvant had poor immunogenicity and demonstrated that only 36% of calves produced SN antibodies following 2 doses of IBR vaccine. To compensate for this poor immunogenicity, inactivated IBR vaccines have been produced with the following adjuvants: Freund’s complete (299); oil (88); adsorbed aluminum hydroxide gels (103); and sodium alginate (127). Neutralizing antibodies were produced after 1 dose of inactivated vaccine (86, 88, 103, 127, 151, 299). However, Matsuoka et al. (151) found that 2 doses of inactivated vaccine were required at 14-21 day intervals before SN antibodies were consistently produced. The SN antibodies were found to persist at least 1 year after vaccination (151). However, duration of immunity was unknown and annual revaccination has been recommended (105). Zuschek and Chow (299) determined that a modified live virus
vaccine induced production of SN antibody titers that were 3 times higher than titers produced by a formalin-killed vaccine. Inactivated IBR vaccines were immunogenic (86, 87, 88, 89, 103, 127, 128, 129, 151, 244, 299) and antigenic inhibition did not occur when they were combined in multivalent inactivated vaccines with PI-3 (151); PI-3 and pasteurella bacterins (129, 151); PI-3 and BVD (127); or, PI-3 and adenovirus types 1 and 3 (88, 89). Hristov et al. (87, 89) demonstrated that inactivated vaccines could be used in pregnant cows without causing abortion and reported that inactivated vaccines possessed immunogenic properties comparable to live virus vaccines.

Cattle that received 1 (299) and 2 doses (151) of formalin inactivated vaccine did not develop disease following intranasal challenge with virulent virus. Juhasz et al. (103) challenged vaccinates by combined intramuscular, intratracheal, intranasal and intravaginal routes and determined that disease was prevented or was less severe than that which occurred in control animals. Challenge of vaccinated animals resulted in elevated temperatures (40 C) and produced characteristic IBR lesions on nasal and vaginal mucosae. Haralambiev (73) reported that inoculation of inactivated IBR vaccine into the nasal submucosa resulted in the production of local SN antibodies which enabled calves to remain free of disease following intranasal challenge with live virus.

Sheffy and Rodman (244) determined that inactivated vaccines did not protect cattle against establishment of latent viral infection following challenge. Vaccinated animals developed elevated
temperatures and shed IBRV for 5-8 days following intranasal challenge. Virus was reactivated with injections of corticosteroids 2-3 months after exposure to live virus.

**Intranasal MLV vaccines**

An intranasally administered IBR vaccine was developed following attenuation of IBRV by serial passage in rabbit cells (272). Intranasal immunization induced local immune protection consisting of secretory antibody (IgA) (272, 274), interferon (272, 274, 275) and cell-mediated immunity (274). Successful immunization depended on virus replication in cells of the upper respiratory tract and resulted in the shed of virus for approximately 13 days following vaccination (274, 275). Circulating antibody was detected on about the 8th day post-vaccination and resulted in the ultimate cessation of shed of virus (272). The transmission of replicating virus from vaccinates to susceptible nonvaccinates was demonstrated (159). However, the vaccine strain did not revert to a virulent form following 10 serial passages in susceptible calves (272).

The SN antibody titer attained on post-vaccination day 14 was higher in intranasally vaccinated calves than in intramuscularly vaccinated calves (159, 274). By 28 days post-vaccination, however, there was no difference between the mean titers of the two groups (159). Moderate SN antibody titers were produced in response to vaccination (42, 159, 274) and 98.6% of vaccinates seroconverted (106). Intranasal vaccination resulted in elevated temperatures
mild signs of upper respiratory tract infection (106, 159), anorexia and decreased milk production (106). The intranasal vaccine has been used in pregnant cows without evidence of vaccine induced abortions (103, 272, 273, 274). Intranasal vaccination protected pregnant cows against abortion when the animals were challenged with virulent virus (251). A newborn calf possessed SN antibodies, which indicated that in utero infection had occurred following intranasal vaccination of its dam (273). Gibson and Zemjanis (62) reported that fetuses at less than 5 months gestation died following in utero inoculation with intranasal vaccine. Fetuses similarly inoculated during the 5th, 7th and 8th months of gestation were born alive and possessed SN antibodies. Schultz and Hall, as quoted in Todd (273), reported 100% survival of calves following in utero inoculation with vaccine virus at 90 to 240 days of gestation.

Challenge of intranasally vaccinated calves with virulent virus resulted in shed of virus (159, 264) and nasal lesions (159). Early protection (40-96 hours) was demonstrated following vaccination of calves with intranasal (132, 274) or intramuscular (20) MLV vaccines. Intranasal vaccination induced interferon production within 40 to 72 hours with interferon titers persisting for approximately 8 days (272, 275). Following challenge with virulent virus, interferon was not induced in intranasal vaccinates but was induced in intramuscular vaccinates (275) and control animals (60). Curtis and Angulo (42) reported that intranasal vaccination of calves 12-24 hours prior to introduction into a feedlot containing IBRV infected calves did not prevent infection.
**Intranasal temperature-sensitive MLV vaccines**

A temperature-sensitive (TS) mutant was selected from wild IBRV following treatment with HNO$_2$ (302). Replication of the TS mutant was restricted at 39 ± 0.5°C with a 5 log$_{10}$ difference in virus production between the TS mutant and parent strain at 39°C. The TS character of the mutant was stable thru nine in vitro passages at permissive temperatures (302). When incorporated into intranasal vaccines (133, 302) the TS character combined the advantages of live and killed vaccines because replication was restricted to the nasal mucosa where local immunity was produced (60, 301, 303). The TS mutant has been combined in vaccines with PI-3 (133) and with TS mutants of PI-3 and adenovirus type 3 (301, 304).

Virus replication occurred following intranasal vaccination and virus was shed for 7-13 days (302) with transmission of virus to contact controls (133, 300, 302). Zygraich et al. (304) demonstrated the establishment of latent infections with TS vaccine; the virus was reactivated with corticosteroid injections at 21 weeks post-vaccination. Cattle vaccinated with TS vaccine remained without clinical signs of disease (60, 133, 300, 302, 304) except for hyperthermia, anorexia and vaginal discharge reported in one herd (140). The vaccine has been used in pregnant cows without evidence of vaccine induced abortion (133, 140). Furthermore, abortion was not produced by intravenous inoculation of pregnant cows (300).

Local antibody responses (60, 201, 304), interferon production (302) and cell-mediated immunity (60) were stimulated by TS vaccine.
In laboratory trials in which cattle were immunized with TS vaccine a seroconversion rate of 100% was achieved (133, 300, 302, 304). However, in field trials the seroconversion rate was approximately 85% (133, 140). Very low SN antibody titers were obtained with TS vaccine (60, 301, 304). Animals that received 2 doses of vaccine achieved peak SN antibody titers at 3 weeks post-vaccination. The SN titers, following 2 doses of vaccine, decreased after 8 weeks but were still detectable at 21 weeks post-vaccination and only one animal became seronegative (304). Nasal secretions contained SN antibodies for approximately 8 weeks following vaccination (301, 304). After challenge with virulent virus, vaccinates did not develop clinical signs (60, 132, 133, 300, 302, 304). Additionally, virus was shed in lower titers and for a shorter time from vaccinates than from control animals (60, 300, 302). Duration of immunity to challenge has not been determined.

Development of Subunit Vaccines

Polypeptide analysis and immunogenic determination have resulted in the characterization and identification of viral antigens capable of eliciting the formation of SN antibodies (182). Surface antigens contained in viral envelopes or in subviral infectious particles induce SN antibody formation. The antigenic subunits of enveloped viruses are the envelope glycoproteins (177). There are three possible sources of viral subunit proteins: (1) crude or purified virion preparations; (2) viral antigens integrated into infected cells; or, (3) soluble viral proteins excreted into
infectious cell culture fluids (256). Infected cell culture cells and cell culture fluids are attractive sources of viral proteins for subunit vaccine production because viral protein synthesis far exceeds requirements for virion assembly. However, vaccines composed of crude cell culture antigens contain more contaminating host cell components than do crude or purified virion vaccines (256).

Whole virus vaccines, either alive or inactivated, contain antigens of higher molecular weight and contain antigenic determinants not present in subunit vaccines (256). The immune response to live virus vaccines is amplified additionally by the replication of viruses in the vaccinate. Although subunit vaccines are safer than whole virus vaccines, their immunogenicity is generally lower (70, 294). The immunogenicity of subunit vaccines is related to the structural form of proteins presented in the vaccine. Monomeric forms of envelope proteins are not suitable for use in subunit vaccines because of poor immunogenicity (97, 177, 234). Water soluble monomers of envelope proteins do not elicit protective antibodies and are clearly not suitable for use in subunit vaccines (93, 234). The immunogenicity of subunit vaccines is preserved by "gentle" solubilization of proteins with nonionic detergents (80) or is accentuated by the aggregation of solubilized monomeric proteins (177).
Detergent solubilization of viral subunits

Membranes play vital biological roles in the functioning and survival of organisms. Extrinsic or peripheral membrane proteins that are water soluble or readily removed from the membrane by alterations in physiologic conditions are poor immunogens (93, 234). The immunogenicity of subunit vaccines is dependent upon intrinsic or integral membrane proteins that are associated with the hydrophobic core of a phospholipid bilayer in membranes (177). Methods that facilitate the "solubilization" of membranes into component parts that retain native biological and antigenic conformational characteristics have been essential to the development of subunit vaccines. "Solubilization" refers to the dissociation, disaggregation, extraction or disruption of the intact membrane into small fragments or component parts that are not removed by centrifugation (145).

Techniques for extraction and analysis of membrane proteins are well established and include the use of organic solvents, protein perturbants and enzymatic digestion (80). However, the resultant irreversible aggregation and denaturation of proteins limit the usefulness of these techniques. Detergents appear to provide the most useful extraction method for membrane proteins. The amphiphilic character of detergents enable them to interact with both hydrophilic and lipophilic regions of proteins (145). Makino et al. (147) state that the chances of obtaining a membrane in its native conformation are greatest if nonionic detergents are used. Amphiphilic detergents, when added to water, dissolve into free monomers that are in equilibrium
with a surface monolayer of detergent monomers (80). Monomers of detergent aggregate into micelles when critical concentrations are attained (critical micellar concentrations, CMC). Nonionic detergents are characterized by low CMC values and high micellar weights. The action of nonionic detergents depends on the interaction of detergent monomers with membrane components and interactions between detergent monomers. The interaction of detergent with itself is significant because effective protein binding depends on free detergent monomers and not on total detergent concentration (145). Detergents with low CMC values have sufficient free monomers to saturate only high affinity protein binding sites. Makino et al. (147) state that concentrations of free monomers are automatically limited by the low CMC value of nonionic detergents such that cooperative binding and denaturation cannot occur at any "reasonable" excess concentration of detergent. Amphiphilic detergents interact with intrinsic membrane proteins by forming partitions between the lipid-protein bilayer and the aqueous solution (268). The detergent monomers bind to the hydrophobic domain of intrinsic proteins (80) and cause solubilization of protein-lipid-detergent complexes (268). The hydrophilic-hydrophobic orientation of the protein is preserved during solubilization with the protein-bound detergent replacing the lipid environment (80).

Simons et al. (247) demonstrated that detergent dissociation of Semliki Forest virus occurred in a stepwise manner: (1) Triton X-100 was bound to the virus envelope at concentrations below CMC values with binding to protein and micelle formation representing a
competitive phenomenon; (2) viral envelopes ruptured with increased detergent binding; and, (3) viral membranes dissociated into lipoprotein-detergent particles and lipid-detergent micelles after about 40,000 monomers of Triton X-100 were bound per viral envelope.

Viral subunit vaccines

Subunit vaccines for influenza virus were introduced in 1969 and have been used to immunize millions of individuals. The vaccines contained hemagglutinin and neuraminidase antigens which were recovered and purified by chromatography following dissociation of influenza viruses with sodium deoxycholate (288), ether (21) or tri(n-butyl)phosphate (224) in the presence of 0.1% Tween 80. Electron microscopic evidence demonstrated that monomeric hemagglutinin subunits aggregated through hydrophobic bonding to form polymerized rosettes (134). The resultant "split vaccine" circumvented the pyrogenic effect characteristic of influenza whole virus vaccines (224). Clinical studies demonstrated that the immune responses in subunit vaccinates were equal to or better than those attained with whole virus vaccines (224).

