CERTAIN CHARACTERISTICS OF INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS: (I) SUSCEPTIBILITY OF GOATS (II) ANTIGEN RELATIONSHIP OF ISOLATES (III) INTERFERON PRODUCTION AND SUSCEPTIBILITY

by

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INTRODUCTION

Infectious bovine rhinotracheitis (IBR) was apparently first observed in the United States in Colorado in 1950 and was recognized in California in 1953. The IBR virus was isolated from cattle affected with respiratory infection in 1955. In 1957 the virus was isolated from cattle with an infectious vaginitis later called infectious pustular vulvovaginitis (IPV), which was found to be the same as Blaschenausschlag, a disease that had been recognized in Central Europe for many years. As the name would indicate, however, the manifestations of IBR in cattle most commonly observed involve the anterior respiratory tract. Occasionally, conjunctivitis is associated with the respiratory form of the disease or it may appear independently. In Australia, the virus was first isolated from calves suffering from an encephalitis. More recently it has also been reported to cause abortions in cattle in the United States.

Vaccines have been developed by attenuating viruses isolated from the respiratory tracts of infected cattle and have been utilized since 1957 to prevent the respiratory form of the disease. The licensed producers of IBR vaccine are required to test its immunizing ability by vaccinating susceptible calves with a prescribed dose and conducting a serum neutralization test with blood collected 14-21 days after vaccination. The serum must neutralize the test dose of virus

(100 to 1000 $TCID_{50}$) at the 1:2 dilution. The vaccination also serves as a safety test.

Testing of commercially produced, licensed biologics is conducted at the National Animal Disease Laboratory, Ames, Iowa (NADL). Due to the cost of calves for testing and space limitations, it would be advantageous to utilize smaller, less expensive animals for testing the IBR vaccines. Since there was a report in the literature that goats are susceptible, it was thought that goats might be useful for this purpose. The report of attempts to infect goats constitutes the first portion of this thesis.

Antigenic comparisons of strains or isolates of IBR virus made by some investigators revealed that they were antigenically the same based upon the ability of antiserums to neutralize viral strains or isolates. More recently some workers had detected apparent antigenic differences. Significant antigenic differences would indicate a need for a vaccine composed of more than one strain of IBR virus. In order to determine if there were antigenic differences, the neutralizing ability of the calf antiserums, collected from susceptible calves vaccinated by the licensed producers, were tested using certain vaccine and virulent viruses. The virulent viruses were recovered from field cases of IBR, IPV, abortion and conjunctivitis. The results and analysis of these serum neutralization tests are reported in the second part of this thesis.

It was noted in the literature that avirulent strains of measles and polio viruses induced a higher yield of interferon (IF) than did virulent strains of these viruses. If the modification of a virulent virus to an avirulent state is related to the production of IF, or susceptibility of the virus to IF, then it should be possible to demonstrate it. If this phenomenon applies to other viruses, it should be possible to show that the IBR virus which had been modified to avirulency induced a higher yield of IF than the virulent strains. Also if this is generally applicable, it could be significant for the evaluation of other vaccines as well as IBR. Therefore, a study was conducted to compare the IF yield or sensitivity of avirulent (vaccine) and virulent strains of IBR virus. Reports of these experiments utilizing several tissue culture cells and variations in temperature, and virus strains constitute the third portion of this thesis.

GENERAL REVIEW OF THE LITERATURE

A disease involving the upper respiratory tract of dairy cattle was reported by Schroeder and Moys (82). The apparent infection, which was characterized by a high fever, up to 108 F. and sudden cessation of lactation, appeared suddenly in the Los Angeles, California area on October 17, 1953. It spread in California during the next few months. The disease was considered by these authors to resemble infectious bronchitis in cattle as described in Hutyra, Marek, and Manninger (43).

McIntyre (58) described the successful reproduction of the disease described by Schroeder and Moys (82) by inoculating calves with a mixture of blood, nasal discharges, sputum and feces from affected cows. He also observed spread by pen contact and reported transmission by the inoculation of materials from chicken embryos which had been previously inoculated with sputum and nasal exudate (treated with penicillin and streptomycin) collected from clinical cases.

McKercher et al. (61) reported that beef cattle were affected in the same epizootic in California. These authors concluded that it was an exotic or new disease entity or an endemic disease that had become so clinically modified as to be unrecognized as such. They were unsuccessful in transmitting the disease using inoculations of blood, spleen and nasal and tracheal curettings from field cases of the disease.

Various routes of inoculation were utilized. One animal inoculated intranasally responded with a temperature rise. Attempts were made to isolate a causative agent by inoculating mice and chicken embryos with spleen, lung, and nasal washings. Guinea pigs inoculated with milk and blood from visibly affected febrile cattle were not infected. These authors explored the relationship of this disease to virus diarrhea (VD) as described by Baker et al. (4). Cross-protection tests were conducted with calves experimentally and naturally exposed to the respiratory disease. Although there appeared to be cross protection among some animals, it was attributed to the general prevalence of VD in the area and to the age of the animals used in the trials. The failure to transmit the disease was also considered to be due to the widespread occurrence of the condition in the area and that therefore the inoculated animals might have been exposed previously.

In 1955 Miller (71) discussed a disease first observed in beef cattle which affected the entire upper respiratory tract and which had been observed in Colorado since 1950. Because of the lesions observed, the condition was known as necrotic rhinotracheitis. He referred to the reports of a similar condition in California (82) and suspected that this was a modified form of the same disease. Beginning in 1953 the disease was observed in younger calves and in dairy herds where no beef cattle were present. Since no drugs were found to be specific

for treating the disease, it was believed to be due to a virus. Therapeutic measures were designed to combat the secondary invaders.

Chow et al. (13) reported the successful experimental reproduction of the disease, now referred to as infectious rhinotracheitis. Routes of inoculation were oral, intranasal, and intravenous. The intranasal inoculations were made with a syringe and as an aerosol. Materials were collected from sick animals and from autopsies. Materials used for inoculation were nasal secretions, feces, tracheal exudates, saliva, splenic suspensions and serums collected from febrile animals. Cattle inoculated were of the beef and dairy breeds, ranging in age from 6 months to 4 years. No evidence of infection resulted from inoculations of mice, rabbits, or chicken embryos. No reaction was noted in cattle which had received inoculations of tissues from the third passage in chicken embryos.

McKercher et al. (62) reported the successful transmission of the disease by inoculating nasal exudate collected from affected cattle. Blood from the same animals did not evoke any reaction. A typical response to the intranasal inoculation included; (1) febrile--24-72 hours postinoculation --temperatures of 105.5 to 107.5 for 3-5 days, (2) serous nasal discharge, (3) shallow rapid breathing, (4) accelerated plus rate, and (5) congested nasal mucosa. After several days, whitish fibrinous exudate could be observed on the mucosa

inside the nares. Salivation increased and was frequently dry and tenacious. Depression and inappetence occurred at the time of maximum fever. Considerable respiratory distress was apparent in the more severely affected cases. The total white blood cell counts (WBC) did not fluctuate beyond normal limits. Lacrimation was sometimes observed but was regarded as coincidental or associated with pinkeye (sic) which some experimental calves developed.

Mature cattle were experimentally infected using the same materials and procedures. There was evidence that the experimentally-produced disease was transmitted to susceptible pen mates through contact. It was however, while typical, a milder response. The possibility that the inoculation did not merely activate a latent infection was tested by inoculating sterile stabilizing fluid. No response was observed. Also, nasal washings from clinically normal mature cattle failed to produce any response when inoculated into calves.

The nature of the agent was established as a virus by producing the disease with bacteria-free filtrates of the nasal washings.

Recovered animals were found to be immune to challenge with the homologous virus and to materials collected from two field cases.

Madin et al. (55) first reported the isolation of the IBR virus (IBRV). They inoculated bovine embryonic kidney (BEK) cells with nasal washings from cattle in the acute phases of the

disease. The IBR virus produced a cytopathic effect (CPE) characterized by rounding and shrinking of the cells and an increased granularity and clumping beginning 24-48 hours after inoculation. The virus also produced CPE in bovine embryonic testicle and lung cells but not in HeLa, KB, L or chick fibroblast cells. Cattle were infected with tissue culture materials collected from the 4th, 7th and 15th passage in BEK cells. They also demonstrated the ability of convalescent serums from experimentally and naturally infected cattle to neutralize the virus.

York and Schwarz (105) reported the successful propagation of the IBR virus in BEK cells with CPE as described in (55) above. They inoculated cattle intranasally with the tissue culture fluid containing virus and observed a typical febrile response and other typical signs of the disease. They challenged the immunity produced in the recovered animals by inoculating them with the homologous virus and heterologous viruses including nasal washings from naturally infected cattle. The previously infected cattle were shown to withstand challenge with all viruses tested. These authors concluded from conducting cross-neutralization tests with serums from convalescent animals inoculated with the homologous and heterologous viruses that the 7 strains or isolates tested were of 1 antigenic type. This report also discusses the development of a modified virus vaccine which is reported fully in (84).

Gillespie et al. (29) confirmed the reports of other workers (55) that (1) the infectious agent was a virus (2) the disease could be transmitted by contact as well as from infected material (13, 62), and infected tissue culture fluid (105), and (3) that immunity was produced by the infection. They were able to recover the virus from nasal exudates, tracheas, mediastinal lymph nodes, lungs, and blood following inoculation intranasally or intratracheally, but not after the animals had recovered. Serum from recovered animals neutralized IBR virus but did not neutralize bovine virus diarrhea virus or react with Mycobacterium bovis or Leptospira pomona by agglutination or lysis.

Schwarz et al. (84) reported the successful modification of the IBR virus by rapid serial passage and terminal dilution procedures in EBK tissue culture. The avirulence of the modified virus was demonstrated by intramuscular and intranasal inoculations of susceptible cattle. The inoculation of the modified virus intramuscularly into cattle elicited a response that protected cattle against an intranasal challenge with virulent virus. The virus did not spread from vaccinated cattle to susceptible contacts and was not re-isolated from the blood or nasal washings of the vaccinated cattle.

Kendrik et al. (47) conducted a field trial of a commercially developed IBR vaccine by vaccinating by intramuscular injection 20 susceptible cattle and maintaining 2 control groups (unvaccinated susceptible cattle), 1 group in contact with and the other isolated from the vaccinates. The challenge

was by an intranasal spray of 10 ml. of tissue culture fluid containing virus. The vaccinated animals all resisted challenge 23 days after vaccination while the contact and isolated controls exhibited typical febrile response and clinical signs of IBR. There apparently was no spread to the contact controls due to the vaccination, since they were completely susceptible when challenged. All vaccinates had antibodies against IBR at the time of challenge. The titers as determined by the serum neutralization (SN) test ranged from 1:3 to 1:50. None of the controls had antibodies detectable by the SN test.

Brown and Chow (9) tested two commercially produced IBR vaccines. The trials were conducted under controlled conditions and in field trials. Of the 20 cattle vaccinated in the first trial, 14 had a mild post-vaccinal reaction but all resisted challenge 4 weeks later although 7 of the 20 showed a mild reaction. Sixteen of 20 unvaccinated cattle that were challenged developed typical IBR. In the field trial 1.08% of the 12,975 vaccinated cattle developed IBR when challenged compared with 5.61% of the 4,623 unvaccinated controls.

McKercher and Straub (64) reported the isolation of a virus designated as the W strain from nasal washings of a range cow. The agent was compared serologically and by cross-protection tests to IBR virus. On the basis of complete reciprocal cross-neutralization tests, with a reference IBR virus, the W strain seemed to be an IBR virus. The authors

stated, "however, this strain might differ in certain antigenic details from the reference strain." The cross-protection tests confirmed the close relationship of the isolate to IBR virus. The delayed temperature responses, 8-9 days post-challenge, observed in 2 inoculated and subsequently challenged animals were not considered to be characteristic of IBR.

McKercher et al. (67) studied the distribution and persistence of IBR virus in experimentally infected cattle. The virus multiplied in the tissues of the upper respiratory tract and extended to the ocular tissues where secondary sites of localization occurred. The virus persisted for 6 days in the ocular tissues and adjacent lymph nodes and in the nasal secretions for 9 days. The virus was last isolated from the larynx on the 12th day following inoculation. The absence of a viremia and the failure to find a primary site of localization of the virus were pointed out as especially significant by the authors because of the recent reports of high incidence of abortion following IBR vaccination.

McKercher et al. (63) reported the successful transmission of IBR to goats and that these animals harbored the virus longer, at least in some cases, than did cattle. They exposed cattle to the virus by ocular inoculation and observed a febrile response, lacrimation, and a slight nasal discharge. The virus was recovered from the nasal secretions but only from the ocular secretions of the inoculated eye. The virus persisted longer in the ocular secretion than it did in the

nasal discharge. Cattle were found to be solidly immune to reinfection for at least one year after clinical recovery.

In 1958 Kendrik et al. (48) reported their studies of an outbreak of infectious pustular vulvovaginitis in a dairy herd. They isolated and cultivated a virus from the vaginal exudate and cultivated it in BEK cells. They were able to reproduce the disease with the tissue-cultured virus. Small foci of necrosis that develop in the vulva and the resulting influx of neutrophils produce pustules that characterize the disease. There is a febrile response and decrease in the neutrophils in the blood. Neutralizing antibodies appeared in the blood in about 2 weeks. Degenerating epithelial cells were characterized by the formation of intranuclear inclusion bodies.

Gillespie et al. (30) compared IBR virus with the virus which causes infectious pustular vulvovaginitis (IPV) by inoculating cattle with both viruses and conducting cross-protection tests and serologic tests of the blood serums. Five heifer calves were inoculated with IBR virus by swabbing the vulva and vagina. Five bull calves were inoculated intranasally with 20 ml of a 1:20 dilution of IPV virus. Each heifer inoculated showed typical signs of IPV beginning 2 to 3 days after inoculation. Of the bull calves inoculated, 3 had a febrile reaction 2 to 3 days later. None showed respiratory signs of illness but virus was isolated from the nasal passages of all bull calves on the 3rd day after inoculation.

All inoculated animals were immunized against subsequent cross challenge with both the IPV virus given vulvovaginally and IBR virus given intranasally. Serum from the inoculated cattle which did not neutralize either virus before inoculation neutralized both viruses after inoculation. The characteristics of both viruses in tissue culture were markedly similar.

Grunder et al. (36) isolated a virus from a steer, believed to have malignant head catarrh, which was found to be IBRV by serologic tests and cytopathic effects.

Liess et al. (51) studied the cultural, serologic, and electron microscopic characteristics of a virus isolated in Germany, designated B_1. On the basis of their studies, they could not differentiate this virus from a virus which caused IPV in the United States and which was considered identical to IBRV. In a subsequent report Liess et al. (52) produced IBR and IPV in 4 bovines with the B_1 isolate referred to in (51).

McKercher (60) made a comparative study of 3 viral isolates recovered from cattle affected with Blaschenausschlag in Austria, East Germany, and Belgium and IBR virus isolated from a dairy herd during a typical respiratory epizootic in California. Through cross immunity studies in calves, the 4 isolates were found to be immunologically homogeneous. The neutralizing indexes of antiserums produced by the inoculation of each of the viral isolates were found to be essentially equal in cross-neutralization tests. Also antiserums from calves inoculated with the Blaschenausschlag viral

isolates and the IBR virus all cross-reacted in complement-fixation tests with the IBR virus to essentially the same extent. The author concludes that on the basis of this study the IBR virus is identical to the Blaschenausschlag virus and that this disease is the same as IPV. He suggests that since Blaschenausschlag occurred in Europe before legislation was enacted in 1930 that prohibited cattle importations from Europe or other countries where foot-and-mouth disease or rinderpest exists, that the virus may have reached the United States before that time.

Straub and Böhm (93) isolated the IBR-IPV virus from the preputial washings of 2 bulls having a purulent preputial discharge. Cows and heifers serviced by the bulls developed a mucopurulent vaginitis but the virus was not recovered from vaginal or nasal swabs of the females. The virus was identified by serum neutralization.

McKercher (68) confirmed the reports of others (30,60) that IBR and IPV are caused by the same agents and that there is complete reciprocal cross-neutralization by their antiserums. Rinderpest antiserums had no effect on the IBR-IPV viruses. The animal transmission experiments gave the same results in that the IBR and IPV inoculated animals were equally resistant to each virus but were susceptible to rinderpest virus. The author reported no distinguishable difference in the CPE with either IBR or IPV viruses. He comments on the realism of grouping the IBR-IPV virus with the Herpes-like

viruses.

McKercher (69) reported further on his studies of the IBR-IPV virus and rinderpest virus using plaque assay. Plaques produced by IBR and IPV viruses were indistinguishable but both differed from rinderpest. The respective antiserums inhibited plaque formation. The plaque production of IBR and IPV viruses was reciprocally inhibited by their antiserums but not by rinderpest antiserum.

Baker et al. (3) studied the effects of the IBR-IPV virus in newborn calves. The calves were inoculated intravenously, orally and through contact exposure. All calves showed signs of illness, one calf inoculated in each way died, and 2 became moribund and were killed. In addition to the typical febrile and respiratory response, extensive lesions were noted in the anterior portions of the alimentary tract. The virus was isolated from the livers, spleens, kidneys and lungs of the intravenously and orally inoculated calves. No significant virus isolations were made from the blood.

Abinanti and Plumer (1) reported the isolation of a viral agent from a herd of beef calves affected with a severe conjunctivitis. Cross-neutralization tests were conducted with IBR antiserum from (1) rabbits and cattle, and (2) serum from a steer convalescing from the conjunctivitis using IBR virus and the virus isolated from one of the affected calves. Each serum gave reciprocal cross-neutralization for each of the viruses. Two calves were inoculated intrapapebrally with the isolated

virus and 2 with known IBR virus. Both calves receiving the field isolate responded with a rise in temperature and typical clinical signs, while only one of the calves inoculated with IBR virus manifested a clinical response. Virus was recovered from the uninoculated eye and nasal secretions of the calf that did not respond clinically to the inoculation. The authors suggest that this may have been contact infection from its pen mate since the virus was not isolated until 9-14 days after inoculation. However, this calf had not developed demonstrable neutralizing antibodies 29 days after the onset of virus secretion. The neutralizing antibodies to both the field isolate and IBR virus developed by the other 3 calves neutralized approximately the same amount of both viruses.

Hughes et al. (42) studied an epizootic of keratoconjunctivitis in calves. They isolated virus from the nasal and ocular secretions of naturally infected animals. By serologic procedures the isolants were identified as IBR virus. They were able by instillation of the virus into the conjunctival sac of both eyes to cause a febrile response, nasal discharge, lacrimation and conjunctivitis. A corneal lesion developed in one calf. They also isolated the virus from nasal washings and ocular secretions of the inoculated calves. The authors report that if keratitis occurs in IBR it is secondary to conjunctivitis, whereas in keratoconjunctivitis it usually occurs first.

McKercher et al. (65) confirmed the findings of Gillespie (30) that the virus causing IBR and IPV are one and the same. However, they state that "critical serological evaluations might possibly reveal minor differences in the nature of strain characteristics, or changes associated with prolonged residence of the virus in different locations in different members of the host species."

Greig (32) detected IBR virus antibodies in the serums of 8.13% of the 1,365 cattle tested. The author states "considering that IBR is not recognized as a clinical disease in the province (Ontario) the results of this test show a surprisingly high percentage of animals with neutralizing titers." The serums having titers of 1:4 to 1:128 represented 18.5% of the herds tested.

Niilo et al. (72) tested 1,000 serums representing 500 herds in Alberta, Canada for antibodies to Padlock and IBR viruses. Of the cattle tested, 22.7% showed antibodies to Padlock virus and 37% to IBR virus. Only 8.2% reacted to both. The occurrence of IBR antibodies is higher than reported from Ontario (32).

Studdert et al. (96) were the first to confirm the occurrence of IBR in Canada. The cattle in the infected herd were first affected with a conjunctivitis. Later a cough, drooling of saliva, and reddening of the muzzle and conjunctiva developed. There was a febrile response in the animals examined. Virus was recovered from the nasal washings and shown to be

IBR by cross-neutralization tests with a known IBR virus and antiserum. The disease was reproduced by inoculating calves intranasally, intrapreputially, and per conjunctiva. Post-inoculation serums from the experimentally infected calves neutralized the isolated agent at a 1:8 dilution as did a specific IBR antiserum obtained from Colorado at the 1:16 dilution.

Dawson et al. (20) made 12 virus isolations from 2 herds of cattle suffering from conjunctivitis and rhinitis. The clinical signs were typical of IBR. One isolate, considered the prototype, was designated "Oxford strain." It was indistinguishable from a Colorado strain by cross-neutralization tests, cytological examination of infected tissue cultures, and transmission experiments.

Darbyshire and Shanks (18) isolated a virus from cattle in Scotland showing typical IBR signs. The isolate caused cytopathic changes typical of IBRV. Cross-neutralization tests showed that the isolate, designated the Aberdeen strain, was closely related to the Colorado and Oxford strains of IBRV.

Dawson and Darbyshire (19) found that 2.1% of 2000 serums collected from Scottish cattle had antibodies against IBRV.

Smith et al. (88) attempted to determine the incidence of IBR and bovine virus diarrhea (BVD) in Massachusetts by conducting serum neutralization tests with random samples of blood serum from cows over 2 years old. Of the 589 serums tested, 12.7% were positive for IBR antibodies and 32 herds

or 18.5% had reactors to the test for IBR antibodies. Only one herd had a clinical history suggestive of IBR based upon a survey of practicing veterinarians. The authors were not able to establish any associations of these viruses with abortions.

French (26) reported that a virus designated N569 isolated from cases of encephalomyelitis, was virtually indistinguishable from IBR virus. The close relationship was based on serologic tests, the biological and physical properties of the viruses, and intranuclear inclusion bodies observed in the tissue culture cells. Animal inoculations with N569 resulted in the reproduction of the encephalomyelitis and in addition a vaginitis was observed.

Johnston et al. (46) reported the transmission of an encephalitis by intracerebral and intravenous inoculations of brain suspensions from natural cases of meningo-encephalitis in calves. The absence of bacteria in the brain and the pathology of the natural and experimental cases suggested that the cause of the disease was a virus. An agent similar to that reported by French (26) was isolated from the brain of one experimentally infected calf. Serologic tests showed that the virus was not a member of the psittacosis-lymphogranuloma group or of group A, B or K arthropod-borne virus.