Spike glycoproteins were selectively released from the envelopes of rhabdoviruses with nonionic detergents. Triton X-100 treatment of vesicular stomatitis virus released glycoprotein G which induced specific SN antibodies in animals (114). Nonidet P-40 (NP-40) solubilized glycoprotein G of rabies virus which elicited a SN
antibody response that protected animals against lethal challenge with virulent rabies virus (291). Rabies virus glycoprotein G purified by isoelectric focusing was considered the ideal human antirabies vaccine because of its purity and potency (39).

Morein et al. (177) demonstrated that lethal Semliki Forest virus infection was prevented with subunit vaccine if the Triton X-100 solubilized antigen (247) was presented in a complexed structural form. Hydrophobically bonded aggregates of monomeric spike proteins (protein micelles) (177) and virosomes composed of spike proteins contained in vesicles of egg lecithin (79) were highly protective. Monomeric spike proteins were only slightly protective.

Subunit vaccines for nonenveloped viruses were not as successful as those for enveloped viruses. Pentons and fibers of adenoviruses induced SN antibodies which affected viral attachment to susceptible cells. However, neutralization of other antigenic sites was required to achieve protection against infection (182). Lithium iodide dissociation exposed adenovirus DNA to enzymatic digestion and the resultant subunit fragments were used to produce specific immunity (182, 224). Bachrach et al. (6) demonstrated that a subunit vaccine composed of VP3 of foot-and-mouth disease virus induced SN antibodies which protected swine against infection upon contact exposure to infected swine.
Herpesvirus subunit vaccines

Inactivated herpes simplex type 1 (HSV-1) vaccines are available in Europe for treatment of patients with recurrent HSV-1 disease (292). Several host-viral relationships unique to herpesviruses have complicated development of HSV-1 vaccines. Potential risks including latency and enhanced oncogenicity following ultraviolet inactivation (209) indicate that live or inactivated homologous or heterologous virus may be unacceptable for use in HSV-1 vaccines (280). The most reasonable approach to immunoprophylaxis is development of subunit vaccines free of viral genetic material (280).

Herpesvirus proteins  Herpesviruses are structurally more complex than the previously discussed viruses. Thirty-three structural polypeptides have been identified in HSV-1 virions (77) and at least 49 viral polypeptides are produced in infected cells (83). Sklyanskaya et al. (249) identified 18 structural proteins in IBRV and determined that 8 proteins were glycosylated. Additional information on IBR viral proteins is lacking, therefore, discussion will be restricted to HSV-1 proteins.

A thorough immunologic characterization of HSV-1 specific antigens has not been accomplished and progress has been slow because of difficulties in preparing purified HSV-1 antigens (280). This difficulty has been compounded by the fact that virus specific proteins were detected not only in the virus (259) but also in cell membranes (78, 258) and in cell culture fluid (111). Kaplan et al. (111) found that more viral proteins were present in the infected cells.
and culture fluid than were present in the virions. Satisfactory
virus purification techniques (259) have been developed to analyze
HSV-1 structural proteins by polyacrylamide gel electrophoresis
(PAGE). However, the sodium dodecyl sulfate (SDS) treatment required
in the procedure denatured proteins so that it was difficult to assign
specific biologic activities to individual polypeptides (37). Alter­
native approaches to the study of viral proteins have utilized water
soluble (37) and Triton X-100 solubilized (283) antigens obtained
from crude extracts of virus infected cells.

All infected cell culture and partially purified virus prepara­
ations of HSV-1 antigens used to induce SN antibodies in animals
(30, 126, 136, 137, 150, 192, 204, 282, 298) have contained glyco­
proteins (280). Honess and Roizman (84) identified 13 glycoproteins
in HSV-1, all of which were localized in the envelope (186, 205, 259)
where they functioned as structural units and contributed to the
biological and immunological properties of herpesviruses (78, 84, 186,
257, 258). Antisera prepared against purified viral envelope proteins
precipitated all major glycosylated viral proteins in infected cell
extracts (84). This demonstrated that viral directed proteins present
in infected cells but absent in the virions were precursors to more
completely glycosylated proteins found in the virions (257). Cohen
et al. (37) initiated the purification of HSV-1 antigens by sepa­
ration of a glycoprotein with calcium phosphate (Brushite) chroma­
tography. Vestergaard and Grauballe (284) demonstrated HSV-1 antigens
by fused rocket immunoelectrophoresis after separation of a crude
antigen preparation by ion-exchange chromatography. Ponce de Leon et al. (200) selectively removed glycoproteins from crude extracts of HSV-1 infected cells by concanavalin A affinity chromatography. Subsequently, five HSV-1 glycoprotein antigens have been identified (204, 283, 285). The apparent discrepancy in glycoprotein numbers, compared to previous determinations (84), was caused by the SDS breakdown of large viral protein entities into polypeptides in the PAGE technique and detection by immunoprecipitation tests of only glycoproteins which provoked a specific antibody response (283). Triton X-100 solubilized glycoproteins (282) produced well-defined immunoprecipitates in crossed immunoelectrophoresis (281, 283) and were used to study the immunological and biochemical characteristics of antigens (184, 185, 285, 286). Vestergaard et al. (282) analyzed SN and precipitating antibody production in rabbits following inoculation with Triton X-100 solubilized HSV-1 antigens and suggested a vaccine potential for these antigens.

Experimental herpesvirus subunit vaccines Martin et al. (150) treated HSV-1 infected cells with NP-40 to obtain envelope antigens and with diethyl ether followed by dialysis in glycine buffer (pH 10.5) to obtain capsid antigens. Envelope antigens were reactive with antisera to whole viruses but did not react with anti-capsid sera. Capsid antigens failed to elicit SN antibodies in rabbits. These findings confirmed electron microscopic results which showed no capsids remaining in envelope antigen preparations. Zaia et al. (298) demonstrated SN antibodies and antigen reactive
lymphocytes in guinea pigs immunized with the envelope antigen. Furthermore, rabbits were protected against a lethal challenge dose of HSV-1 and demonstrated an anamnestic SN response following challenge.

Lesso et al. (137) obtained envelope antigen from purified HSV-1 virions by NP-40 treatment. Immunized rabbits produced higher titers of SN antibodies against envelope antigen than against whole virus. Convalescent HSV-1 serum produced two precipitation zones against whole virus and envelope antigen in gel immunodiffusion. Cappel (30) demonstrated that 2 doses of NP-40 solubilized envelope antigen stimulated humoral (SN antibodies) and cell-mediated (lymphocyte stimulation) immune responses in vaccinated rabbits. The immune responses produced were lower than the response evoked by live HSV-1 vaccine but a protection rate of 80% against challenge with virulent virus was not statistically different from the protection rate produced by live virus vaccine.

Kitces et al. (126) produced a subunit HSV-1 vaccine by treatment of HSV-1 infected cells with sodium dodecyl sulfate and Sarkosyl. The vaccine did not evoke detectable SN antibodies in mice. However, when challenged with virulent virus, immunized mice were protected from fatal encephalitis and had reduced incidence of lesion production and latent infections.

Vestergaard et al. (282) solubilized HSV-1 infected cells with Triton X-100 and recovered an antigen containing five membrane bound HSV-1 glycoproteins. Vaccinated rabbits developed SN antibodies
as effectively as with an active HSV-1 infection. Sera from rabbits receiving live virus vaccine reacted more strongly and with a higher number of HSV-1 antigens than did sera from rabbits immunized with Triton X-100 solubilized antigen. Vaccinated rabbits were not challenged.

Lesnik and Ross (135) produced a vaccine using NP-40 to solubilize antigens from cells infected with Marek's disease virus (MDV). The subunit vaccine produced an immune response in vaccinated chickens which suppressed systemic spread and feather follicle replication of MDV but did not affect viremia.

Papp-Vid and Derbyshire (192) used Rexol 25J (an analogue of NP-40) to solubilize envelope antigens from partially purified equine herpesvirus type 1 (EHV-1) virions. Envelope antigen preparations were further subjected to PAGE analysis after solubilization with SDS and the resultant gel was divided into four equal sections. Hamsters were vaccinated with envelope antigen, whole gel and each quarter section of gel. Challenge of immunized hamsters with EHV-1 demonstrated protection in animals that had received envelope antigen and to a lesser extent those that had received whole gel. Protection was induced only by the top gel quarter section which contained peptides of high molecular weight including a glycoprotein component of EHV-1 envelope.
PART I. EVALUATION OF THE RABBIT AS A LABORATORY MODEL FOR INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS INFECTION

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EVALUATION OF THE RABBIT AS A LABORATORY MODEL FOR INFECTIOUS

BOVINE RHINOTRACHEITIS VIRUS INFECTION

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ABSTRACT

Experimental infection of rabbits with infectious bovine rhinotracheitis virus (IBRV) produced diverse manifestations of disease which included abortion, conjunctivitis, dermatitis, vulvovaginitis, systemic infection, neonatal death and respiratory tract infection. Each disease syndrome was studied using virus isolation, fluorescent antibody examination and histologic examination.

Conjunctivitis, dermatitis and vulvovaginitis lesions were characterized by edema, infiltration of mucosa and submucosa with inflammatory cells and ulceration of epithelium. Systemic infection resulted in severe necrosis of liver and adrenal glands with large numbers of cells containing intranuclear inclusions. Pregnant rabbits aborted within 48 hours following inoculation of IBRV. Virus infection and viral lesions were not demonstrated in aborted fetuses.
Introduction

Infectious bovine rhinotracheitis virus (IBRV) is a cause of respiratory, genital and nervous system disease of cattle (105). Natural infections with IBRV have been described in goats (175), swine (50, 187, 233), water buffalo (263), wildebeest (113), mink and ferrets (201). Experimental infections have been established in mule deer (34), goats (162), swine (181, 293), ferrets (253). Serologic studies indicate that IBRV infections may occur in additional species (2, 12, 59, 76, 110, 135, 208, 225, 226, 260, 270).

The rabbit is susceptible to infection with IBRV (4, 15, 28, 117, 195, 254). Bindrich (15) produced vulvovaginitis and conjunctivitis in rabbits with IBRV but was unable to serially transfer infection. Armstrong et al. (4) produced dermal inflammation and testicular necrosis with IBRV but concluded that the reactions were a toxic response and not due to virus replication. Bwangamoi and Kaminjolo (28) isolated IBRV from dermal and testicular lesions in rabbits. Kelly (117) provided electron microscopic evidence of herpesvirus replication in the liver and the adrenal glands of IBRV infected rabbits.

Studies of IBRV pathogenesis and vaccine evaluation currently are limited to cattle. Efforts to find a suitable laboratory model for IBRV infection have been unsuccessful. Smith (253) studied the pathogenesis of IBRV in English ferrets and stated that ferrets
are a potential laboratory model of respiratory infection. Kelly (117) suggested that the neonatal rabbit may be useful as an experimental host for studies on the pathogenesis of IBRV infection. Van Houweling (277) determined that the goat was unsatisfactory as a laboratory model of infection.

The objectives in this research were to determine the pathogenicity of IBRV for rabbits and to evaluate the rabbit as a suitable model of infection.

Materials and Methods

**Virus** - The Cooper strain of IBRV (kindly supplied by Dr. A. Strating, National Veterinary Services Laboratory, Ames, Iowa) was received at the eighth passage level and then passed two times in bovine lung cells (BLU). The stock virus pool used for inoculation contained $1.0 \times 10^8$ plaque forming units (PFU)/ml. Stock virus was maintained at -70 C until used.

**Experimental Animals** - Adult white rabbits were purchased from a local commercial source and were held in individual cages throughout the experiment. Neonatal rabbits were purchased at 2 days of age, transported in the litter box and maintained with the doe.