Barenfus et al. (5) reported the isolation of a cytopathic agent designated as LAC from various tissues of dairy calves suffering from a fatal meningoencephalitis which had

occurred sporadically in the area for several years. The isolation was accomplished in primary tissue culture of BEK cells. Inoculation of a 5-month-old calf resulted in a febrile response, leukopenia, and conjunctivitis. The agent was recovered from several tissues. There was no apparent pathogenicity for adult and suckling mice or guinea pigs.

The isolate was identified as an IBR virus by reciprocal serum-virus neutralization tests. The antiserums used were from hyperimmunized rabbits. By electron microscopic examination, the morphologic and developmental features of the virus were reported to be similar to the herpes virus group.

McKercher (59) gives several reasons why he does not think IBRV is the cause of abortion in cattle. He states that the final answer must await the results of further study. He also suggests that until further critical work is done that IBR vaccination be limited, when practicable, to non-pregnant animals.

Lukas et al. (53) isolated cytopathogenic agents from bovine fetal tissues by methods commonly used for the isolation of IBR virus. Comparative serologic studies by virus and serum neutralization tests, and reciprocal cross-neutralization serum studies, demonstrated the fetal viral isolate, designated as Fresno #2294, to be identical to IBR virus. In addition, they found that the Fresno #2294 isolate could cause infection resulting in abortion, pustular vulvovaginitis, rhinitis and conjunctivitis. These authors report that abortions occurred

under several conditions; (1) in IBR vaccinated as well as unvaccinated herds, (2) herds where there were signs of respiratory disease alone and in conjunction with ocular signs, and (3) herds where there were no clinical manifestations of IBR. Furthermore, that McKercher had reported in a personal communication and in (59) that the injection of an IBR virus, recovered from a bovine respiratory tract, into pregnant cattle did not result in abortion. These field observations and negative findings support the consideration that this isolate may possess infective and antigenic properties not shared by other strains of IBR virus.

Crane et al. (16) reported that IBR has been proved conclusively to be a major cause of abortion in beef cattle in California. The authors suspect that postnatal colibacillosis and prenatal IBR were intimately associated in several "abortion storms." IBR vaccine given after the disease was diagnosed proved to be of only transitory value in preventing abortions. The authors acknowledge that the level of nutrition in beef cattle played "an extremely important part in the prevalence of abortion in any given year in the herds studied."

McKercher and Wada (66) reported the recovery in tissue culture of 6 viral isolates from body organs and fluids of fetuses aborted by dairy heifers between the 6th and 7th month of gestation. These heifers had shown no signs of illness previously, but calves on the same premises were affected several months earlier with a mild respiratory disease. The

herd had not been vaccinated against IBR. Six of 7 heifers inoculated intravenously, intramuscularly, and intranasally with a representative isolate shown to be free of virus diarrhea virus (VD) aborted 18-64 days after inoculation. The virus was isolated from all aborted fetuses. All heifers became febrile and most developed conjunctivitis but only those inoculated intranasally had respiratory illness. This same isolate was shown by cross-immunity and reciprocal serum neutralization tests to be indistinguishable from a known IBR virus and an isolate from aborted fetuses in Ohio.

An IBR isolate was also recovered from fetuses of cattle which aborted following vaccination against IBR. These findings incriminate both field and modified strains of the IBR virus as a cause of abortion. The authors believe that abortion due to this virus is a relatively new syndrome and that there is experimental evidence that suggests the possibility of an enhanced viral invasiveness as the factor responsible for this new manifestation of disease.

Sattar et al. (80) recovered IBR virus from 6 of 28 aborted bovine fetuses from 3 herds of 13 herds that were examined for viruses. A myxovirus, parainfluenza-3 (MP-3), was isolated from 1 fetus in a 4th herd. Of 101 serum samples originating from 17 herds where abortion was a problem, 37 (36.6%) had IBR antibodies.

In 1 herd frank signs of IBR were observed prior to abortion and IBR virus was isolated from 3 aborted fetuses.

In 2 herds there was no history of IBR yet IBR virus was isolated from some aborted fetuses. Significant titers of IBR antibodies were demonstrated in most of the cows tested in one of these herds. Abortions and the isolation of IBR virus from an aborted fetus after vaccination of a herd with IBR vaccine provides further evidence that this vaccine may cause abortion when administered to pregnant cattle.

Owen et al. (74) caused abortions by inoculating heifers in strict isolation with IBRV. The virus was isolated from the foetal tissues and the uterus of the dams. The authors believe that it may be possible to prevent abortion by vaccinating cattle before pregnancy with the IBR vaccine.

Crane (15) in a further report of IBR abortions again emphasizes the importance of adequate nutrition in the prevention of bovine abortions. He says, "Vaccination procedures for brucellosis, IBR, leptospirosis, and bovine virus diarrhea, without attention to proper supplemental feeding of the breeding stock on ranches in this area have not reduced the incidence of abortion, premature calves, and diarrhea of newborn calves to a satisfactory level."

Greig and Bannister (33) infected the quarters of lactating cows with 10^6 to 10^7 $TCID_{50}$ of the bovine herpes (IBR-IPV) virus. In 4 of 7 experiments the inoculated quarters showed marked evidence of the infection and there were profound changes in the physical appearance of the milk. In all cases virus was recovered from the milk of the inoculated quarters

following infection but uninfected quarters remained normal and virus could not be recovered from the milk. One experiment involving 2 animals showed that about 1000 TCID of virus were required to produce infection. One cow having a pre-inoculation serum titer for the IBR-IPV virus proved resistant. The authors state "the experiments indicate that the bovine udder is readily susceptible to bovine herpes virus."

Straub (92) experimentally infected 12 cattle, between 1-1/2 and 2 years old, with IBRV by inoculating 6 of them intracranially and the others into the cerebrospinal duct. Five died during the first 14 days after inoculation, and 2 were killed 3 days postinoculation to study the distribution of the virus. The surviving animals were challenged intranasally with the same virus and the shedding of the virus from the respiratory tract determined. It was also observed that the virus propagated in the central nervous system. The surviving animals had a high level of antibody for the virus.

Fastier and Smith (24) reported the successful isolation of the IBR-IPV virus from naturally occurring cases of bovine rhinitis and vaginitis. They produced hyperimmune serum with 2 of the vaginal and 2 of the nasal isolates. Reciprocal neutralization tests using these 4 isolates and a confirmed IBR virus and their respective antiserums showed a high degree of antigenic similarity between the 5 strains. The confirmed IBR strain, modified using the selection technique of Schwarz et al. (84) was used as a vaccine. Vaccinated cattle were

protected against either nasal or vaginal challenge with virulent virus. The vaginal challenge resulted in the appearance of pustules on the mucosa, swelling and edema of the labiae, and micturition appeared to be accompanied by some pain. There was no definite temperature response, however.

Cheatham and Crandell (11) noted and reported the consistent presence of distinct intranuclear inclusions in tissue culture cells infected with 3 different strains of IBR virus. These inclusions were observed in conjunction with the cytopathogenicity caused by the virus in BEK and human amnion cells. They were also able to demonstrate similar intranuclear inclusions in necropsy material from calves inoculated intranasally with the same 3 strains of IBR. The cells were fixed in Bouin's fluid and necropsy material was fixed in Zenker's and Bouin's fluids. After fixation hematoxylin-and-eosin stained preparations were made.

Tousimis et al. (100) reported that the IBRV particles associated with infectivity have a spherical diameter smaller than 175-211 mμ but equal to or larger than 148-151 mμ as determined by centrifugation. The electron microscopical examination of infectious fluids revealed particles of 136 ± 10.8 mμ in diameter. Similar particles were observed intracellularly in ultrathin sections of the infected human amnion tissue culture cells.

Stevens and Groman (91) reported that the IBRV at 37° C. had a half-life of 10 hours. They found that the inactivation

rate is clearly a first order reaction over the range studied. IBRV was found to be ether sensitive. Other characteristics studied such as serologic, growth rate, and plaque formation offered no contradiction to the suggestion that IBRV be classified as a member of the herpes group.

Orsi and Cabasso (73) observed inclusions in HeLa and primary bovine kidney cells (PBK) infected with IBRV that resembled in form and general staining properties the hematoxylin-and-eosin (H & E) stained and human amnion cells reported by Cheatham et al. (11). The same similarity was demonstrated by use of Feulgen technic. The absence of DNA was invariably demonstrated in the inclusions of both cells and regardless of the staining method used.

Armstrong et al. (2) concluded based upon their electron microscopic studies that the size, morphology, and apparent mode of formation of IBRV support the view that it is closely related to the herpes-virus group. Further evidence of its relation to the herpes group is its ether sensitivity and the visible cytopathic effect in tissue cultures including the appearance of type A intranuclear "inclusions" in the host cells. The authors also cite evidence which suggests that it is a DNA virus. They suggest that since it can cause a central nervous system disturbance that it be considered a bovine member of the herpes virus group.

Grinyer et al. (35) confirmed the findings of Armstrong (2). They could not demonstrate intranuclear inclusion bodies

in IBR or IPV infected cells. No nucleoid structures devoid of outer membranes could be found even after prolonged searching of a large number of nuclei. They state that there is ample evidence of a cycle of development beginning within the nucleus where particles consisting of a nucleoid and a single outer membrane can be demonstrated. In cell cultures receiving heavy inoculums intracytoplasmic particles were numerous and frequently were situated within large, abnormal, cytoplasmic vacuoles.

Stevens and Chow (89) studied the effects of some fixatives on the demonstration of intranuclear inclusion bodies reported by Cheatham and Crandell (11). They found that Bouin's and Zenker's gave the best demonstration of the characteristic inclusion bodies. Modifications of these stock solutions reduced or eliminated entirely the ability to demonstrate the inclusions.

Griffin et al. (34) reported that IBRV is remarkably stable at the pH 6-9 range, that it lost titer rapidly at the lower pH of 5.0 and 4.4, and that it was inactivated promptly by ether, alcohol and acetone. Temperature studies revealed a marked stability at -60° C. and at 4° C. Even at 22° C. no loss in infectivity was noted until after 3 days. At 37° C. titer loss began after 1 day and was complete in 10 days. At 56° C. the virus was inactivated in 22 minutes.

Sabina and Parker (79) developed a plaquing procedure for IBRV in an established bovine kidney cell line. They found

through studies of one-step growth curves that the eclipse period for IBRV lasts approximately 4 hours and that the infectious virus increases at a logarithmic rate for 12-14 hours. Only 1-9% of the total virus is realeased at 24 hours postinfection. The half-life at 37° C. and 42° C. was found to be 16 and 3.5 hours, respectively. Hyperimmune bovine and rabbit serums neutralize 92% of the infective particles within 30 minutes.

Cruickshank and Berry (17) examined the fine structure of the particles of IBRV by the negative staining technic. They found that the virus is identical in fine structure with herpes simplex and that all 4 types of particles seen in herpes virus preparations are seen also in IBR. Other viruses having similar structure are pseudorabies, varicella, infectious laryngotracheitis, and human cytomegalovirus.

Hahnefeld and Hahnefeld (37) reported that the growth, cytopathic effect and plaque formation in calf kidney cell culture of IBR and IPV viruses was inhibited by 5-iodo-2-desoxyuridine. In this respect these viruses resembled Aujeszky's disease virus, which contains desoxyribonucleic acid (DNA) but not ECBO virus which contains ribonucleic acid (RNA). They concluded that IBR and IPV viruses contain DNA which confirms their affinity to the herpes group.

Stevens and Groman (90) reported the inhibition of IBRV by 5-bromodeoxyuridine (BUDR) and 5-fluorodeoxyuridine (FUDR). They state "this strongly suggests that this is a DNA virus."