**Experimental Design** - Rabbits (n=37) were divided into nine groups and inoculated as detailed (Table 1). The IBRV was administered to rabbits by inoculation of 0.1 ml intradermally, 0.2 ml in each conjunctival sac or 1.0 ml by intravenous, intraperitoneal
Table 1. Experimental infection of rabbits with infectious bovine rhinotracheitis virus (IBRV)

<table>
<thead>
<tr>
<th>Group</th>
<th>Disease syndrome</th>
<th>Route of inoculation</th>
<th>Number of rabbits</th>
<th>Virus dose (PFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Systemic disease, neonatal</td>
<td>intraperitoneal</td>
<td>5</td>
<td>$10^{8.0}$</td>
</tr>
<tr>
<td>2</td>
<td>Systemic disease, adult</td>
<td>intravenous</td>
<td>6</td>
<td>$10^{8.0}$</td>
</tr>
<tr>
<td>3</td>
<td>Systemic disease, adult</td>
<td>intravenous,</td>
<td>2</td>
<td>$10^{8.0}$</td>
</tr>
<tr>
<td></td>
<td>dexamethasone treated</td>
<td>conjunctival sac</td>
<td></td>
<td>$10^{7.3}$</td>
</tr>
<tr>
<td>4</td>
<td>Abortion</td>
<td>intravenous</td>
<td>4</td>
<td>$10^{8.0}$</td>
</tr>
<tr>
<td>5</td>
<td>Conjunctivitis</td>
<td>conjunctival sac</td>
<td>4</td>
<td>$10^{7.3}$</td>
</tr>
<tr>
<td>6</td>
<td>Dermatitis</td>
<td>intradermal</td>
<td>4</td>
<td>$10^{7.0}$</td>
</tr>
<tr>
<td>7</td>
<td>Respiratory disease</td>
<td>intratracheal</td>
<td>2</td>
<td>$10^{8.0}$</td>
</tr>
<tr>
<td>8</td>
<td>Vulvovaginitis</td>
<td>intravaginal</td>
<td>2</td>
<td>$10^{8.0}$</td>
</tr>
<tr>
<td>9</td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a.</td>
<td>Neonatal</td>
<td>intraperitoneal</td>
<td>1</td>
<td>none</td>
</tr>
<tr>
<td>b.</td>
<td>Adult, pregnant</td>
<td>intravenous</td>
<td>2</td>
<td>none</td>
</tr>
<tr>
<td>c.</td>
<td>Adult, dexamethasone treated</td>
<td>intravenous</td>
<td>1</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td></td>
<td>conjunctival sac,</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>intradermal,</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>intratracheal, and</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>intravaginal</td>
<td></td>
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</tbody>
</table>
intratracheal or intravaginal routes. Control rabbits were inoculated with the same volumes of Eagle's minimum essential medium (MEM) supplemented with 10% γ-irradiated fetal calf serum (FCS). Neonatal rabbits were inoculated at 4 days of age. Pregnant rabbits were inoculated at 17 and 22 days of gestation. Dexamethasone (Azium, Schering Corporation, Kenilworth, New Jersey) treated rabbits received 4 mg/day intramuscularly for 7 days prior to virus inoculation and 2 mg/day intramuscularly following virus inoculation. Rabbits were observed carefully for clinical signs of illness following virus inoculation and rectal temperatures were recorded daily. Rabbits were killed by electrocution at 6 days post inoculation (PID) except as indicated in the text.

Virus Isolation Procedures - For virus isolation, cotton-tipped swabs were saturated with conjunctival, vaginal or nasal secretions. Swab samples were immersed into 1.0 ml MEM containing antibiotics (100 IU penicillin, 100 µg kanamycin sulfate and 100 µg streptomycin sulfate per ml) and frozen at -70 °C until processed. Serial 10-fold dilutions of swab samples were titrated on BLU monolayer cell cultures in 24-well plastic tissue culture plates. After a 90 minute adsorption period, monolayers were washed with MEM and an overlay containing 1% agarose (Seakem ME, Marine Colloids Division, FMC Corporation, Rockland, Maine), MEM, 5% FCS, 0.5% lactalbumin hydrolysate and antibiotics was added. Cultures were incubated at 37 °C in a 5% CO₂ atmosphere. After 72 hours incubation, the cultures were fixed with 10% buffered neutral formalin
(BNF), the agarose overlay was removed and the cell monolayers were stained with crystal violet. Plaques were enumerated and the virus titer was determined.

Tissue specimens for virus isolation were collected at necropsy and frozen at -70 C. Tissue suspensions (10% in MEM) were prepared by homogenization with TenBroeck tissue grinders. Homogenized suspensions were cultured as described above. Nerve fibers were cut into 1- to 2-mm fragments, washed with MEM and co-cultivated with BLU monolayer cell cultures. Virus was detected by cytopathic effect in the monolayer.

Immunofluorescence Procedures - Frozen sections of tissues were cut, air dried and fixed in acetone. Sections were flooded with fluorescein-conjugated bovine anti-IBRV serum and incubated for 30 minutes at 37 C in a moist chamber. After washing with 0.01 M phosphate buffered saline (pH 7.4) and distilled water, sections were mounted with buffered (pH 8.6) glycerol and examined by fluorescence microscopy. Known IBRV positive and negative controls were included.

Histologic Procedures - Samples of tissues were collected and fixed in 10% BNF. Tissues were prepared by routine paraffin techniques, cut at 3 to 4 µ and stained with hematoxylin and eosin for light microscopy by standard methods (143).

Electron Microscopic Procedures - Tissues for thin section examination were fixed with 3.5% gluteraldehyde in a 0.1 M sodium cacodylate buffer (pH 7.2) for 4 hours at 4 C. Specimens were rinsed
in 0.1 M sodium cacodylate buffered sucrose solution (pH 7.2), postfixfixed with buffered 2% OsO$_4$, dehydrated in a graded alcohol series and embedded in an epon-araldite epoxy resin according to standard methods (74).

Results

*Systemic Infection of Neonates* - Neonatal rabbits died or were moribund 43 hours following intraperitoneal inoculation of IBRV. No gross lesions were observed at necropsy. Virus was isolated from lungs, kidney, spleen, brain and adrenal glands.

Fluorescent antibody examination of frozen tissue sections of liver and adrenal glands demonstrated large multiple foci of IBRV-specific fluorescence (Fig. 1). Specific fluorescence was observed in the spleen, brain and lungs but involved only individual cells or small foci of 4 to 6 cells.

Microscopic examination revealed an acute fibrino-necrotic pleuritis and peritonitis with adhesions to the liver, kidney, pancreas, intestine, spleen and adrenal glands. The liver contained randomly arranged, interconnecting, multifocal to diffuse areas of necrosis that involved approximately 50% of the parenchyma. Cells in necrotic foci were characterized by karyorrhexis and cytoplasmic fragmentation. Necrotic areas were surrounded by a zone of degenerating virus infected cells which contained large intranuclear inclusions and marginated chromatin. Very few heterophils had infiltrated the necrotic areas. Adrenal glands contained extensive well-
Figure 1. Systemic IBR virus infection in neonatal rabbit 2 days after intraperitoneal inoculation. Focus of IBRV-specific fluorescence in adrenal gland. Fluorescence micrograph x 150.
demarcated foci of necrosis in the zona fasciculata and zona reticularis (Fig. 2 A). The heart had multifocal areas of acute myocardial degeneration composed of hyaline fibers without striations. Scattered necrotic cells occurring individually or in small groups were found in the spleen. Lesions were not observed in other tissues.

**Systemic Infection of Adults** - At 24 to 48 hours following intravenous inoculation of IBRV, rabbits had a febrile response (to 40.6 °C) of less than 24 hours duration. No gross lesions were observed at necropsy on PID 6. Virus was isolated from the adrenal glands (4.3 log_{10} PFU/gm) and spleen (3.0 log_{10} PFU/gm). Multiple foci of IBRV-specific fluorescence were observed in the adrenal glands. Specific fluorescence in the spleen and liver was limited to scattered individual cells.

Microscopic examination revealed multiple foci of coagulative necrosis in the zona fasciculata and zona reticularis of the adrenal glands (Fig. 2 B). Each focus contained a central mass of cell debris surrounded by virus infected cells containing intranuclear inclusion bodies. The lesions were infiltrated by a few heterophils. Other cells in the adrenal cortex were swollen and contained a finely granular cytoplasm.

Microscopic examination of adrenal glands obtained on PID 30 contained foci with large "clear" cells (Fig. 2 C). "Clear" cells appeared to be large, space occupying cells without any cellular detail. Cells in the adjacent areas were enlarged which resulted in disarrangement of the sinusoidal architecture.
Figure 2. Lesions of adrenal glands in systemic IBR virus infection of rabbits. H&E stain; x 60.

A. Focus of coagulative necrosis in zona fasciculata and zona reticularis of IBR virus infected neonatal rabbit 2 days after intraperitoneal inoculation.

B. Focus of coagulative necrosis in zona fasciculata and zona reticularis of IBR virus infected adult rabbit 6 days after intravenous inoculation.

C. Space occupying "clear cells" in resolved focal lesion of IBR virus infected adult rabbit 30 days after intravenous inoculation.

D. Uninfected control. Following 7 days dexamethasone treatment.
Systemic Infection of Dexamethasone Treated Adults - After intravenous and conjunctival inoculation with IBRV, dexamethasone treated rabbits developed severe conjunctivitis, labored respiration, anorexia and severe depression. One rabbit died on PID 4 and the second rabbit was killed on PID 7 in a moribund state. A control rabbit remained clinically normal during 14 days of dexamethasone treatment.

Gross lesions observed at necropsy included enlarged adrenal glands with extensive pale areas in the cortex, ecchymotic hemorrhages in the kidney, multiple diffuse pale and red foci in the liver and severe conjunctivitis.

Virus was isolated from daily swab samples of nasal and ocular secretions and from tissue homogenates of adrenal glands (4.7 log_{10} PFU/gm), liver (7.0 log_{10} PFU/gm) and spleen (2.3 log_{10} PFU/gm). Additionally, IBRV was isolated from explants of trigeminal and optic nerve fibers. Multiple focal to diffuse zones of IBRV-specific fluorescence were observed in the adrenal glands and liver. Hepatocytes located near the peripheral margin of necrotic foci had IBRV-specific fluorescence which was limited primarily to enlarged nuclei (Fig. 3). Diffusely scattered specific fluorescence was observed in the spleen.

Microscopic examination revealed multiple focal to diffuse areas of coagulative necrosis in the liver (Fig. 4). Virus infected cells surrounding necrotic foci contained large intranuclear inclusion bodies (Fig. 5). Numerous areas of focal necrosis were hemorrhagic
Figure 3. Systemic IBR virus infection in dexamethasone treated adult rabbit. Focus of IBRV-specific fluorescence in liver. Nuclei of hepatocytes near peripheral margin of lesion have IBRV-specific fluorescence. Fluorescence micrograph x 150.
Figure 4. Systemic IBR virus infection in dexamethasone treated adult rabbit. Multiple foci of coagulative necrosis in the liver. H&E stain; x 75.

Figure 5. Systemic IBR virus infection in dexamethasone treated adult rabbit. Intranuclear inclusions in hepatocytes. H&E stain; x 200.
and mineralized. Diffuse coagulative necrosis of the adrenal glands was limited to the zona fasciculata and zona reticularis. Lesions were infiltrated with heterophils. Conjunctival tissue was edematous and contained multiple focal areas of necrosis. The limbus was infiltrated with lymphocytes and had increased vascularization which extended into the corneal stroma. The corneal stroma was edematous and contained scattered inflammatory cells. Ballooning degeneration of corneal epithelial cells was evident in focal areas and one rabbit had focal areas where the epithelium was denuded. Lesions were not observed in other tissues.

Electron microscopic examination of liver tissue revealed hepatocytes with large intranuclear inclusion bodies that resulted in margination of chromatin (Fig. 6). Naked herpesvirus capsids were observed in the nuclei and enveloped virions were observed in perinuclear cisternae and cytoplasm of infected cells (Fig. 7).

Cells of the adrenal glands and liver from dexamethasone treated control rabbits were uniformly enlarged and had a granular cytoplasm (Fig. 2 D).

**Abortion** - Intravenous inoculation of four pregnant rabbits with IBRV resulted in either complete or incomplete abortion within 48 hours. Incomplete abortions in three rabbits were manifested by expulsion of two live fetuses (rabbit 1) and discharge of fetid vaginal fluids (rabbits 2 and 3). Incomplete abortion was preceded by a febrile response (40.4 C to 40.7 C) that lasted for approximately 24 hours. Complete abortion (rabbit 4) on PID 2 was not accompanied
Figure 6. Systemic IBR virus infection in dexamethasone treated rabbit. Electron photomicrograph of hepatocyte with intranuclear inclusion body and marginated chromatin. Herpes virions are present in the nucleus and the perinuclear cisternae. x 10,000.

Figure 7. Systemic IBR virus infection in dexamethasone treated rabbit. Electron photomicrograph of enveloped herpesvirus particles in cytoplasm of hepatocyte. x 100,000.
by a febrile response. No clinical sign other than abortion was observed.

When killed on PID 3, rabbit 3 contained 5 live and 7 dead fetuses. When killed on PID 6, rabbits 1 and 2 contained decomposed fetuses. Adrenal glands of aborted rabbits were 2 to 3 times normal size with pale friable cortices evident on section.

Virus was isolated from the adrenal glands of all rabbits (titers $3.0 \log_{10}$ PFU/gm to $6.3 \log_{10}$ PFU/gm) and from all placentae (titers $1.9 \log_{10}$ PFU/gm to $4.0 \log_{10}$ PFU/gm). Virus was isolated from the uteruses collected on PID 6. The uteruses and placentae did not contain IBRV-specific fluorescence. Virus was not isolated from pools of fetal tissues collected from aborted or retained fetuses.

Mild histologic changes were observed in the placentae and uterus of aborted rabbits. Microscopic changes in the placentae included a light infiltrate of heterophils and vacuolization of trophoblasts. An uneven perfusion of the placental labyrinth was found in several sections. The uterus contained focal edema and a sparse infiltrate of heterophils. Diffuse coagulative necrosis of the adrenal glands involved the zona fasciculata and zona reticularis. No lesions were observed in fetal tissues.

Conjunctivitis - Rabbits developed conjunctivitis within 24 hours after inoculation of IBRV into the conjunctival sac. Ocular lesions were characterized by hyperemia and edema of the conjunctiva, injected scleral vessels, muco-purulent discharge and chemosis (Fig. 8). Rabbits exhibited photophobia and blepharospasm. Conjunctivitis
persisted for 10 to 14 days. The eyes of control rabbits remained normal.

Samples of ocular secretions yielded IBRV on PID day 1 to 13. Virus was isolated from nasal secretions on PID 1 to 11. Virus titers from ocular secretions exceeded $3.0 \log_{10} \text{PFU/swab}$ for 7 days and then declined. Virus was isolated from optic and trigeminal nerves collected at necropsy on PID 11.

Microscopic examination revealed conjunctival inflammation with infiltration of lymphocytes and heterophils. Ulceration of the conjunctival epithelium was evident. The limbus region had an accumulation of inflammatory cells and increased vasculature. The cornea was vascularized and edematous. A focus of ballooning degeneration of the corneal epithelium was observed in one rabbit.

Dermatitis - Twelve hours post inoculation, the intradermal site of IBRV inoculation was surrounded by an intense zone of hyperemia. At 24 hours post inoculation, the lesion had a 5 mm hemorrhagic center surrounded by a 15 mm edematous wheal (Fig. 9). On PID 4, a dry crust was adhered to an underlying area of dermal ulceration. By PID 6 the edematous swelling had subsided and the central lesion appeared to be healing.

Virus was recovered from serous exudate collected from dermal lesions on PID 2, 4 and 6. Skin biopsies collected on PID 2 and 6 had IBRV-specific fluorescence restricted to the area immediately surrounding the epithelial ulcer.

Skin biopsies collected from the site of intradermal inoculation
Figure 8. IBR virus induced conjunctivitis in adult rabbit 6 days after inoculation. Rabbit had hyperemia of conjunctiva, injected scleral vessels, mucopurulent discharge and chemosis.

Figure 9. IBR virus induced dermatitis in adult rabbit 24 hours after intradermal inoculation. Upper markers refer to control sites. Lower markers refer to dermal lesions. Lesion consists of a hemorrhagic center surrounded by an edematous wheal.
on PID 2 had lesions characterized by edematous swelling of the papillary layer of the dermis. The swollen dermal papillae were anchored peripherally by hair follicles (Fig. 10 A). At the inoculation site there was ballooning degeneration of epidermal cells, small epidermal ulcerations (Fig. 10 B) and an acute cutaneous cellulitis characterized by edema, infiltration of lymphocytes and a few heterophils. The underlying cutaneous muscle was infiltrated with lymphocytes and heterophils. Coagulative necrosis and edema of the dermis resulted in fragmentation of collagen fibers.

On PID 6, a fibrino-purulent crust covered epidermal ulcerations at the inoculation site (Fig. 10 C). Epidermal acanthosis occurred peripheral to the ulcer. The cellulitis in the dermis was resolving but still contained accumulations of lymphocytes and heterophils. A cutaneous myositis was present with a heavy infiltratin of lymphocytes. Control sites of inoculation remained unchanged (Fig. 10 D).

Respiratory Disease - Intratracheal inoculation of two rabbits with IBRV resulted in a febrile response (40.4 C and 40.6 C) on PID 1. The temperature returned to normal within 24 hours. On PID 5, one rabbit had a second febrile response (to 41.5 C) that lasted for 48 hours. Rabbits remained clinically normal throughout the experiment.

Nasal secretions contained 1.2 log_{10} PFU/swab at 24 hours post inoculation. Virus titers increased to 3.3 log_{10} PFU/swab on PID 3 and persisted at that level until rabbits were killed on PID 7. Virus was isolated from swab samples of ocular secretions on PID 3 to 7 and PID 5 to 7 respectively. Virus was isolated from trachea, lungs and nasal mucosa.
Figure 10. Dermal lesions in IBR virus infected rabbits.
H&E stain; x 65.

A. Edematous swelling of the papillary layer of the dermis 2 days after intradermal inoculation.

B. Small epidermal ulcerations at site of intradermal inoculation 2 days post inoculation.

C. A fibrino-purulent crust covering epidermal ulcerations at the IBR virus inoculation site 6 days post inoculation.

D. Control site 6 days after inoculation.
Microscopic examination revealed a mild pneumonitis that was indistinguishable from lesions observed in control rabbits. Lesions were not observed in other tissues.

**Vulvovaginitis** - Vaginal inoculation of IBRV resulted in an intense hyperemia of vulvar tissues. The vulva had scattered 1- to 3- mm pustular lesions that had an adherent muco-purulent exudate. No other clinical signs were observed.

Virus was isolated from vaginal swab samples for 3 days post inoculation and was isolated from vulvar and vaginal tissue at necropsy. The epithelial layer of vulvar tissue had IBRV-specific fluorescence.

Microscopic examination revealed hyperemia, hemorrhage in the submucosa and pustules in the epithelium of the vulva. The vulvar epithelium was diffusely infiltrated with heterophils many of which had accumulated on the epithelial surface.

**Discussion**

Results of these experiments indicate that IBR viral infection can be established in rabbits when inoculated by intraperitoneal, intravenous, intradermal, intratracheal or conjunctival routes of administration. The diverse clinical manifestations of IBRV infection produced in rabbits were similar to those reported in cattle (105) and included neonatal systemic infection, conjunctivitis, vulvovaginitis, dermatitis, abortion and respiratory tract infection.

Although previous studies (4, 15, 28, 117, 195, 254) have demonstrated the susceptibility of rabbits to IBRV infection, only
limited virus isolation studies have been conducted (28, 195). In the present study virus isolation and fluorescent antibody studies demonstrated that IBRV was associated with lesions produced in each of the disease syndromes. Furthermore, isolation of IBRV from optic and trigeminal nerves provided evidence of neural translocation of the virus from primary sites of infection which indicates the potential for latent infections in the rabbit.

Systemic infection following intraperitoneal inoculation of neonates and intravenous inoculation of adults resulted in lesions that were more extensive in the neonate than in adult rabbits. However, dexamethasone treatment exacerbated infection in adults and resulted in a systemic infection similar to neonatal infection. Severe necrosis of the adrenal glands was consistently produced in all systemic infections. There was focal hepatic necrosis in neonates and in adults treated with dexamethasone. Hepatic and adrenal gland lesions found in rabbits were similar to those reported in bovine fetuses aborted by IBRV infection and young calves with systemic IBRV infection (10, 68, 118, 124, 171, 211). There was, however, a significant difference in that IBRV inclusion bodies were easily found in the liver and adrenal glands of systemically infected rabbits. The demonstration of IBRV-specific fluorescence of nuclei in IBRV infected tissue has not been previously reported. Previous reports (67, 68, 236) have described nuclear fluorescence as limited to the nuclear membrane at the nucleo-cytoplasmic junction. Virus has been isolated from the adrenal glands of calves after experimentally induced IBRV
infection (45, 68) and after reactivation of IBRV by corticosteroid treatment (242).

The cause of IBRV induced abortion in rabbits appeared to differ significantly from what has been reported in cattle (121, 122, 176, 191). In cattle, virus infection of the placenta (122, 188, 191) was accompanied by viremia and multifocal coagulative necrosis of liver and spleen in the fetus (118, 122, 124, 191). Abortions occurred 15 to 30 days following IBRV infection of the dam and several days after fetal death. In contrast, virus infection of the placenta occurred in rabbits but virus infection and microscopic lesions were not found in rabbit fetuses. In rabbits, live fetuses were expelled as early as 18 hours post inoculation and evidence of impending abortion was observed in all rabbits within 48 hours following inoculation of IBRV. The cause of abortion in rabbits has not been proven but may be related to the extensive coagulative necrosis observed in the adrenal cortex and the resultant inhibition of the pituitary-ovarian axis due to release of steroids from the adrenal glands.

The variety of clinical syndromes produced in rabbits by IBRV infection suggests that the rabbit may be well-suited as a laboratory model for the study of IBRV disease pathogenesis, viral latency, pathogenicity of virus strains or evaluation of vaccine safety and efficacy.
PART II. EXPERIMENTAL INFECTION OF EASTERN COTTONTAIL RABBITS
(Sylvilagus floridanus) WITH INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS

This manuscript has been accepted for publication in the American Journal of Veterinary Research
EXPERIMENTAL INFECTION OF EASTERN COTTONTAIL RABBITS (*Sylvilagus floridanus*) WITH INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS

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SUMMARY

Experimental infection of eastern cottontail rabbits (*Sylvilagus floridanus*) with infectious bovine rhinotracheitis (IBR) virus caused acute keratoconjunctivitis and a fatal systemic infection. The clinical syndrome was characterized initially by blepharospasm and ocular discharge. The rabbits were markedly depressed on postexposure day (PED) 5 and were dead or moribund on PED 6. The virus was readily recovered from liver and adrenal gland tissue on PED 6 and from conjunctival swabs on PED 1 to 6.

Histopathologic studies revealed a few necrotic foci in the liver and multiple focal to diffuse necrosis of the adrenal glands. Viral isolation and immunofluorescence tests were used to demonstrate a direct association between IBR viral antigens and the lesions.
Infectious bovine rhinotracheitis (IBR) virus is a herpesvirus with a worldwide distribution. The diverse clinical manifestations of IBR in cattle include respiratory tract disease, pustular vulvovaginitis, abortion, conjunctivitis, infertility, meningoencephalitis, balanoposthitis, and systemic infection (105). The IBR virus has been isolated from goats (175), swine (50, 233), water buffalo (263), and mink and ferrets (201). Infection has been experimentally induced in mule deer (34), goats (162), swine (181), ferrets (253), and rabbits (4, 15, 28, 117) and subclinically in sheep and horses (162). Serologic studies indicate that IBR infections may exist in additional species (2, 110, 225).

The objective in the present research was to determine the pathogenicity of IBR virus in wild eastern cottontail rabbits (Sylvilagus floridanus).

Materials and Methods

**Virus** - The Cooper strain of IBR virus used had been passaged eight times before receipt and then was passaged two times in Georgia bovine kidney (GBK) cells. The stock preparation used for inoculation contained $1.0 \times 10^8$ plaque forming units (PFU)/ml.

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Animal Inoculation - Adult rabbits were inoculated by intravenous administration of 1.0 ml of IBR stock virus and by flooding each eye with 0.2 ml of the virus preparation. Control rabbits were inoculated in the same manner with Eagle's minimum essential medium (MEM).

Viral Isolation Procedures - Cell cultures were grown in MEM supplemented with 10% γ-irradiated bovine fetal serum and antibiotics (100 u of penicillin, 100 μg of kanamycin sulfate, and 100 μg of streptomycin sulfate per ml). Conjunctival swabs were collected daily and immersed in 1.0 ml of MEM containing antibiotics. Swab samples were serially diluted and titrated on Georgia bovine kidney monolayer cultures in 24-well plastic tissue culture plates. After a 90-minute adsorption period, MEM containing 5% bovine fetal serum and antibiotics was added and the cultures were incubated at 37 C. Cultures were examined daily for cytopathic effect.