Their finding that IBRV is able to produce plaques in the presence of an excess of antiserum has also been reported by others as a characteristic of the herpes group. Their findings indicated that the inclusion bodies associated with IBR consist mainly of virus or protein material that has become coagulated and rendered visible by fixation procedures.

Plummer (75) found no cross neutralization among the herpes viruses from different species of animals with the exception of neutralization of herpes simplex by B-virus antiserums. There were a number of cross-complement fixations noted particularly between the non-human herpes viruses. IBRV antiserum fixed complement with equine herpes virus type I to the same titer as with IBRV i.e. 1:16.

Schulze et al. (83) using the electron microscope found no detectable difference between an IBRV and the Jena strain of coital vesicular exanthema virus. Structurally, these viruses resembled viruses of the herpes group.

Schimmelpfennig and Liess (81) studied the development and histochemistry of nuclear inclusions produced by IBRV in bovine testicular cells.

The strain of virus utilized, which had been isolated in Western Germany, produced changes in tissue culture similar to those produced by a variety of virus isolated and studied in America. They reported the formation of amphophile inclusions similar to those observed in the early stages of herpes virus. They concluded that the nuclear inclusions demonstrated by

different methods of fixation represent the effect of the virus.

Straub et al. (94) attempted to distinguish between the IBR and IPV viruses by means of ultracentrifugation and carrier-free zone electrophoresis in glucose density gradient. They were found to have the same sedimentation constants whereas parallel electrophoretic studies showed characteristic and different mobilities of the 2 viruses which would indicate that they are not identical but sub-types of the same virus.

Studdert et al. (97) reported 2 epizootics of vaginitis observed in a herd of cattle in California. Clinically, the lesions were typical of IPV and a virus isolated from the cattle in the 2nd epizootic was shown by serologic and transmission studies to be IPV virus.

Studdert et al. (95) experimentally infected 18 bulls with IPV virus by inoculating the mucosae of the penis and prepuce. The bulls manifested a clinical response and antibodies were detectable 2-weeks postinoculation. The IPV and IBRV both produced nasal lesions in bulls following intranasal inoculation. The lesions observed in the bulls inoculated intrapreputially and intranasally with IPV and IBR virus were indistinguishable. Reciprocal cross-neutralization tests established that the agents were serologically indistinguishable.

Van Kruiningen and Bartholomew (102) diagnosed IBR in a 10-day-old calf which died during an epizootic in a dairy herd.

The diagnosis was based upon lesions observed at necropsy and the intranuclear inclusion bodies observed in the epithelium of the rumen. The cytopathic effect produced by inoculating suspensions of the kidney tissue from the calf into primary BEK cells was typical of IBRV. The isolate was neutralized by known IBR antiserum.

Kennedy and Richards (49) reported the occurrence of focal necrosis in the liver of fetuses and placenta as well as other organs as the consistent lesion characterizing IBRV abortions. The pattern of abortions in 1 herd vaccinated for IBR was: (1) abortions occurred 23-52 days following vaccination (average 36 days), (2) of the 16 cows in 6th, 7th, and 8th month of gestation, 9 aborted (statistically highly significant), and (3) none of the cows in the first 5-1/2 months of gestation aborted.

Chow et al. (12) induced abortion in 2-year-old heifers in the 1st and 3rd trimester of pregnancy by intramuscular inoculations of 5.0 ml. of IBRV. In addition to the abortions which occurred 3-5 weeks after inoculation, typical respiratory and vaginal signs were observed in the heifers immediately following inoculation. The IBRV was isolated from the fetuses and the antibody response of the heifers followed the expected pattern for IBR.

Chow and Davis (12) reported a high incidence of antibody to IBRV in captive deer at the Veterinary Foothills Research Unit of Colorado State University. The virus was isolated

from nasal, lacrimal and rectal swabs of experimentally infected deer. The symptoms seen following inoculation and the antibody response in the experimentally infected deer were similar to that observed in cattle.

PART I. SUSCEPTIBILITY OF GOATS TO INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS

Introduction

The primary objective of these experiments was to find a smaller, less expensive animal that could be used for assaying the immunizing ability of IBR vaccines. The Standard Requirement of the Veterinary Biologics Division (VBD) of the Agricultural Research Service of the U.S. Department of Agriculture for this product is described in the second paragraph of the general introduction.

The testing of the IBR vaccines at the National Animal Disease Laboratory (NADL) has been limited to the determination of the $TCID_{50}$ in a prescribed dose by inoculating embryonic bovine kidney (EBK) tissue culture cells. The vaccine titer (number of $TCID_{50}$) has not been correlated with animal protection tests. One of the objectives of the biologics testing at NADL is to correlate the laboratory tests, i.e., vaccine titer with host animal protection or the immunizing ability of the vaccine. If a smaller less expensive animal could be used for this purpose, it would reduce the cost of the testing and the facilities required for this testing and correlation. Therefore, it was decided to undertake experiments with the objective of finding such a test animal.

Review of Literature

A literature review relative to the susceptibility of animals other than bovines revealed that McKercher et al. (61), Chow et al. (13), Madin et al. (55), McKercher et al. (63),. Armstrong et al. (2), French (26), and Chow and Davis (12) had attempted to infect various animals and chicken embryos with IBRV. As shown in table 1 except for McKercher et al. (63) and Chow and Davis (12) who reported the experimental infection of goats and of deer, respectively, others were unable to establish the infection in species other than the bovine. The goats responded with some elevation in temperature and mild clinical signs. There were no detectable IBRV antibodies in the serums of the goats prior to inoculation. Twenty days after inoculation the titers ranged from 1-7 to 1-26 and 10 days later were slightly higher.

Materials and Methods

Source of viruses

The strains or isolates of IBR virus utilized were received from Dr. D. G. McKercher of California and Dr. T. L. Chow of Colorado. Dr. Chow supplied the Cooper 13th passage level and Dr. McKercher the others. The viruses were propagated and titered on primary embryonic bovine kidney (EBK) tissue culture cells. Titers are expressed as $TCID_{50}$ calculated by method described by Reed and Muench (76). The

Table 1. Species of animals other than bovine tested for IBR susceptibility and inoculation routes

Species	Literature reference	IN	IC	IV	IM	SC	IP	ID
Horses	13,62	-	-	-				
Sheep	13,62	-	-	-				
Rabbits	13,25,62,2[a]	-	-	-	-	-		-
Guinea Pigs	13,25,62,69,55	-	-	-			-	
Hamsters	13	-	-	-				
Mice[b]	13,25,69,55,62	-[c]	-	-			-[d]	
Ferrets	13	-	-	-				
Goats[e]	62	+		+				
Swine	62	-		-				
Chicks	25		-					
Deer	14	Intratracheal inoculations caused typical clinical manifestations						
Chicken embryos	13,25,69,55,62	Yolk sac, allantoic cavity, chorioallantoic membrane, amniotic cavity, inoculations all -						

IN - Intranasally
IC - Intracerebrally
IV - Intravenously
IM - Intramuscularly
SC - Subcutaneously
IP - Intraperitoneally
ID - Intradermally
\- = Attempted without establishing infection

[a]Also inoculated the cornea and testicles of rabbits. Hardening of dermis and testicle was noted but there was no antibody response. Believed to be toxic reaction.

[b]Some mice showed incoordination 12-18 hours after intracerebral inoculation.

[c]1-5 day old and adult mice inoculated.

[d]24 hour old and adult mice inoculated.

[e]Temperature and antibody response with mild clinical signs.

strains used, passage level, and titers are shown in table 2. In addition, 2 goats in the 2nd trial were inoculated with EBK tissue culture fluid from the 2nd passage of the agent recovered from the nasal washing from goat #15 in the 1st trial. The titer of this agent was 10^4 per 0.1 ml.

Inoculation procedures

With the exception of 3 goats in the 2nd trial which were inoculated intracerebrally, all goats and the 1 calf were inoculated intranasally. In the 1st trial 4 goats were also exposed by contact to the inoculated goats. In the first 2 trials, except for the 2 goats which were inoculated with 2 ml. of the agent from the nasal washings of goat #15, 5 ml. of tissue culture fluid containing the virus was inoculated. In the 3rd and 4th trials, 5 ml. of the infected tissue culture fluid was diluted with 5 ml. of stabilizing fluid[1]. The calf in the 3rd trial was inoculated with 10 ml. of the infected culture fluid mixed with 10 ml. of the stabilizing fluid. All intranasal inoculations were made with a DeVilbiss atomizer operating at approximately 15 lb. p.s.i. and the inoculum was sprayed into both nares in equal amounts. The intracerebral inoculations were made by injecting 2 ml. of the undiluted virus-containing tissue culture fluid into the cerebrum after trephining the skull.

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[1]See section on media and fluid.

Table 2. Passage level and titers of viruses used

Trials	Viruses and passage level	Titers[a]
First	LA - 6th	$10^{7.3}$
Second	Cooper - 13th	$10^{6.8}$
Third	LA - 4th	$10^{6.5}$
Fourth	Cooper - 4th, Dixon and Wennermuch - 1st	Cooper $10^{7.3}$ Dixon $10^{5.3}$ Wennermuch $10^{5.5}$

[a]Per 0.2 ml.

Experimental animals

The first 15 goats were purchased in Missouri, were Angora type, males and females, and ranged from approximately 3 months to about 1 year of age. The 2nd group of 8 goats were from Central Iowa, were milk type, males and females and were from 6 to 9 months old. The calf, a Holstein male approximately 4 months old, was produced at NADL. None of the goats were known to have been associated with cattle, and insofar as could be determined, had not been vaccinated for any disease prior to purchase.

Preinoculation procedures

Temperatures were recorded daily for all animals. Blood was collected from all animals and total white cell counts were made at least twice. A serum neutralization test was conducted, with serum from each animal utilizing IBR-LA-6th passage virus and EBK primary cells.

In attempts to isolate virus, the nasal cavity of each animal was flushed with buffered saline solution or stabilizing fluid containing antibiotics[1] and the washings collected in sterile beakers. Following centrifugation of the nasal washings, each of 10 tubes of EBK primary cells were inoculated with .1 ml. of the supernatant fluid.

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[1]See section on media and fluids.

Postinoculation procedures

The temperature of each animal was recorded twice daily.

The animals were observed twice daily for any clinical manifestation of infection. The nares were examined daily with the aid of a flashlight for any evidence of hyperemia or other lesions during the period from 3-7 days after inoculation.

The nasal cavity of each animal was flushed 5, 7 and 9 days after inoculation and the washings collected, and the EBK cells inoculated as described above.

Total white cell counts were made daily usually beginning 4 days after inoculation and continued until 9 days postinoculation. Also white cell differential counts were made as part of the 3rd and 4th trials.

Blood for serum neutralization tests was collected 14, 18, 25 and 32 days after inoculation in the 1st trial, and on the 26th day postinoculation in the 2nd trial. Serum was not collected from the goats in the 3rd trial but was collected 14, 25 and 32 days after inoculation from the calf. Serum was tested from all goats 14 days after inoculation in the 4th trial.

Serum neutralization procedures

The constant virus-decreasing serum method of serum neutralization was used throughout this experiment. The serum was diluted with serum-free tissue culture medium by making

two-fold dilutions up to 1:16. Infected tissue culture fluid containing 100-500 $TCID_{50}$ of virus per 0.1 ml. was mixed with an equal quantity of each undiluted serum and each serum dilution and allowed to stand at room temperature for 30 minutes. Five tubes with a confluent sheet of EBK cells were used for each dilution. Each tube was inoculated with 0.2 ml. of serum-virus mixture. The tubes were incubated at 37° C. and observed for CPE on the 4th and 5th days after inoculation. Five uninoculated tubes served as controls. For a determination of titer of the virus used, 5 tubes of cell cultures were inoculated with 0.2 ml. of each of the ten-fold virus dilutions ranging from 10^{-1} to 10^{-7}.

Preparation of EBK tissue cultures

Primary cells After removing the capsule from a 3-6 month bovine embryonic kidney, the lobes and the cortex between lobes are placed in a 100-mm. petri dish. The renal tissue is scraped into a mincing tube supported in a 150-ml. beaker. The mincing is accomplished by cutting the tissue with a scalpel. The washing, trypsinizing and centrifugation is carried out at 4° C. The minced tissue is put into a sterile powder funnel in the top of a trypsinizing flask and washed with 200 ml. of PBS. The trypsinizing flask is placed on a magnetic stirrer and agitated for 5 minutes to wash away the excess debris, blood, and toxic materials. After the tissue has settled, the supernatant is decanted. The washing is re-

peated 3 or 4 times until the blood and debris appear to be removed, after which 200 ml. of 0.25% (1:300) trypsin solution is added. The trypsinizing flask is placed on a magnetic stirrer and left overnight in a refrigerator. The tissue is agitated in the flask as fast as possible without causing foaming. After the overnight trypsinization, the fluid is decanted and poured into 125-ml. centrifuge tubes through sterile gauze. The fluid is centrifuged at 1,000 r.p.m. for 5 minutes in a refrigerated centrifuge. After decanting the supernatant fluid, the cells are resuspended in 100-150 ml. of PBS and again centrifuged at 600 r.p.m. for 5 minutes. After again decanting and adding PBS, it is centrifuged at 400 r.p.m. for 5 minutes. The packed cell volume is determined and the concentrated cell suspension diluted to 1:150 or 1:200 (number of cells per ml.) with Hanks' medium enriched with 10% specific pathogen free (SPF) calf serum. One ml. of the cell suspension is added to each roller tube and placed in a 37° C. incubator. The medium is removed on the 2nd day and replaced with Earle's medium with 10% SPF calf serum added. When the cell sheet is confluent, usually the 5th day, the medium is removed and replaced with Earle's medium to which 5% calf serum is added.

Secondary cells Primary cells as described above are grown in 32-oz. prescription bottles until confluent. The cells are removed from the glass with versene-trypsin prepara-

tion. After the old medium has been decanted, the suspension of cells is centrifuged at 1000-1200 r.p.m. for 5 minutes and the supernate is decanted after which 5 ml. of medium is added to the packed cells. The cell clumps are dispersed by aspirating and expelling the suspension in a syringe or pipette. Sixty ml. of Hanks' medium with 10% SPF calf serum is added to the suspension. After gentle mixing, 1 ml. of the suspension is dispensed in each roller tube. The medium changes as described for the primary cells are made on the 3rd and 5th day.

Fluids and media

1. Stabilizing fluid

Sucrose	74.621 grams
Monopotassium phosphate	0.517 grams
Dipotassium phosphate	1.254 grams
Monopotassium l-glutamate monohydrate	0.956 grams
Bovine albumin Fraction V (Armour)	10.000 grams
Distilled water	1,000 ml.

Seitz filtered and refrigerated at 4-6 C.

2. Earle's basic salt solution

NaCl	68.00 grams
KCl	4.00 grams
$CaCl_2$	2.00 grams
$MgSO_4.7H_2O$	2.00 grams
$NaH_2PO_4.H_2O$	1.25 grams
Dextrose	10.00 grams
$NaHCO_3$	22.00 grams
Lactalbumin enzymatic hydrolysate	25.00 grams
5% phenol red solution	5.00 ml.
Glass distilled water	10,000 ml.

Sterilized by filtration using Horman filter press

3. Earle's medium

NaCl	68 grams
KCl	4 grams

$CaCl_2$	2 grams
$MgSO4.7H_2O$	2 grams
$NaH_2PO_4.H_2O$	1.25 grams
Glucose or dextrose	10 grams
$NaHCO_3$	22 grams
LAH	50 grams
Phenol red (5% stock culture)	5 ml.
Distilled demineralized water	10 L.

4. Hanks' Medium

NaCl	80 grams
KCl	4 grams
$CaCl_2$	1.4 grams
$MgSO_4.7H_2O$	2 grams
$Na_2HPO_4.2H_2O$	0.6 grams
KH_2PO_4	0.6 grams
Glucose or dextrose	10 grams
$NaHCO_3$	3.5 grams
LAH	25 grams
Phenol red (5% stock solution)	5 ml.
Distilled demineralized water	10 L.

5. "ATV solution"

NaCl	8.0 grams
KCl	.4 grams
Dextrose	1.0 grams
$NaHCO_3$	.58 grams
Trypsin	.5 grams
Versene	.2 grams
q.s.	1,000.0 ml.

6. Eagle's medium

NaCl	6.8 grams
KCl	.4 grams
$NaH_2PO_4.H_2O$	.15 grams
$NaHCO_3$	2.0 grams
$CaCl_2$	.2 grams
$MgCl_2.6H_2O$	.2 grams
Dextrose	1.0 grams
5% phenol red (stock solution)	.5 ml.
Vitamins 100X	10.0 ml.
Amino acids 50X	20.0 ml.
Glutamine (200 mM)	6.0 ml.
q.s.	1,000.0 ml.

Results

The manifestations observed following the exposure of 23 goats and 1 calf to IBRV are summarized in tables 3, 4, 5 and 6. No symptoms suggestive of IBR were observed in any of the goats inoculated intranasally. No inappetence or depression was observed with the exception of 1 goat, No. 9, which was inoculated intracerebrally and displayed some depression on the 6th day after inoculation. The temperatures of 2 of the 3 goats inoculated intracerebrally, No. 9 and No. 13, respectively, went down to 100.8° F. and to 100.0° F. during a 12-hour period on the 6th day. After that they returned to normal except that the temperature of goat No. 13 went down to 100.4° F. on the next day (Chart No. 1). The same goats demonstrated slight roughness of hair coat on the 6th and 7th days and in all 3 a very slight nasal discharge was observed at the same time.

The temperatures of a number of the goats went above 104.0° F. occasionally, but only for 1 or 2 recordings. No clinical manifestations were observed at the time of elevated temperatures. Since the elevations in temperature were of a transitory nature and were not correlated or consistent with the time after inoculation, they were not considered significant. Furthermore, there were proportionately as many recordings above 104.0° F. prior to inoculation as afterward.

The calf inoculated in the 3rd trial, at the same time and with the same virus as the 3 goats, had a temperature rise

Table 3. Summary of first attempt to infect goats with IBRV

Goat #	Virus strain	Exposure route	Preinoculation			Postinoculation								
			Antibody level	Virus isolation	Temp. peak °F.	Temp peak °F.	Clinical response	Virus isolation				Antibody level		
								Days post-inoculation						
								5	7	9	14	25	32	
6	LA-6	Intranasal	-	-	103.6	104.0	-	-	-	-	-	-	-	
12	LA-6	Intranasal	-	-	103.3	104.2	-	-	-	-	-	-	-	
14	LA-6	Intranasal	-	-	103.4	104.0	-	-	-	-	-	-	-	
15	LA-6	Intranasal	-	-	103.8	103.8	-	+	-	-	-	-	-	
8	LA-6	Contact	-	-	103.0	103.8	-	-	-	-	-	-	-	
10	LA-6	Contact	-	-	104.2	103.8	-	-	-	-	-	-	-	
5	LA-6	Contact	-	-	103.3	104.4	-	-	-	-	-	-	-	
3	LA-6	Contact	-	-	104.0	104.4	-	-	-	-	-	-	-	

Table 4. Results of attempts to infect goats with IBRV - second trial

Goat #	Virus strain	Exposure route	Preinoculation			Postinoculation					
			Antibody level	Virus isolation	Temp. peak °F.	Temp. peak °F.	Clinical response	Virus isolation Days post-inoculation 5	7	9	Antibody level 27
9	Cooper 13	Intracerebrally	-	-	105.8	103.5	Slight depression and roughness of hair coat on 6th and 7th day and slight nasal discharge	-	-	-	-
13	Cooper 13	Intracerebrally	-	-	104.6	104.4		-	-	-	-
4	Cooper 13	Intracerebrally	-	-	104.0	104.0	-	-	-	-	-
7	Cooper 13	Intranasal	-	-	104.4	104.2	-	-	-	-	-
2	Cooper 13	Intranasal	-	-	104.0	103.6	-	-	-	-	-
1	Isolated from Goat #15 first trial	Intranasal	-	-	103.6	103.8	-	-	-	-	-
11	Isolated from Goat #15 first trial	Intranasal	-	-	104.6	103.4	-	-	-	-	-

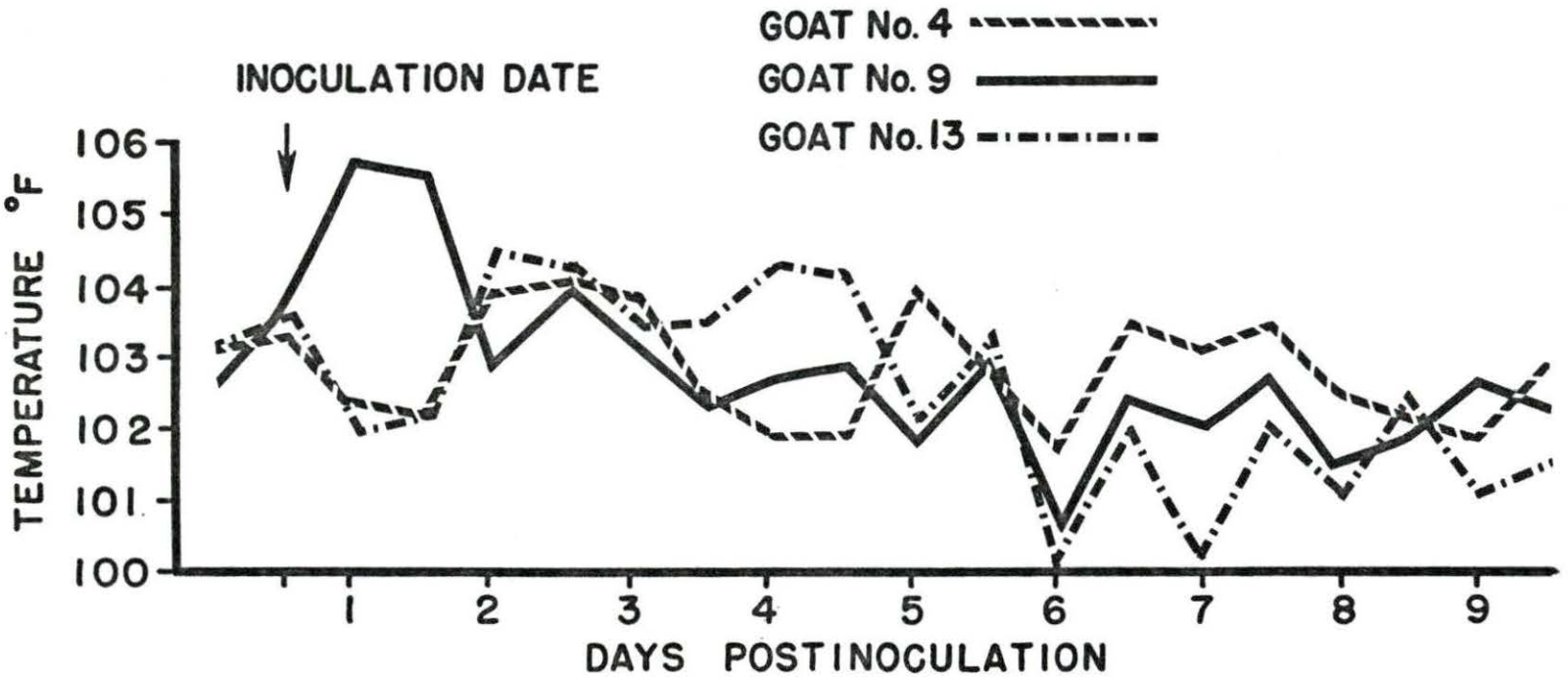
Table 5. Results of attempts to infect goats and a calf with IBRV - third trial