Tissues from lung, liver, spleen, and adrenal gland were taken from rabbits at necropsy. Tissue suspensions (10%) in MEM were prepared by homogenization with TenBroeck tissue grinders. The homogenized suspensions were cultured as described earlier.

Fluorescent Antibody and Histopathologic Examinations - Frozen sections of lung, liver, spleen, and adrenal gland were cut, air-dried and fixed in acetone. The sections were flooded with fluorescein-conjugated bovine anti-IBR serum and incubated for 30 minutes at 37 C in a moist chamber. After the sections were washed with 0.01 M
phosphate-buffered saline solution (pH 7.4) and distilled water, they were mounted with buffered glycerol (pH 8.6) and examined by fluorescent microscopy. Cell cultures of virus isolated from infected tissues were similarly examined. Known IBR virus-positive and -negative controls were included. Various tissue specimens collected at necropsy were fixed in 10% buffered formalin for microscopic examination.

Results

**Infection of Rabbits** - Rabbits developed mild conjunctivitis within 24 hours after inoculation. By postexposure day (PED) 3, the conjunctivitis had increased in severity with intense hyperemia and marked edema, which caused the conjunctiva and nicitating membrane to protrude between the eyelids. Rabbits exhibited blepharospasm, photophobia, and serous ocular discharge. The scleral blood vessels were injected, and the sclera was hyperemic. The eyelids were markedly swollen and rigidly fixed on PED 5 (Fig. 1). There were dehydration of the exposed tissues and clouding of the cornea. Rabbits 2 and 3 had a foamy, purulent discharge from the eyes and nasal cavity. All rabbits were severely depressed on PED 5. On PED 6, rabbits 1 and 3 died and rabbits 2 and 4 were killed because of their moribund state.

**Viral Isolation** - Results of IBR viral isolation from swab samples and tissue homogenates are summarized in Table 1. The titers of IBR virus isolated from daily conjunctival samples are shown in Figure 2. Nasal swab samples yielded IBR virus on PED 5. The eye and nasal
Figure 1. Eastern cottontail rabbit 5 days after inoculation with $2 \times 10^7$ PFU of Cooper IBR virus. Rabbit had severe conjunctivitis with accumulation of mucous exudate and excessive lacrimation.

Figure 2. Average virus titers (TCID/swab) in conjunctival swabs from rabbits inoculated in the conjunctival sac with $2 \times 10^7$ PFU of IBR virus.
Table 1. Results of viral isolation and fluorescent antibody procedures on cottontail rabbits inoculated with IBR virus

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Spleen</th>
<th>Liver</th>
<th>Lung</th>
<th>Adrenal gland</th>
<th>Corneal smears</th>
<th>Conjunctival swabs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (♂, day 6)</td>
<td>-</td>
<td>+ 0.5</td>
<td>-</td>
<td>+ 5.0</td>
<td>+</td>
<td>+ (day 1-5)</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>+ 1.0</td>
<td>-</td>
<td>+ 4.5</td>
<td>+</td>
<td>+ (day 1-6)</td>
</tr>
<tr>
<td>3 (♂, day 6)</td>
<td>- 1.0</td>
<td>+ 1.5</td>
<td>-</td>
<td>+ 2.5</td>
<td>+</td>
<td>+ (day 1-5)</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+ 2.0</td>
<td>+</td>
<td>+ (day 1-6)</td>
</tr>
<tr>
<td>5 (control)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6 (control)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*a = fluorescent antibody test

*b In viral isolation, titer is expressed as log₁₀ TCID₅₀/ml of 10% tissue homogenate.

*c = death.
discharges from rabbits 2 and 3 yielded virus and *Bordetella* sp on culture. Viral isolates, were identified as IBR virus by immunofluorescent tests of infected cell cultures.

**Fluorescent Antibody and Histopathologic Examinations** - Results of immunofluorescence tests of tissues are summarized in Table 1. Adrenal gland tissue contained multiple focal areas of specific fluorescence (Fig. 3) which corresponded with microscopic lesions. Specific fluorescence of liver tissue was limited to individual cells with few foci present. Corneal impression smears collected on PED 3 had IBR virus-specific fluorescence.

Pathologic changes in the adrenal glands consisted of multiple foci and diffuse areas of necrosis (Fig. 4) which involved 30% to 80% of the adrenal cortex in rabbits 1, 2, and 4. The necrotic foci involved only the adrenal medulla in rabbit 3. These foci contained a central mass of necrotic cellular debris that was surrounded by a zone of degenerating cells and was infiltrated by numerous heterophilic leukocytes. Cells located peripherally to the necrotic foci were hyperchromatic with margination of chromatin around large intranuclear inclusions. Rabbits 1, 2, and 3 had hepatic lesions which primarily consisted of randomly distributed hyperchromatic hepatocytes with pyknotic nuclei. Foci of coagulative necrosis were observed infrequently.

Examination of ocular tissues revealed an acute keratoconjunctivitis and iridocyclitis characterized by infiltrated heterophilic
Figure 3. Fluorescence microscopy of section of adrenal gland from a rabbit 6 days after intravenous inoculation of $1 \times 10^8$ PFU of IBR virus. Two foci of fluorescence specific for IBR virus are visible. x 235.

Figure 4. Section of adrenal gland from a rabbit 6 days after intravenous inoculation of $1 \times 10^8$ PFU IBR virus. A focus of coagulative necrosis is evident. H&E stain; x 235.
leukocytes. The cornea was denuded of epithelium, except in areas adjacent to the corneo-scleral junction. Intranuclear inclusions were observed in the intact corneal epithelium. There was marked edema of the corneal stroma with a light infiltrate of heterophilic leukocytes. There was mild pneumonitis in the lungs, characterized by an infiltrate of mononuclear cells in alveolar septae. The tracheal epithelium consisted of flattened stratified squamous cells devoid of goblet cells. The few cilia remaining were short, thick, and club-like. Pathologic changes were not observed in control rabbits.

Discussion

Results of these experiments indicate that IBR viral infection can be established in eastern cottontail rabbits with a resultant severe keratoconjunctivitis and death. According to an earlier report (117), IBR viral infection of laboratory rabbits under 10 days of age resulted in death and microscopic focal necrotic lesions of adrenal glands and liver. Infection of adult laboratory rabbits produced mild conjunctivitis (15, 117), dermal lesions (4, 28), and necrotic foci in the liver (1 of 7 rabbits) and the adrenal glands (2 of 7 rabbits) (117). In the present study IBR infection in adult eastern cottontail rabbits was more severe than that reported in adult laboratory rabbits and was similar to that reported in neonatal laboratory rabbits.

Infection of cattle with IBR virus produced conjunctivitis
with or without the classic signs of respiratory tract involvement (105). Bovine fetuses aborted due to IBR viral infection (105) have microscopic necrotic foci in adrenal glands and liver that are similar to the lesions found in cottontail rabbits. Focal necrosis of the liver (10, 211) and the adrenal glands (68) has been reported in young calves infected with IBR virus. Additionally, IBR virus has been isolated from adrenal glands after experimentally induced IBR viral infection (45, 68) and after reactivation of IBR virus by corticosteroid treatment (242).
PART III. CLEARANCE AND SHED OF INFECTIONOUS BOVINE RHINOTRACHEITIS VIRUS FROM THE NASAL MUCOSA IN IMMUNE AND NONIMMUNE CALVES

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CLEARANCE AND SHED OF INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS FROM THE NASAL MUCOSA IN IMMUNE AND NONIMMUNE CALVES

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SUMMARY

Infectious bovine rhinotracheitis (IBR) virus was rapidly cleared from the nasal mucosa of calves following intranasal aerosol exposure. Nonimmune calves cleared $10^9$ plaque forming units (PFU) virus from the nasal mucosa in less than 4 h and $10^6$ PFU virus in 1 h. An eclipse phase followed the clearance of virus inoculum. Replicating virus was first detected 9 h after inoculation. Virus titers increased in a step-wise manner until maximum titers were attained on post inoculation day 4. Virus persisted in the nasal mucus until day 12. Clinical signs corresponded with virus shed. In contrast to nonimmune calves, immune calves cleared $10^9$ PFU virus in 1 hr and $10^6$ PFU virus in less than 5 min. An abortive reinfection occurred following challenge of immune calves with $10^9$ PFU virus. Virus was first detected in these calves at 15 h after inoculation and was not detected beyond 24 h post inoculation. Immune calves that received $10^6$ PFU virus did not shed virus after clearance of inoculum. Clinical signs were not observed in immune calves following virus challenge. There was no detectable residual virus beyond 4 hr post inoculation.
Bovine Herpesvirus 1 infection is manifested by several clinically distinct disease syndromes including respiratory tract disease, vulvovaginitis, balanoposthitis, abortion, conjunctivitis and meningoencephalitis. These clinical disease syndromes, commonly known as infectious bovine rhinotracheitis (IBR), were recently reviewed (105). The respiratory form of IBR is a significant component of the bovine respiratory disease complex.

Pulmonary clearance techniques have been used to study the physiological clearance mechanisms of the respiratory tract and clearance rates for bacteria have been determined in several animals species (138). The pulmonary clearance of bacteria in nonimmune animals has been utilized to study the effect of the environment, infectious agents and immunologic mechanisms on respiratory disease. Similar pulmonary clearance data are lacking for viral agents due to the absence of techniques for observing the behavior of dispersed virus particles on mucous membranes. Immune clearance of viruses from the respiratory tract is attributed to specific immunologic mechanisms, phagocytosis and interferon activity (279). The shed of virus from respiratory tract infections commonly has been determined using daily nasal, pharyngeal or tracheal swab samples.

The objective of the present research was to investigate the clearance and shed of IBR virus from the nasal mucosa of immune and nonimmune calves. Knowledge of the normal physiological clearance rate of virus from the nasal mucosa is essential when evaluating the effect of immune mechanisms on the clearance rate.
Materials and Methods

**Virus.** - The Cooper strain of IBR was received at the eighth passage level, and then was passed two times in bovine lung cells (BLU). The stock preparation of virus used for inoculation contained $1.0 \times 10^9$ plaque forming units (PFU)/ml.

**Animals** - Cross-bred calves (3-6 months old) of both sexes were obtained, divided into 2 groups and housed in isolation units throughout this study.

**Animal Inoculation** - Group 1 calves (n=3) received a high titer virus inoculum ($4 \times 10^9$ PFU). Calves were inoculated with 2 ml of stock virus into each nostril. A gas-powered atomizer was used to deliver the inoculum as previously described (248). Group 2 calves (n=4) were inoculated in the same manner with a standard challenge virus inoculum ($1 \times 10^6$ PFU).

After 30 days recovery, the inoculations were repeated in the respective groups (immune animals) using identical inocula. One calf in group 1 was necropsied for other studies.

**Virus Isolation** - Nasal secretions were collected on swabs for 3 days prior to exposure and at various intervals (See Figure 1 and 2) through 12 days post-exposure. Nasal secretions were collected by

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*b* Protocol, IBR vaccine potency test, Challenge virus, National Veterinary Services Laboratory, Ames, Iowa.
Figure 1. Clearance of IBR virus from the nasal mucosa of immune (□—□) and nonimmune (●—●) calves following intranasal inoculation with (A) $1 \times 10^9$ PFU IBR virus and (B) $1 \times 10^6$ PFU IBR virus.

Figure 2. Isolation of IBR virus from the nasal mucosa of immune and nonimmune calves following intranasal inoculation with IBR virus.

Symbols:

- ● = Nonimmune calf inoculated with $1 \times 10^9$ PFU virus
- △ = Immune calf challenged with $1 \times 10^9$ PFU virus;
- ○ = Nonimmune calf inoculated with $1 \times 10^6$ PFU virus;
- ■ = Immune calf challenged with $1 \times 10^6$ PFU virus.
Hours after inoculation

Days

A

B

log₁₀ PFU/swab

log₁₀ PFU/swab

Hours

Days

A

B

log₁₀ PFU/swab
inserting a 15 cm cotton-tipped swab to its full length into the left ventral nasal meatus. Swabs were submerged in 1 ml Eagle's minimum essential medium (MEM) supplemented with 5% gamma irradiated fetal calf serum (FCS) and antibiotics (100 units penicillin, 100 µg streptomycin sulfate and 100 µg kanamycin sulfate per ml) and were frozen at -70 C until tested for virus. Swab samples were serially diluted and titrated in quadruplicate on BLU monolayer cultures in 24-well plastic tissue culture plates. After 90 minutes adsorption, the cultures were washed with MEM, and overlaid with 1% agarose containing MEM, 5% FCS and antibiotics. Cultures were incubated at 37 C for 72 hours and then fixed with 10% buffered formalin. The agarose layer was removed and the cell sheet was stained with crystal violet. Plaques were enumerated and the virus titer was recorded as the average of 4 replicates.