Animals	Virus strain	Exposure route	Preinoculation			Postinoculation								
			Antibody level	Virus isolation	Temp. peak °F.	Temp. peak °F.	Clinical response	Virus isolation			Antibody level		
								Days postinoculation					
								5	7	9	14	25	32
Goat 18	LA-4	Intranasal	-	-	104.0	103.6	-	-	-	-	NC	NC	NC
Goat 20	LA-4	Intranasal	-	-	104.0	103.0	-	-	-	-	NC	NC	NC
Goat 21	LA-4	Intranasal	-	-	103.4	104.2	-	-	-	-	NC	NC	NC
Calf	LA-4	Intranasal	-	-	102.6	106.2	Typical of exp. IBR	+	+	+	1:4	1:8	1:16

Table 6. Results of attempts to infect goats with IBRV - fourth trial

Goat #	Virus strain	Exposure route	Preinoculation			Postinoculation					
			Antibody level	Virus isolation	Temp. peak °F.	Temp. peak °F.	Clinical response	Virus isolation			Antibody level
								Days post-inoculation			
								5	7	9	14
23	Cooper 4	Intranasal	-	-	105.0	104.8	-	+	+	-	-
16	Dixon	Intranasal	-	-	105.8	106.8	-	+	-	-	-
17	Dixon	Intranasal	-	-	104.6	103.2	-	+	+	-	-
19	Wennermuch	Intranasal	-	-	103.6	102.4	-	+	-	-	-
22	Wennermuch	Intranasal	-	-	103.8	103.2	-	+	+	-	-

Chart 1. Temperatures of 3 goats inoculated intracranially with IBRV.


GOAT No. 4
GOAT No. 9
GOAT No. 13
INOCULATION DATE
TEMPERATURE °F
106
105
104
103
102
101
100
1
2
3
4
5
6
7
8
9
DAYS POSTINOCULATION

in 36 hours to 105.2° F. and it remained above 104.0° F. for 4 days (Chart No. 2). Marked clinical symptoms were also observed. Labored, fast respirations, rales, depression, roughness of hair coat, lacrimation and hyperemia of the nasal mucosa was accompanied by serous nasal discharge. The clinical syndrome observed was interpreted as typical of experimentally-induced IBR infection.

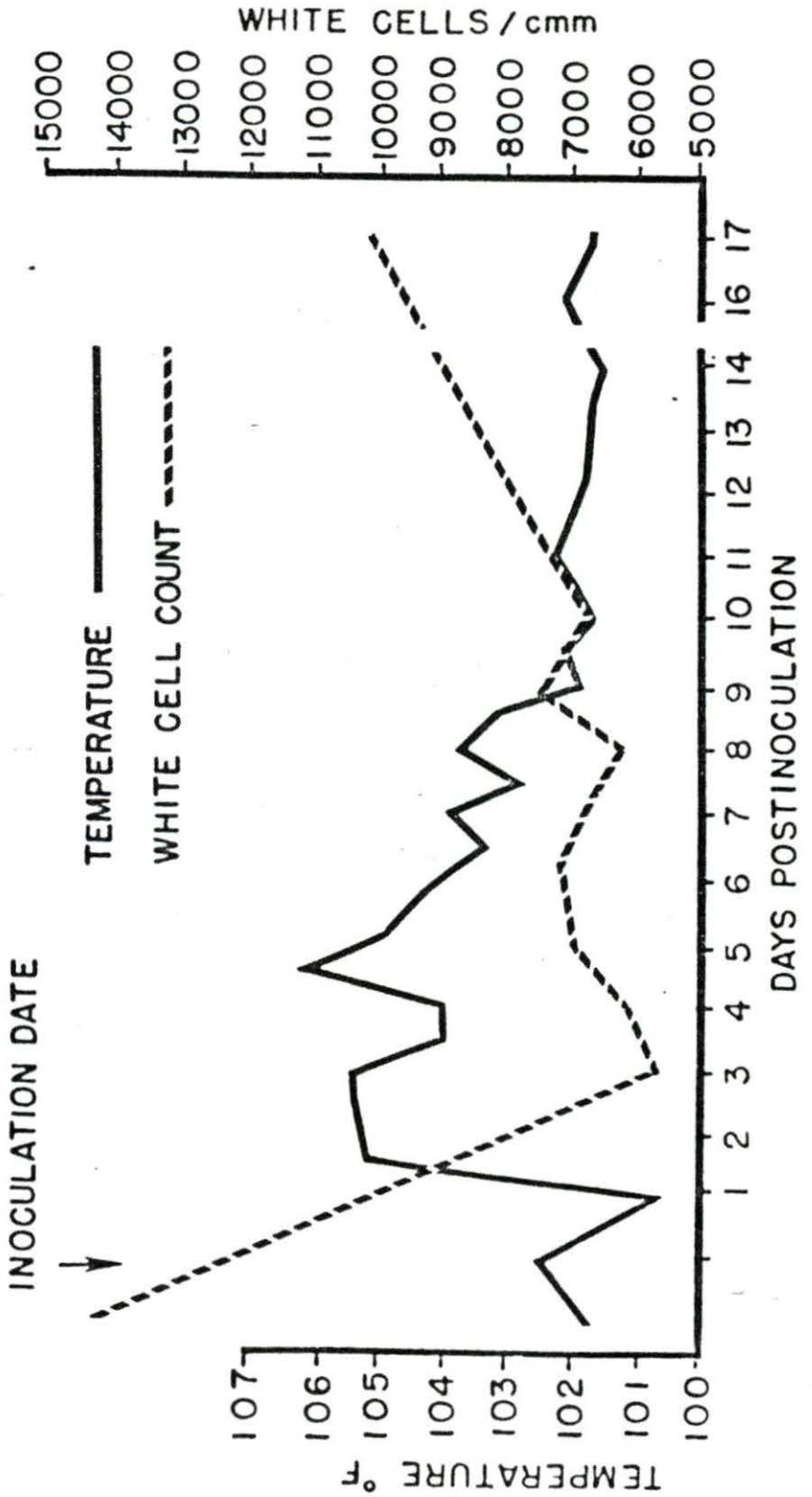
Attempted virus isolations

No agent that produced CPE was isolated from any of the preinoculation nasal washings collected. An agent that produced CPE and which was neutralized by IBR antiserum was recovered from the nasal washings collected from goat No. 15 in the 1st trial, from all 5 goats 5 days postinoculation and from 3 goats 7 days after inoculation in the 4th trial. No agent was isolated from subsequent washings.

Serum neutralization

No antibodies were detected by serum neutralization tests conducted with the preinoculation serums collected from each goat and the calf. No antibodies were detected in the postinoculation serums from any of the goats. The serum collected from the calf on the 14th and 25th day postinoculation neutralized virus at the 1:4 and 1:8 dilutions, respectively. The titer of the serum collected 32 days after inoculation was 1:18.

Chart 2. Temperatures and white cell counts of calf inoculated with IBRV.


WHITE CELLS / cmm
15000
14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
TEMPERATURE
WHITE CELL COUNT
INOCULATION DATE
TEMPERATURE °F
107
106
105
104
103
102
101
100
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
DAYS POSTINOCULATION

Hematology

There was quite a wide range in the total white cell counts of the "normal" goats as indicated by counts made prior to inoculation. Although there were marked variations between goats, there were also wide variations in the same goat from day to day. However, there was no pattern of changes in the counts that could be correlated with the time of inoculation, variations in temperature, or any other clinical observations. The lowest white cell count was 7,000 per cmm. Several counts were in the 8,000-9,000 range and the highest count was 25,000.

The white cell count in the calf showed a marked reduction after inoculation (Chart No. 2). The preinoculation count was 14,400 per cmm. Three days postinoculation it dropped to 5,800 and remained in the 6,000-8,000 range for the next 7 days. By the 16th day postinoculation it had returned to 10,200.

Discussion

Based upon the results reported above, none of the 23 goats utilized in these experiments which were from farms in Iowa and Missouri were susceptible to IBRV. This is in contrast to the report of McKercher et al. (63) that goats were susceptible to IBRV. This provokes the obvious question as to why the difference? Several factors can be considered in discussing this question.

The first might be, had the goats acquired resistance before they were purchased? No IBRV antibodies were detected in serum collected from the goats before or after the inoculations. Therefore, there is no serological evidence of acquired resistance before or as a result of the inoculations. Another question might be asked in regard to the differences in viruses used or methods of inoculation. However, the strains of virus used and the inoculation procedures employed were the same as reported by McKercher et al. (63).

The virulence of the viruses used could also be questioned. The strains of the virus used were acquired from McKercher and Chow and considered by them to be fully virulent. In addition, 2 isolates from field outbreaks of IBR that had not been passaged in the laboratory were included. Also the typical disease syndrome observed in the calf inoculated at the same time and with the same virus as the goats indicates that the virus was virulent. The tissue culture titers were evidence that the virus strains were infective.

The infection in the calf resulted in a titer of 1:18. No antibody was detected in the serum of any of the goats exposed.

However, it is possible to produce antibodies to IBRV in goats by parenteral injections. Dr. C. E. Phillips, NADL, Ames, Iowa (private communication) has produced goat IBRV antiserum by injecting the virus in an adjuvant intra-

muscularly followed by 2 intravenous injections at 6-8 week intervals. The titer of the serum produced in this way was 1:10 against 100-500 $TCID_{50}$ of virus. McKercher et al. (63) on the other hand reported that 20 days after intranasal inoculation the goats had serum titers of 1:6 and 1:26 which increased slightly in another 10 days. It should be noted this was following 1 intranasal inoculation and not after hyper-immunization.

In these trials virus was isolated from the nasal washings of some of the inoculated goats. In considering this it is difficult to explain why these goats did not show any signs of infection. The absence of detectable antibody in the serum of the inoculated goats supports the contention that the goats were not actually infected but were refractory to the virus and did not undergo a sub-clinical or inapparent infection. Possibly the virus survived on the nasal mucosae and had not become established. The fact that no virus was isolated after the 7th day postinoculation in any of the goats also supports this possibility. This, too, is in contrast to the trials reported by McKercher et al. (63) in which the virus was isolated as long as 20 days after inoculation from 1 goat.

The difference in the susceptibility of goats is another aspect to consider. The goats used by McKercher et al. (63) were milk type but both milk type and Angora type goats were utilized in these trials. There could also be a difference due to geographical area of origin of the goats. However, it

does not seem likely that goats from one area would be completely resistant and from another area show marked and consistent response to inoculation.