**Serologic Procedures** - Anti-IBR serum antibody titers were determined by plaque-reduction-neutralization tests. Two-fold serial dilutions of heat inactivated serum (56 C for 30 min) were mixed with equal volumes of MEM containing 1000 PFU IBR virus/ml and incubated for 1 hour at 37 C. Monolayers of BLU cells in 35 mm, 6-well plastic tissue culture plates were inoculated with 0.2 ml of serum-virus mixture. After adsorption of the serum-virus mixture for 1 h at 37 C, the cultures were processed as described in "virus isolation". The serum-neutralization titer was the reciprocal of the highest serum dilution that reduced the plaque count by at least 50%.
Results

Disease Production - Calves developed IBR infection that was characterized by temperatures to 41.7°C, reduced appetite, increased respiration rate, slight dyspnea, cough and a mucopurulent nasal discharge. Initially, the nasal mucosa was hyperemic with numerous, small herpetic pustules observed between 12 and 24 hr. The pustules enlarged and coalesced by 72 h to form adherent fibrino-necrotic plaques involving approximately 70% of the visible nasal mucosa. Clinical signs subsided on post inoculation day (PID) 7 and calves recovered without complications. At post mortem examination of one calf on PID 8 there were necrotic plaques on the nasal and turbinate mucosae. Histologic lesions consisted of multifocal to diffuse necrosis of the nasal cavity mucosae and the tonsils. Re-exposure of calves to IBR virus on PID 30 did not produce clinical signs or lesions.

Serology - Plaque-reduction serum neutralization antibody to IBR was not detected in calves prior to virus inoculation. Calves had anti-IBR serum antibody titers of 1:16 to 1:64 when challenged on PID 30. No differences were observed in antibody titers produced following inoculation with $10^6$ or $10^9$ PFU virus.

Virus Clearance - Figure 1 illustrates the clearance time for IBR virus from the nasal mucosa in nonimmune and immune calves following inoculation with $10^9$ PFU virus (Figure 1A) and $10^6$ PFU virus (Figure 1B).
Virus Shed - An eclipse phase, during which virus could not be isolated from nasal swabs, followed the clearance of virus inoculum. Figure 2 illustrates the shed of virus after challenge of immune and nonimmune calves. Virus was first detected in the nasal mucus of nonimmune calves 9 h after intranasal inoculation with $10^9$ PFU virus. When nonimmune calves were inoculated with $10^6$ PFU IBR virus, virus was first detected in the nasal mucus at 14 h. Immune calves challenged with $10^9$ PFU shed IBR virus for approximately 24 h after challenge. No virus was shed from immune calves following challenge with $10^6$ PFU IBR virus.

Discussion

The virus isolation data in calves given $10^9$ PFU IBR virus intranasally have several properties which correlate with in vitro single-step growth curves of IBR virus. An eclipse phase of 9 h after inoculation corresponds with the first appearance of extracellular virus in in vitro studies (26, 261). The subsequent step-wise increase in virus titers indicate that several cycles of virus replication occurred prior to the attainment of maximum virus titers on PID 4. This was to be expected because the cells of the respiratory tract initially received a low multiplicity of infection and because virus infection occurred in the presence of respiratory tract clearance mechanisms.

It is generally accepted (220) that herpesvirus vaccines only protect against disease and not infection. Previously vaccinated
calves shed IBR virus following intranasal challenge despite evidence of local immunity in the respiratory tract (60, 159, 251, 274, 302). Humoral antibody plays a crucial role in mediating specific immune protection against viral reinfection (220). Additionally, many nonspecific humoral substances such as complement and interferon are involved in antiviral immunity (220). In this study IBR virus was not detected following challenge of immune animals with $10^6$ PFU virus, whereas an abortive reinfection was detected following challenge with $10^9$ PFU. The shed of virus following challenge of immune animals was at lower titers and for a shorter period of time than that found in primary infections as previously reported (60, 302).

Interpretation of virus isolations following intranasal challenge of vaccinated animals has been confounded by the lack of knowledge of the fate of virions in the inoculum. Virus present in nasal secretions during the first 6 days following intranasal challenge exposure has been described as residual challenge virus and not replicating virus (60, 159). The data presented herein establish that the virus contained in an intranasal aerosol inoculum was rapidly cleared from the upper respiratory tract of both immune and nonimmune calves. The time of virus clearance was directly related to both the immune status of the recipient calves and the quantity of virus administered. Virus isolated after the eclipse phase of IBR virus infection (9 h after inoculation) was the result of virus replication and was not residual virus.
PART IV. EXPERIMENTAL SUBUNIT VACCINES FOR INFECTIOUS BOVINE RHINOTRACHEITIS

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EXPERIMENTAL SUBUNIT VACCINES FOR INFECTIOUS BOVINE RHINOTRACHEITIS

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SUMMARY

Subunit infectious bovine rhinotracheitis (IBR) antigens were prepared by solubilization of IBR virus infected cell cultures with nonionic detergents Triton X-100 and NP-40. When used with Freund's incomplete adjuvant, Triton X-100 and NP-40 subunit antigens induced high serum neutralization titers in vaccinates. Two doses of Triton X-100 and NP-40 subunit vaccine prevented clinical signs of disease in all vaccinates following intranasal challenge with Cooper strain IBR virus. Furthermore, two doses of NP-40 subunit vaccine prevented infection in all vaccinates following challenge.
Infectious bovine rhinotracheitis (IBR) was first recognized as a distinct disease of cattle during the 1950's (170, 238). Consistent with the characteristics of other herpesviruses, IBR virus (IBRV) replicates in a wide range of cell types and produces diverse disease manifestations which include respiratory tract disease, conjunctivitis, vulvovaginitis, abortion, balanoposthitis, meningoencephalitis, alimentary tract disease and fatal systemic infection. The literature of IBR has been extensively reviewed (25, 51, 53, 105, 154, 160, 173, 215, 232, 252).

The first IBRV vaccine consisted of a parenterally administered modified live virus (MLV) that had been attenuated by rapid passage in bovine cell culture (239). Subsequent parenterally administered MLV vaccine strains were attenuated by adaptation to porcine (240) or canine (299) cell cultures; by adaptation to cell cultures at 30 C (96); or by selection of heat stable mutants (56 C for 40 minutes) (13). Intranasally administered MLV vaccine strains have been attenuated by serial passage in rabbit cell culture (272) or modified by treatment with HNO\textsubscript{2} followed by selection of temperature-sensitive mutants (302). Inactivated IBRV vaccines (hereafter inactivated vaccine) have been produced by formalin treatment (299), ethanol treatment (73), and heat or ultraviolet light inactivation (86). Inactivated vaccines without adjuvant have poor efficacy (237). Efficacy has been established using Freund's complete adjuvant (299), oil (88), saponin (73), adsorbed aluminum hydroxide gel (103), and sodium alginate (127). Administration of two doses of adjuvanted,
inactivated vaccine to cattle consistently induced the production of serum neutralizing antibodies (151).

Intranasal MLV vaccine induced local immunity involving interferon (60, 272, 274, 275, 302), secretory (IgA) antibody (60, 272, 301, 304) and cell-mediated immunity (60). Local-intranasal immune protection also was induced following parenteral administration of MLV vaccine (60) or inoculation of inactivated vaccine into the nasal mucosa (73). Early protection (40 to 96 hours) was demonstrated following vaccination of calves with intranasal (132, 274, 275) or intramuscular (20) MLV vaccines.

The extensive use of MLV vaccines has reduced the incidence of IBR disease (31, 202) but there are reports where vaccination has failed to prevent IBRV induced respiratory tract disease and conjunctivitis in cattle (42, 94, 99). The intranasal vaccination of cattle with MLV vaccines resulted in the shed of virus from vaccinated animals (159, 272, 274, 275, 302) and subsequent transmission of IBRV to nonvaccinated animals (133, 159, 300, 302). Periodic shed of IBRV and development of mild clinical signs has been described following vaccination of cattle with intranasal MLV vaccine (94). Corticosteroid treatment of animals that had been vaccinated with MLV vaccine resulted in the reactivation of latent virus infection (43, 242, 244, 304).

Subunit vaccines composed of only the necessary antigenic subunits (viral structural components) required for evoking protective immunity have been developed for influenza virus (224), vesicular stomatitis virus (114), rabies virus (71) and Semliki Forest virus
Several investigators (30, 126, 136, 192, 282, 298) have demonstrated that subunit components of herpesviruses are immunogenic and have demonstrated a vaccine potential for these antigens.

Viral proteins are present not only in the virus (259) but also in cell membranes (78, 258) and in cell culture fluids (111). Viral protein synthesis exceeds requirements for virion assembly; therefore, far more viral proteins are present in infected cells than in virions (111, 280). The protective antigenic subunits of enveloped viruses are the envelope glycoproteins (177). Herpes simplex virus type 1 (HSV-1) contains 13 glycoproteins, all of which are localized in the envelope (186, 205, 259). There have been 8 glycosylated proteins identified in IBRV (249). All subunit HSV-1 immunogens obtained from infected cell cultures and partially purified virus preparations and used to induce serum neutralizing antibodies in animals have contained glycoproteins (30, 126, 136, 137, 150, 192, 204, 282, 298).

The immunogenicity of subunit vaccines is dependent upon intrinsic or integral membrane proteins that are associated with the hydrophobic core of the phospholipid bilayer in membranes (177). The amphiphilic character of nonionic detergents enables them to dissociate the membrane into small fragments (80, 268). This "gentle solubilization" of proteins with nonionic detergents (140) facilitates the extraction of protein antigens which retain their native conformation (143), biologic activity and immunogenic properties. It has been demonstrated that nonionic detergent extracted antigens from HSV-1 infected cell cultures are immunogenic in animals (30, 136, 282).
In the present report we have evaluated nonionic detergent extracted IBRV antigens as subunit vaccines for IBR.

Materials and Methods

**Virus** - The Cooper strain of IBRV was received at the eighth passage level. It was passed two times in bovine lung cells (BLU) and a stock pool containing $1.0 \times 10^8$ plaque forming units (PFU) per ml was frozen at -70 C.

**Cell Culture Methods** - Cell cultures utilized in this study (BLU cells at passage level 15 to 33) were grown in Eagle's minimum essential medium (MEM) supplemented with 10% γ-irradiated fetal calf serum (FCS) (heat inactivated at 56 C for 30 minutes), 0.5% lactalbumin hydrolysate and antibiotics (100 units penicillin, 100 μg streptomycin sulfate per ml). The medium was buffered with 0.16% sodium bicarbonate and 8 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES). Cultures were incubated at 37 C in a 5% CO₂ atmosphere.

**Virus Isolation** - Nasal secretions were collected for virus isolation by insertion of a 15 cm cotton-tipped swab to its full length in the left ventral nasal meatus and manipulation of the swab in a rotary motion until saturated. Swab samples were immersed in 1.0 ml MEM containing antibiotics (200 units penicillin, 200 μg kanamycin sulfate, 200 μg streptomycin sulfate and 15 μg amphotericin B per ml) and held at 4 C for 30 minutes. Swabs were then removed.

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from the medium and the medium was frozen at -70 C until cultured.

Plaque forming units of virus in specimens were determined by inoculation of duplicate cultures of BLU monolayers with serial 10-fold dilutions (in MEM) of each specimen. The inocula were adsorbed for 60 minutes, monolayers were washed with MEM and overlaid with 1% agarose containing MEM, 5% FCS and antibiotics. Cultures were incubated at 37 C for 72 hours in a 5% CO₂ atmosphere. The cultures were fixed with 10% buffered formalin, the agarose layer was removed and the cell sheet was stained with crystal violet. The virus titer was determined by enumeration of PFUs.