In a personal communication Dr. McKercher said that he could not explain the differences in the results but that they had discontinued their work with some aspects unresolved.

In reviewing all the variables and possible differences there does not seem to be any completely satisfactory explanation for the differences in the results. Even though there must be an explanation, a comparison of the viruses used, methods employed, and animals tested, does not seem to provide the explanation. There must, therefore, be some more subtle difference which possibly would be revealed only as the result of additional investigations.

These trials did demonstrate that these goats which may be representative of goats from the Midwest were not sufficiently susceptible to IBRV to be satisfactory animals for IBR vaccine testing. This still leaves the bovine as the only satisfactory animal for testing the immunizing ability of the IBR vaccines.

Conclusion and Summary

An attempt to utilize goats for the testing of IBR vaccine was unsuccessful due to the inability to infect goats with IBR

virus by intranasal and intracerebral inoculations and by contact exposure. Nineteen goats were inoculated with 4 strains, 2 at different passage levels, all of which were believed to be fully virulent. Four goats were exposed by contact to those inoculated intranasally. No virus was isolated from the nasal washings collected prior to inoculation. The IBRV was recovered 5 and 7 days postinoculation from the nasal washings of 6 goats but since no antibodies were detected in the blood of any goat, the virus was considered to have survived on the nasal mucosae. No clinical signs of IBR were noted in any of the goats. However, 1 calf inoculated with the same inoculum as a group of goats did respond with symptoms typical of experimentally induced IBR and postinoculation serum from this calf neutralized IBRV at the 1:16 dilution. It is concluded that goats are not sufficiently susceptible to IBRV to serve as test animals for vaccine evaluation.

PART II. ANTIGENIC COMPARISONS

Introduction

Ideally, a virus vaccine should immunize animals against all strains of the virus. Therefore, if there are strains of the virus that are antigenically distinct, they should be incorporated into the vaccine.

Numerous investigators have made antigenic comparisons of IBR viruses isolated from field outbreaks of IBR and IPV. Generally they have not found serologic differences, at least not detectable by serum neutralization (SN) tests utilizing the Beta procedure (constant virus-decreasing serum).

However, tests conducted for the evaluation of IBR vaccinates revealed that there were some apparent antigenic differences in that some viruses were not completely neutralized by IBR antiserum produced in rabbits. Similar observations have been reported by others. It is also reported that certain antiserums from IBR vaccinated calves did not neutralize IBR virus.

As a result of these varying reports an additional study of the antigenicity of the IBR vaccine viruses and viruses isolated from field cases was undertaken. This part is a report of this comprehensive study using the Alpha procedure (constant serum-decreasing virus) for SN testing.

Literature Review

Madin et al. (55) reported that the original IBRV strains isolated in Colorado and California were antigenically identical based upon their reciprocal cross-neutralization tests.

York and Schwarz (105) challenged the immunity produced by 1 IBRV with other isolates and concluded that the 7 strains or isolates compared were of 1 antigenic type.

McKercher and Straub (64) compared an isolate from a range cow with a known IBRV by serologic and cross-protection tests. They reported complete reciprocal cross-neutralization, but stated that the strain isolated from the cow might differ in certain antigenic details from the reference strain.

Gillespie et al. (30) found that the IPV virus immunized cattle against IBRV and vice versa and that postinoculation serums neutralized both viruses. Liess et al. (51) found a virus isolated in Germany to be serologically indistinguishable from an IBR-IPV virus from the United States. McKercher (60) found 3 viruses isolated from cattle affected with Blaschenausschlag and an IBRV to be immunologically homogenous. He found the neutralizing indexes of the antiserums to be essentially equal in cross-neutralization tests and that all antiserum cross-reacted equally in complement-fixation tests with IBRV. McKercher (68,69) and McKercher et al. (65) while confirming the results of others relative to the complete reciprocal cross-neutralization of the IBR and IPV antiserums

stated that critical serological evaluations might possibly reveal minor strain differences. Abinanti and Plumer (1) reported cross neutralization of a virus isolated from a herd affected with conjunctivitis by the antiserum from the herd and by a known IBRV antiserum.

Studdert et al. (96) in Canada, Dawson et al. (20) in England and Darbyshire and Shanks (18) in Scotland isolated viruses from cattle which on the basis of cross-neutralization tests were found to be closely related to a Colorado strain of IBRV. French (25) and Barenfus et al. (5) found virus isolates from calves suffering from encephalomyelitis and meningoencephalitis, respectively, to be serologically indistinguishable from IBRV. Lukas et al. (53) and McKercher and Wada (66) found that viruses isolated from aborted bovine fetuses and a known IBRV were on the basis of reciprocal serum neutralization tests indistinguishable.

Straub et al. (94) using ultracentrifugation and a glucose density gradient determined that IBRV and IPV virus had the same sedimentation constants. Electrophoretic studies revealed that the viruses had characteristic and different mobilities and therefore are not identical but represent subtypes of the same virus group.

Maré and van Rensburg (56) found that 6 virus isolates from herds having outbreaks of "epivag" and vaginitis, which were grouped serologically and on the basis of the intranuclear inclusions produced, were serologically identical with

IPV virus and IBRV.

Segre (85) compared 6 strains of virus by hyperimmunizing rabbits and checking the antiserums against the homologous and heterologous virus strains by cross-neutralization tests. Using the Beta method of serum neutralization he found marked antigenic differences between the strains compared. He stated that more antigenic relationship might have been shown if a higher titer antiserum and smaller amounts of virus had been used.

Dr. Phillips was unable to completely neutralize 1 IBRV with rabbit antiserum produced from another strain of IBRV. Dr. T. L. Chow, Colorado State University, Ft. Collins, Colorado (private communication) found that rabbit antiserums produced in his laboratory did not completely neutralize the 3 strains of virus sent to him by Dr. Phillips. Dr. D. G. McKercher, University of California, Davis, California (private communication) was not able to corroborate Dr. Chow's and Dr. Phillips' results using rabbit and bovine antiserums. He suggested, based upon his experience with rabbit serums, that the apparent antigenic differences might be due to nonspecific neutralization by rabbit antiserums.

Dr. Phillips tested antiserums from calves that had been vaccinated with several serials or lots of commercially-

produced vaccine, and observed that at the 1:2 dilution, the serum of some calves did not neutralize a Cooper strain of IBRV. With each vaccine tested there were individual animals with little or no detectable antibody. One vaccine produced no detectable antibody at the 1:2 dilution of the serum in any of the calves vaccinated. Another vaccine produced detectable antibody in only 3 of the 6 calves vaccinated and tested.

The testing referred to above using the Beta procedure of SN revealed that the vaccination of calves with IBR vaccine results in antiserums that neutralize virus at low dilutions, usually not higher than 1:16. Most often the titers of the serums are only 1:4 to 1:8. With such low titers it was doubtful if minor antigenic differences would be detected using the Beta procedure.

Langer and McEntee (50), McKercher (60) and Gillespie et al. (28) had all used the Alpha (constant serum-decreasing virus) serum neutralization procedures for making antigenic comparisons of bovine viral isolates. Mascoli and Burrell (57) in a discussion of Alpha and Beta procedures of serum neutralization stated that the Beta procedure is less precise and has other disadvantages. Specifically mentioned is the difficulty encountered in achieving an equal amount of virus in each test and how the results are not comparable when the amount of virus varies from test to test.

Materials and Methods

Serums

Each licensed producer of IBR vaccine was requested to send serum collected from calves before and after they were vaccinated with a specified serial of vaccine.

An antiserum designated as NADL^{+} that had been collected from the calf inoculated in the 3rd trial of Part I was used throughout as the standard antiserum or as a positive control in all SN testing and a known negative serum from a NADL calf was used as a negative control serum.

Viruses

(1) LA-6, (2) Cooper 15, (3) PM 89 a high passage Cooper strain, (4) isolates from field cases of conjunctivitis designated Hiemstra-6th, 79th and 21st tissue culture passage (TCP), Keo-2, 54th TCP and ISU 239, 15th TCP, (5) isolates from New York, one an IBRV designated as Cornell I, and another IPV isolate designated as Cornell K-22, (6) a virus isolated from aborted fetuses in Ohio, (7) 2 isolates, Dixon and Wennermuch, from field cases of IBR in California, and (8) each licensee was instructed to submit the virus used for conducting SN tests for antibody measurement unless the same virus as used for vaccine production was used.

Tissue cultures and media

Primary and secondary EBK tissue cultures and media pre-

pared as described in Part I were used for SN testing.

Serum neutralization procedures

Calf serums were inactivated by heating to 56° C. for 30 minutes.

The viruses used were diluted from 10^{-1} through 10^{-7}. To each tube containing 0.6 ml. of the positive and postvaccination serums, 0.6 ml. of the virus dilutions 10^{-1} through 10^{-4} was added. To each tube containing 0.6 ml. of negative and prevaccination serum, 0.6 ml. of 10^{-4} to 10^{-7} virus dilution was added. The serum-virus mixtures were held at 37° C. for 1 hour. After this, each of 5 tubes of EBK cells was inoculated with 0.1 ml. of each of the virus-serum mixtures. The inoculated tubes were incubated at 37° C. and observed for CPE after 5 days and again at 6 days. The neutralization index (NI) was calculated by subtracting the titer of the positive and postinoculation serums from that of the negative and prevaccination serums.

Each serum was tested with the same 3 viruses, LA-6, Cooper 15, and PM 89 as well as the virus used by the licensee for SN testing. The nonvaccine field isolates were tested using the NADL^{+} and negative control serums and selected serums and selected serums from calves vaccinated by licensees.

Results

The results of the SN testing with the 14 serums and 22

viruses are summarized in the following graphs and tables.

Graph #1 summarizes the neutralization indexes of the NADL$^+$ serum against the LA-6 virus which was used routinely as the control serum.

Graph #1 shows that there was a high degree of consistency in regard to the NI of the NADL$^+$ serum against the LA-6 virus and the titer of the virus. The standard deviation (s) from the mean for the virus titer is 0.355. All but one of the titer endpoints is less than 2 s from the mean and only 3 are more than 1 s from the mean.