Attempts to isolate infectious IBRV from the Triton antigen were made by inoculating BLU monolayers (75 cm² flasks) with 5 ml of 1:10, 1:20, 1:30 or 1:50 dilutions of the antigen or with 1 ml of antigen that had been treated with copolymer beads as described previously (82). Following a 60 minute adsorption period, the monolayers were washed three times with MEM, and maintenance medium was added. At 12 and 24 hours incubation, the medium was removed from cultures which received detergent containing inocula, the monolayers were washed with MEM and the maintenance medium was replenished. Cultures were considered negative for IBRV when cytopathic effect (CPE) was not observed during three serial passages of the inocula in cell cultures.

\(^{b}\)Seakem ME Agarose, Marine Colloids Division, FMC Corp., Rockland, Maine.

\(^{c}\)BioBeads SM-2, Bio-Rad Laboratories, Richland, California.
Electron-Microscopic Procedures - The negative staining procedure of Brenner and Horne (22) was utilized to examine virions remaining in the cell precipitates after vaccine production.

Plaque Reduction-Neutralization Tests - Anti-IBRV serum neutralization titers were determined by plaque-reduction neutralization tests. Two-fold serum dilutions of heat inactivated serum (56 C for 30 minutes) were mixed with equal volumes of MEM containing 1000 PFU IBRV per ml and incubated for 60 minutes at 37 C. Monolayers of BLU cells in 35-mm, 6-well plastic tissue culture plates were inoculated with 0.2 ml of serum-virus mixture. After adsorption of the serum-virus mixture for 60 minutes at 37 C, the cultures were washed with MEM, and overlaid with 1% agarose containing MEM, 5% FCS and antibiotics. Cultures were incubated at 37 C for 72 hours and then fixed with 10% buffered formalin. The agarose layer was removed and the cell sheet was stained with crystal violet. The serum neutralization titer was the reciprocal of the highest serum dilution that reduced the plaque count by at least 50%.

Vaccine Production - The BLU cells were propagated in 490 cm² roller bottles until monolayers were formed. The monolayers were infected by removing the medium and adding 5 ml of stock IBRV. After 60 minutes adsorption the inoculum was removed, the monolayer was washed with MEM and 30 ml of maintenance medium (MEM containing 5% FCS) was added. The cells were incubated at 37 C with rolling (1 rpm) until CPE was complete (approximately 24 hours). The infected BLU cells were scraped from the roller bottle surface with a rubber policeman.
The cell-medium mixture was centrifuged at 100,000 x g for 1 hour. The resultant pellet was solubilized using the following modification of the procedure described by Westergaard et al. (282). The pellet was suspended in 0.010 M glycine - 0.038 M tris (hydroxymethyl) aminomethane (Tris) (pH 9.0) containing 0.5% Triton X-100 (1 ml per roller bottle). The resuspended pellet was homogenized with a Dounce homogenizer and sonically treated for two-20 second cycles with a sonicator\(^d\) at a setting of 90% efficiency. The homogenate was then stirred for 1 hour at 4°C. The mixture was centrifuged at 100,000 x g for 1 hour and the supernatant fluid was used as the Triton antigen. The antigen was stored at -70°C until used. The vaccine was prepared by mixing equal volumes of Freund's complete or Freund's incomplete adjuvant and emulsifying with 200 strokes in an adjuvant mixing apparatus.\(^e\) A second subunit vaccine was produced using 2.5% Nonidet P-40 (NP-40) in distilled water (150) instead of the buffered Triton X-100 solution. The extraction procedures used were as described above and the supernatant fluid was used as the NP-40 antigen. Cell control antigens were produced as described above using Triton X-100.

**Protein Determination** - Protein content was assayed by the method of Lowry et al. (141) using bovine serum albumin as the standard. Protein content of samples containing nonionic detergent was determined

\(^d\)Biosonik IV, Bronwill VWR Scientific, Denver, Colorado.

\(^e\)Mulsi Churn apparatus, Mulsi Jet Inc., Division of Particle Data Inc., Elmhurst, Illinois.
using a modified Lowry procedure (197).

**Agar-Gel Immunodiffusion Test** - A variation of the Ouchterlony two-dimensional immunodiffusion technique (189) was utilized in testing for precipitating antibodies. Tests were performed in 100-x 11-mm plastic dishes. A base layer of 2 ml of 2% agarose was overlaid with 5 ml of 1% agarose containing 0.5% Triton X-100 (19) buffered with 0.01 M glycine and 0.0038 Tris (pH 9.0). Six 8-mm wells were cut around an 8-mm center well, leaving 4.0 mm of agar between wells. The Triton X-100 antigen (100 µl) was placed in the center well. Test sera (IBRV subunit vaccinate, cell control vaccinate and FCS) and IBRV positive reference serum (100 µl) were placed in alternating peripheral wells as described by Hayward and Augustin (75). Plates were incubated at room temperature for 48 hours. Precipitin lines were visible after 24 hours but became more distinct at 48 hours when results were recorded.

**Experiment 1: Determination of Subunit Antigen Immunogenicity** - Triton X-100 solubilized IBRV subunit antigens and cell control antigens were administered to 10 IBR seronegative cattle in two doses injected deep in the neck muscles at 21 day intervals as detailed (Table 1). Animals were observed daily throughout the study for clinical signs of disease. Rectal temperatures were recorded for 3 days prior to vaccination and for 10 days after vaccination.

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Provided by Beecham Laboratories, White Hall, Illinois.
Table 1. Experiment one: Inoculation schedule for determining the immunogenicity of Triton X-100 solubilized IBRV antigens

<table>
<thead>
<tr>
<th>Calf number</th>
<th>Inoculum</th>
<th>Dose (ml)/ inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cell control antigens without adjuvant(^a)</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Cell control antigens with adjuvant</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Triton antigen without adjuvant</td>
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<tr>
<td>4</td>
<td>&quot;</td>
<td>1</td>
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<tr>
<td>5</td>
<td>&quot;</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>Triton antigen with adjuvant</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>&quot;</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>&quot;</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>&quot;</td>
<td>4</td>
</tr>
</tbody>
</table>

\(^a\)Freund's complete adjuvant
Nasal secretions were collected for virus isolation prior to vaccination and on days 2, 3 and 7 post-vaccination. Serum samples were collected at weekly intervals.

**Experiment 2: Determination of Immune Protection** - Twenty IBRV seronegative calves, 3 to 6 months of age, were randomly divided into 5 groups (4 in each group) and housed in separate, enclosed, isolation rooms throughout the study. Groups of calves, designated 1 to 5, were vaccinated intramuscularly as illustrated (Table 2). Thirty days after vaccination, the calves were administered a standard challenge virus inoculum\(^h\) (1 x 10\(^6\) PFU Cooper strain IBR, 10th passage). A gas-powered atomizer was used to deliver 2 ml of inoculum into each nostril as previously described (248).

Calves were examined daily for clinical signs of disease from 7 days prior to vaccination until the study was terminated. Rectal temperatures were recorded for 3 days prior to vaccination, for 10 days following each vaccination and for 14 days following challenge. Nasal secretions were collected for virus isolation three times prior to vaccination and daily for 7 days following the initial vaccination in each group. Following challenge, nasal secretions were collected at the following times: 5 minutes post-challenge; 15 minute intervals thru 1 hour post-challenge; hourly intervals thru 15 hours post-challenge; at 18, 20, 22, 24 hours post-challenge; and at 24 hour

\(^h\)Protocol, IBR Vaccine Potency Test, Challenge Virus, National Veterinary Services Laboratory, Ames, Iowa.
Table 2. Experiment two: Vaccination schedule for determining the efficacy of Triton X-100 and NP-40 solubilized IBRV subunit vaccines

<table>
<thead>
<tr>
<th>Group number</th>
<th>Calf numbers</th>
<th>Vaccinea</th>
<th>Doses of vaccine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 to 4</td>
<td>Triton X-100 solubilized cell control antigen</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>5 to 8</td>
<td>Triton X-100 antigen</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>9 to 12</td>
<td>NP-40 antigen</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>13 to 16</td>
<td>Triton X-100 antigen</td>
<td>2b</td>
</tr>
<tr>
<td>5</td>
<td>17 to 20</td>
<td>NP-40 antigen</td>
<td>2</td>
</tr>
</tbody>
</table>

a Each vaccine consisted of 1 ml antigen and 1 ml Freund's incomplete adjuvant.

b Second dose of vaccine administered at 30 days.
intervals thru 14 days post-challenge. Serum samples were collected three times prior to vaccination and at weekly intervals following vaccination.

Results

**Examination of Subunit Vaccine - Triton X-100 and NP-40**

Solubilized antigens did not contain cytopathogenic virus as determined by virus isolation procedures. Electron microscopic examination of negative stained preparations of the pellet which remained after treatment with Triton X-100 and NP-40 revealed that the envelope had been dissociated from all herpes virions examined (approximately 500 virions) (Figure 1).

In Experiment 1, the Triton X-100 subunit vaccine contained 4.4 mg protein per ml. In experiment 2, the Triton X-100 subunit vaccine contained 3.5 mg protein per ml and the NP-40 subunit vaccine contained 5.8 mg protein per ml.

In the agar-gel immunodiffusion test, 4 precipitin lines were formed between the Triton X-100 solubilized antigen and serum from animals vaccinated with the Triton X-100 subunit vaccine (Figure 2). Precipitin lines were not visible between the antigen and fetal calf serum or sera from cattle vaccinated with cell control antigens.

**Experiment 1. Determination of Subunit Antigen Immunogenicity -**

All 10 animals in experiment 1 remained clinically normal throughout the study. Rectal temperatures following vaccination were within ±1 C of temperatures recorded prior to vaccination. Virus was not
Figure 1. Electron photomicrograph of naked herpesviruses from the pellet obtained following treatment of IBR virus infected cells with Triton X-100. PTA negative stain. x 100,000.

Figure 2. Agar-gel immunodiffusion plate. Note 4 precipitin lines formed between central well (A) containing Triton X-100 solubilized IBRV antigen and well C containing serum from calf vaccinated with Triton X-100 subunit vaccine. Hyperimmune IBRV reference serum was placed in B wells. No lines are formed against fetal calf serum (D) or serum from calf vaccinated with Triton X-100 extracted bovine lung cells (E). x 2.75.
isolated from nasal secretions prior to or following vaccination. Serum neutralization titers are detailed (Table 3).

**Experiment 2: Vaccine Challenge Study** - Following vaccination, 4 of 4 control calves and 5 of 12 vaccinates developed indurated swellings at the site of vaccine inoculation and febrile responses (40 to 41.1 C) that persisted for 1 to 3 days. Deep intramuscular inoculation and avoidance of "inoculum drag" prevented reaction to the second vaccination.

Table 4 details the serum neutralization titers following vaccination and challenge.

The average temperatures of calves in each of the 5 groups following intranasal challenge with Cooper strain IBRV are illustrated (Figure 3). Following intranasal challenge with IBRV, all calves in groups 2, 3, 4 and 5 remained clinically normal except calves 5, 10 and 11 which developed mild respiratory tract infection. Clinical signs of disease included elevated temperature, intranasal herpetic pustules and fibrino-necrotic nasal exudate. The clinical course of disease observed in calves 10 and 11 was characterized by an elevated temperature which persisted beyond resolution of intranasal lesions and nasal exudation. The temperature returned to base-line values 24 hours after antibiotic therapy was initiated on post-challenge day 9. Calf 20 died from causes unrelated to experimentation (rumen stasis) on post vaccination day 38. Post mortem examination did not reveal evidence of IBRV disease or any lesion at the site of vaccination.
Table 3. Experiment one: Serum neutralization titers following intramuscular administration of Triton X-100 solubilized IBRV subunit vaccine

<table>
<thead>
<tr>
<th>Calf number</th>
<th>Vaccine</th>
<th>Dose (ml)</th>
<th>Serum neutralization titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Weeks post vaccination&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
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<tr>
<td>1</td>
<td>Cell control without adjuvant</td>
<td>1</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>2</td>
<td>Cell control with adjuvant&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
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<td>IBRV subunit without adjuvant</td>
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<td>&lt;2</td>
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<tr>
<td>4</td>
<td>&quot;</td>
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<td>&lt;2</td>
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<td>&quot;</td>
<td>2</td>
<td>&lt;2</td>
<td>8</td>
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<td>4</td>
<td>&lt;2</td>
<td>64</td>
</tr>
</tbody>
</table>

<sup>a</sup>Titer expressed as the reciprocal of the highest serum dilution that reduced the plaque count by at least 50%.

<sup>b</sup>Second dose of vaccine administered at 3 weeks.

<sup>c</sup>Freund's complete adjuvant.
Figure 3. Average temperature response of calves following intranasal challenge with Cooper strain IBRV 30 days after vaccination with Triton X-100 and NP-40 solubilized subunit vaccines.

Symbols:

——— = Group 1; cell control

———— = Group 2; Triton X-100, 1 dose

——— = Group 3; NP-40, 1 dose

——— = Group 4; Triton X-100, 2 doses

———— = Group 5; NP-40, 2 doses
Temperature °C

Days post-challenge

Antibiotics
Table 4. Experiment two: Serum neutralization titers following intramuscular administration of Triton X-100 and NP-40 solubilized IBRV subunit vaccines

<table>
<thead>
<tr>
<th>Group/Animal number</th>
<th>Subunit vaccine</th>
<th>Serum neutralization titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Weeks post-vaccination</th>
<th>Weeks post-challenge&lt;sup&gt;c&lt;/sup&gt;</th>
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<td></td>
<td></td>
<td></td>
<td>0 1 2 3 4 5 6 7 8 1 2</td>
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<td><strong>Group 1</strong> Cell Control</td>
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<tr>
<td>4</td>
<td></td>
<td></td>
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<td>32</td>
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<tr>
<td><strong>Group 2</strong> Triton X-100</td>
<td>1 dose</td>
<td></td>
<td>&lt;2 &lt;2 16 16 32</td>
<td>128 512</td>
</tr>
<tr>
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<td></td>
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<tr>
<td>6</td>
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<td>8</td>
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<td>128 512</td>
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<td><strong>Group 3</strong> NP-40</td>
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<td></td>
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<td>128 256</td>
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<tr>
<td>13</td>
<td>2 doses</td>
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</tr>
<tr>
<td>14</td>
<td>&lt;2 2 16 64 32 256 256 512 512 512 512</td>
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<td>15</td>
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<td>&lt;2 2 8 8 8 8 256 256 128 128 128 128</td>
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<table>
<thead>
<tr>
<th>Group 5</th>
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<tbody>
<tr>
<td>17</td>
<td>2 doses</td>
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<tr>
<td>18</td>
<td>&lt;2 2 8 32 32 256 512 512 512 256 512</td>
</tr>
<tr>
<td>19</td>
<td>&lt;2 &lt;2 32 32 256 256 256 256 128 128 256</td>
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<tr>
<td>20</td>
<td>&lt;2 2 16 16 16 16 128 d d d d</td>
</tr>
</tbody>
</table>

\( ^{a} \) Titer expressed as the reciprocal of the highest serum dilution that reduced plaque count by at least 50%.

\( ^{b} \) Freund's incomplete adjuvant.

\( ^{c} \) Intranasal challenge with \( 1 \times 10^6 \) PFU Cooper stain IBRV virus administered 30 days after last dose of vaccine.

\( ^{d} \) Calf died on post-vaccination day 58.
Clinical signs of disease and certain data relating to shed of virus from control calves has been reported previously (144). Challenge exposure of control calves produced a severe respiratory disease characterized by elevated temperature (to 41.7°C) and formation of extensive fibrino-necrotic plaques on the nasal mucosa.

Virus was not recovered from nasal secretions prior to or following vaccination. In groups 2 and 3 challenge inoculum virus was isolated at 5 min and 15 min post challenge but could not be isolated from nasal secretions of calves at 30 minutes after challenge exposure. Challenge inoculum virus could not be isolated from nasal secretions of calves in groups 4 and 5 at 5 minutes after challenge inoculum. Following the initial clearance of challenge virus inoculum, virus was isolated from nasal secretions 18 hours after challenge in groups 2 and 3 and at 22 hours in group 4 (2 of 4 calves). Table 5 details shed of virus in nasal secretions for days 1 to 14 post challenge. Virus was not isolated from calves in group 5 following intranasal challenge exposure.

Discussion

This study has demonstrated that subunit vaccines prepared by Triton X-100 and NP-40 solubilization of IBRV infected cell cultures are immunogenic and efficacious. Triton X-100 subunit antigen was poorly immunogenic when administered without adjuvant; however, high serum antibody titers were induced in all animals vaccinated with Triton X-100 and NP-40 subunit vaccines containing adjuvants. One
Table 5. Experiment two: Virus isolation following intranasal challenge of calves vaccinated with Triton X-100 and NP-40 solubilized IBRV subunit vaccines

<table>
<thead>
<tr>
<th>Group/Animal number</th>
<th>Subunit vaccine</th>
<th>Days post challenge</th>
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<td>Cell control</td>
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<td>Triton X-100</td>
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<tr>
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\[^a\text{Virus titer as log}_{10} \text{ plaque forming units/nasal swab.}\]

\[^b\text{No virus detected.}\]
dose of Triton X-100 subunit vaccine prevented clinical signs of disease in 3 of 4 calves challenged with Cooper strain IBRV and NP-40 subunit vaccine protected 2 of 4 challenged calves. All calves were protected from challenge after two doses of either vaccine. Results demonstrated that one dose of subunit vaccine markedly modified the infection that developed in calves following challenge. Serologic data indicated that the first dose of vaccine acted as a "priming" dose and subsequent vaccination or exposure to challenge virus resulted in at least a 4-fold increase in serum antibody titer. Additionally, it was demonstrated that two doses of Triton X-100 subunit vaccine prevented shed of virus from 2 of 4 challenged calves and two doses of NP-40 subunit vaccine prevented shed of virus from 3 of 3 challenged calves.

Attenuation of IBRV for use in MLV vaccines has been reported to reduce immunogenicity of the virus (96, 239, 240). Several investigators have reported low serum antibody titers following vaccination with IBRV vaccines (42, 60, 115, 159, 240, 274, 299, 301, 304). Zygraich et al. (304) reported that two doses of temperature-sensitive IBRV vaccine did not prevent reinfection when vaccine strain virus was administered a third time.

Challenge of IBRV vaccinated cattle with virulent virus resulted in transient elevated temperature (24, 103, 123, 159, 229), mild nasal lesions (103, 123, 159, 229) and shed of virus (60, 96, 123, 159, 275, 300, 302). The shed of virus following intranasal challenge of IBRV vaccinated cattle has been attributed to residual challenge
inoculum (60, 159). In this study, challenge virus inoculum was isolated from calves in groups 2 and 3 at 5 and 15 minutes after challenge but could not be isolated 30 minutes after challenge. In groups 4 and 5, virus could not be isolated from nasal secretions 5 minutes after challenge. These findings are in agreement with previous findings that demonstrated clearance of virus inoculum from the nasal mucosa of nonimmune animals required approximately 1 hour; whereas, clearance from immune animals required less than 5 minutes (144).

The immunogenicity of nonionic detergent solubilized herpesvirus protein has been demonstrated by other workers. Vestergaard et al. (282) demonstrated that Triton X-100 was capable of solubilizing 90% of the protein content of HSV-1 infected cells and identified 11 precipitating HSV-1 antigens (281), including 4 glycoprotein antigens (283). Spear and Roizman (259) reported that NP-40 treatment quantitatively removed envelope glycoproteins from HSV-1 virions. Antibodies against HSV-1 envelope glycoproteins are capable of neutralizing the virus (200). Cappel (30) reported that NP-40 solubilized HSV-1 subunit vaccine induced both humoral and cell-mediated immune responses. In the present study, Triton X-100 and NP-40 solubilized IBRV vaccine induced precipitating antibodies, induced high neutralizing antibody titers, and protected challenged vaccinates against disease and infection.

Kahrs (104) stated that IBRV vaccination should prevent severe manifestations of IBRV infections but could not be expected to prevent
infection of the superficial mucosa, establishment of latent infections or reactivation of existing latent infections. The high efficacy obtained with vaccines in the present study could be attributed to the following facts: (1) The use of virulent of "wild" type virus precluded loss of viral antigens through attenuation or repeated cell culture passage (186, 241) and (2) the use of nonionic detergents which solubilize membrane proteins with minimal disruption of native conformational and immunogenic structure (268).

Utilization of subunit vaccines in the prevention of IBRV infection would provide the following advantages: (1) Subunit vaccine does not contain live virus and therefore cannot be transmitted to other animals, cause abortion in pregnant cows or establish latent infections. (2) The subunit vaccine preparation technique removes capsid proteins; therefore, different antigens are present in natural infections. This provides a potential for development of serologic procedures to differentiate vaccinates from naturally infected animals. (3) Subunit vaccine could be used in young calves possessing maternal antibodies (104, 198). (4) Prevention of infection by use of the subunit vaccine offers potential for eradicating IBR.
SUMMARY AND CONCLUSIONS

This study consisted of two parts: (1) an evaluation of the rabbit as a laboratory model for the study of IBRV infection and (2) an evaluation of the safety and efficacy of nonionic detergent extracted IBRV subunit vaccines.

Experimental infection of laboratory rabbits with IBRV produced disease manifestations which included abortion, conjunctivitis, dermatitis, vulvovaginitis, systemic infection, neonatal death and respiratory tract infection. Each disease syndrome was studied using virus isolation, fluorescent antibody examination and histologic examination.

Experimental IBRV infection of eastern cottontail rabbits caused acute keratoconjunctivitis and a fatal systemic infection. Infection in eastern cottontail rabbits stressed by captivity was similar to infection experimentally produced in dexamethasone treated laboratory rabbits.

Following intranasal aerosol exposure of calves with IBRV, virus inoculum was rapidly cleared from the nasal mucosa. Nonimmune calves cleared \(10^9\) PFU IBRV from the nasal mucosa in less than 4 hours and \(10^6\) PFU IBRV in 1 hour. In contrast, immune calves cleared \(10^9\) PFU IBRV in 1 hour and \(10^6\) PFU IBRV in less than 5 minutes. The data show that there was no residual virus inoculum on the nasal mucosa beyond 4 hours post inoculation.

Subunit IBRV antigens were prepared by solubilization of
IBRV infected cell cultures with nonionic detergents Triton X-100 and NP-40. Immunogenicity of subunit antigens was demonstrated by induction of high serum neutralization antibody titers in vaccinates. Two doses of Triton X-100 and NP-40 solubilized IBRV subunit vaccine prevented clinical signs of disease in all vaccinates challenged with Cooper strain IBRV at 30 days post vaccination. Furthermore, two doses of NP-40 subunit vaccine prevented infection in all vaccinates challenged with Cooper strain IBRV at 30 day post vaccination.

The following conclusions can be made:

1. The rabbit is well suited as a laboratory model for the study of IBRV disease pathogenesis and may be suited for studying IBRV latent infection, determining pathogenicity of virus strains and evaluating vaccine safety and efficacy.
2. Virus inoculum administered by intranasal aerosol exposure is rapidly cleared from the bovine nasal mucosa. Residual IBRV inoculum cannot be detected on the nasal mucosa beyond 4 hours post inoculation.
3. Experimental subunit IBRV vaccines are safe and efficacious. Subunit IBRV vaccine prevents disease in challenged vaccinates. Furthermore, subunit IBRV vaccine containing NP-40 solubilized antigens can prevent infection when vaccinates are challenged.

Results obtained in these studies have answered some questions. However, these studies have identified the following additional
problem areas for future research:

1. Further study is needed to elucidate the cause of IBRV-induced abortion in the rabbit.

2. The rabbit model of IBRV infection needs additional study to determine whether latent infection is established and whether the latent infection can be recrudesced.

3. The subunit IBRV antigens need to be characterized and purified. The immunogenicity of individual IBRV glycoproteins needs to be determined.

4. The subunit vaccines described in this study need to be studied further to verify these findings in larger numbers of calves and to evaluate the vaccine under field conditions. The duration of immunity induced by subunit IBRV vaccine needs to be determined.

5. Serologic test procedures need to be developed to distinguish subunit IBRV vaccinates from calves vaccinated with while virus vaccines and from calves naturally infected with IBRV.

6. Additional research is needed to elucidate the humoral and cell-mediated immune mechanisms involved in clearance of virus inoculum and clearance of replicating virus from the nasal mucosa in calves.

7. Additional research is needed to determine if subunit IBR vaccines will prevent latent infection.
REFERENCES


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I have come to realize that it has been through the forbearance of those closest to me that this project has been completed. Therefore, I offer thanks to my wife, Nancy, who made my graduate education endurable, to my parents who made it possible, and to my children, Max, Russell and Martina, who made it all worthwhile.