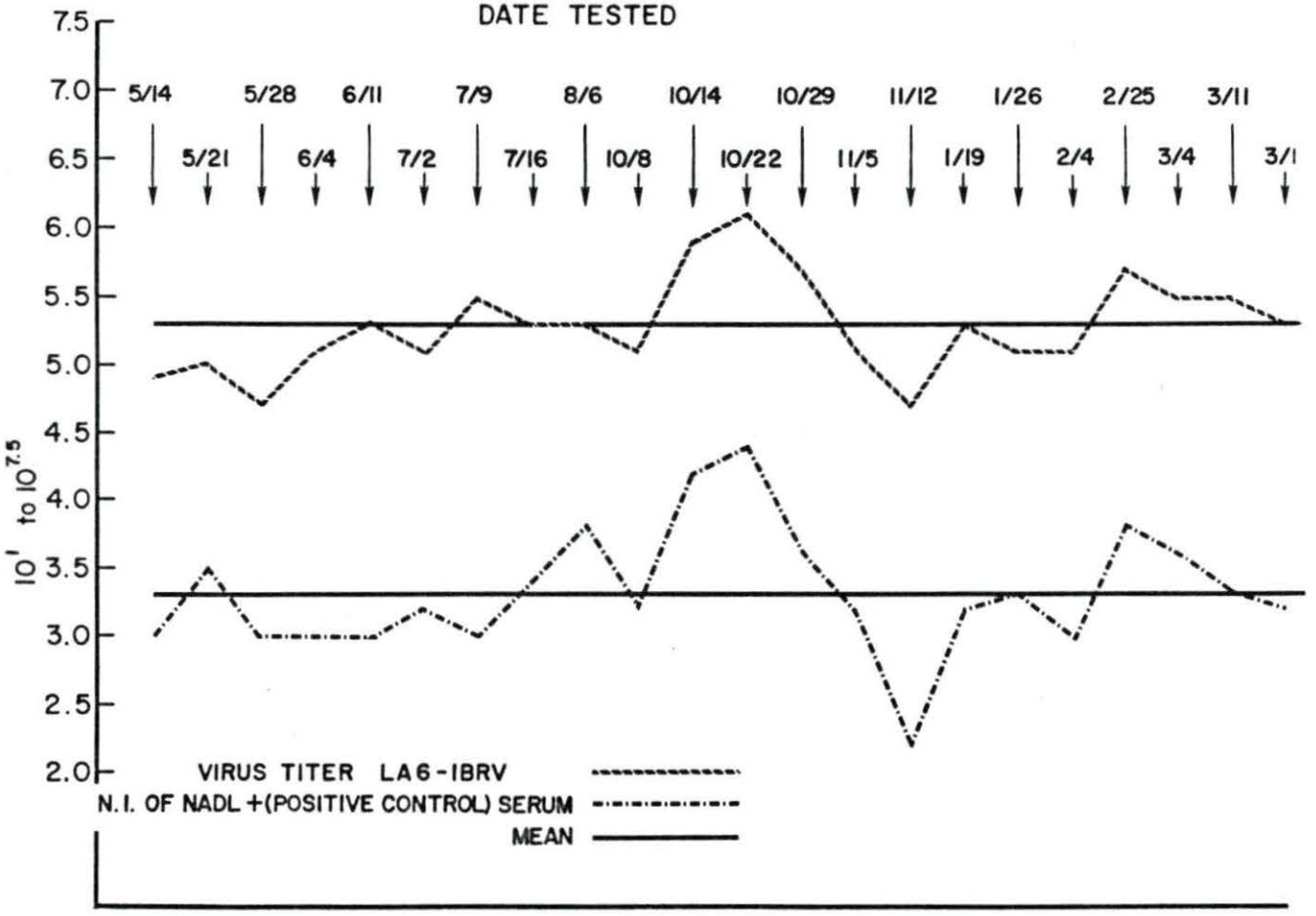
The s for the NI is 0.465. Of the 22 NI determinations, only 1 falls outside of 2 s and only 2 others are more than 1 s from the mean. These variations from the mean are well within the normal distribution pattern.

This graph also shows that there is a correlation between the virus titer and NI of a serum on a given date. The correlation coefficient between the two is 0.82. Further calculations reveal that 67.2% of the variability in one is expressed in the other, or that 67.2% of the variability of the NI is reflected by or accounted for by the virus titer.

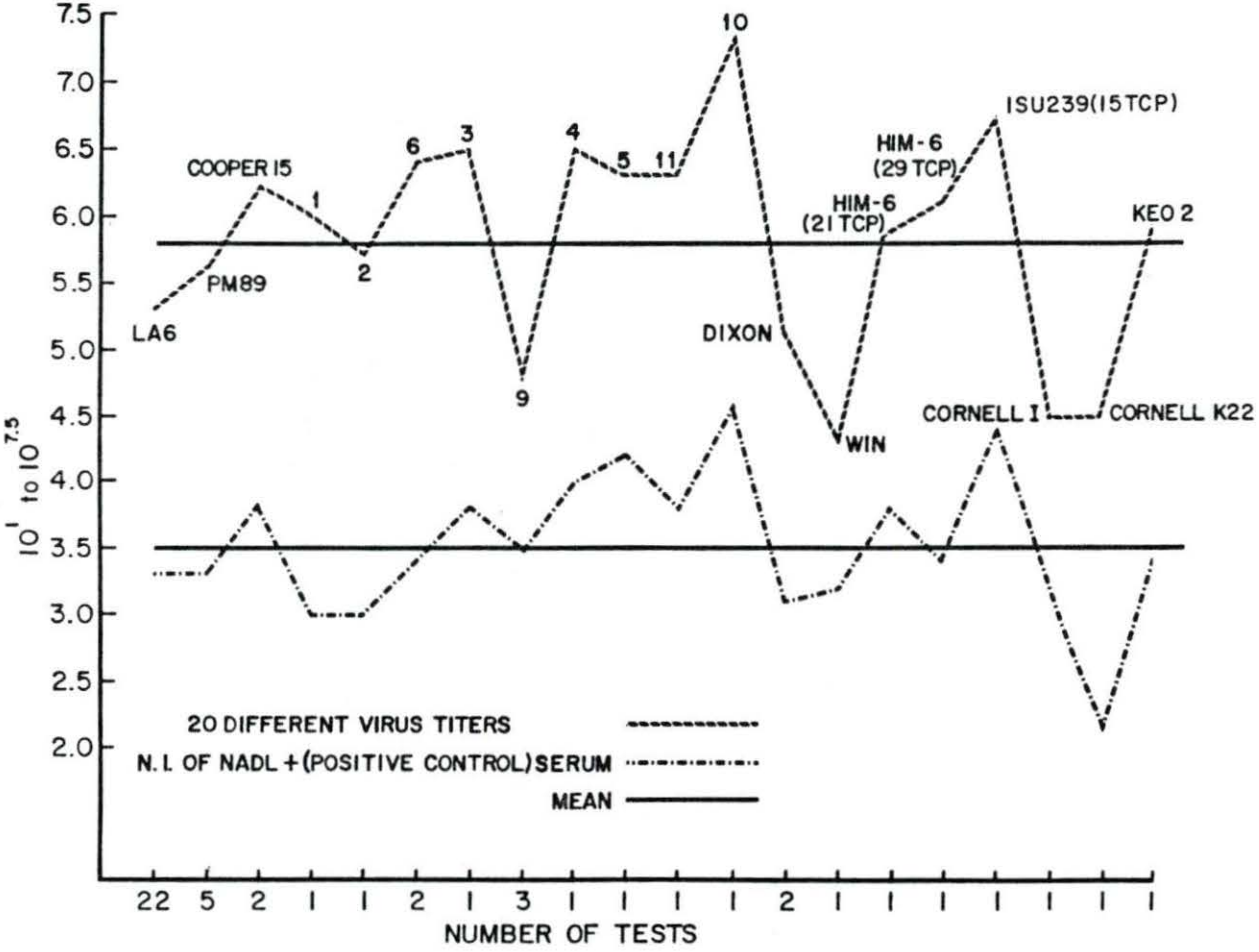
Graph #2 summarizes the NI of the NADL$^+$ serum against the 20 viruses tested. When a virus was tested more than once against this serum, the NI is the average of all tests conducted with a virus and this serum.

This graph also reflects the correlations between virus titer and NI shown in graph 1.

Graph 1. Repeated titrations and neutralizing indexes using LA-6 virus against NADL^{+} serum.


DATE TESTED
5/14
5/21
5/28
6/4
6/11
7/2
7/9
7/16
8/6
10/8
10/14
10/22
10/29
11/5
11/12
1/19
1/26
2/4
2/25
3/4
3/11
3/1
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
10^{1} to $10^{7.5}$
VIRUS TITER LA6-IBRV
N.I. OF NADL +(POSITIVE CONTROL) SERUM
MEAN

Graph 2. The virus titers of 20 IBR viruses and the NI of the NADL^{+} serum when tested against each virus. Numbers 1-11 refer to coded number of licencees.


7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
10^1 to $10^{7.5}$
LA6
PM89
COOPER 15
1
2
6
3
9
4
5
11
10
DIXON
WIN
HIM-6 (21 TCP)
HIM-6 (29 TCP)
ISU239(15TCP)
KEO 2
CORNELL I
CORNELL K22
20 DIFFERENT VIRUS TITERS
N.I. OF NADL + (POSITIVE CONTROL) SERUM
MEAN
22 5 2 1 1 2 1 3 1 1 1 1 2 1 1 1 1 1 1 1
NUMBER OF TESTS

The NI of this serum against these viruses is also consistent and grouped closely to the mean of 3.5. The results are within the normal distribution in that 13 out of 20 are within 1 s of the mean, 5 are less than 2 s and the NI against K-22 virus and the virus from licensee #10 are the only ones more than 2 s from the mean but neither of them are 3 s from the mean. One might expect in a normal distribution pattern to have 1 out of 3 of the observations to be outside of 1 s, and 1 out of 20 to be more than 2 s from the mean.

The correlation coefficient of the virus titer and NI in graph 2 is 0.75 which means that 56.3% of the variability in the NI may be reflected by the virus titer. Therefore, taking into account the correlation shown between the virus titer and NI in graph 1 and graph 2, it is highly unlikely that there is any significant difference between the NI of the $NADL^{+}$ serum against any of these viruses.

Tables 7 through 15 show the results of all of the testing of the serums with each virus that it was tested against and the dates when the tests were conducted. This "detailed" information is included for the purpose of providing a complete report of all the tests conducted and they are summarized in tables 16 and 17.

The NI of a known negative serum included in each test is not reported since it served only as a negative control. When a serum submitted by a licensee was from a calf, which had a NI prior to vaccination, the results of the NI testing with

these serums were not included. The known positive serum designated as NADL+ was always included in the tests conducted on each date and served as the positive control in the system. The results of these tests serve as a basis for comparison for the other serums. The homologous virus is the vaccine virus used to produce the serums except for licensees 1, 3, 5, and 11 where the virus used by them for their SN tests was furnished and utilized in these SN tests.

Table 7 shows the NI of serum #1 and NADL+ against 6 viruses. There is no significant difference between the NI of serum #1 against any of the viruses tested. It has a titer that is comparable to the NADL+ serum.

Table 7. Neutralization indexes of serum from licensee #1 tested with 6 IBR viruses

Viruses	Dates tested						Average[a]	
	5-14		5-21		10-14			
	#1	NADL+	#1	NADL+	#1	NADL+	#1	NADL+
Homologous	...	...	3.2	3.0	...	...	3.2	3.0
LA-6	2.8	3.0	4.2	3.5	3.3	4.2	3.4	3.6
PM 89	3.0	3.0	2.5	2.7	...	...	2.75	2.85
Cooper 15	...	...	4.0	3.5	...	...	4.0	3.5
Wennermuch	...	...	...	...	3.5	3.2	3.5	3.2
Dixon	...	...	...	...	3.6	3.0	3.6	3.0

[a]When more than 1 test has been conducted with a serum and virus the average is shown, otherwise the result of the single test is shown.

In table 8 the NI of serum #2 is shown to be 0.4 and 0.8 against LA-6 virus. Although the titer of this serum is low for all viruses, this seems to be unusually low, and is about 1 log below the average for this serum against the 4 viruses with which it was tested. However, the virus titer for LA-6 was also low, $10^{4.7}$, on this date. The other serums, #4 and #11, do not show any appreciable difference in their ability to neutralize these viruses.

Table 8. Neutralization indexes of 3 serums tested with 4 viruses

Viruses	Dates tested and serums[a]							
	5-27		6-3		7-2		10-8	
	#2	NADL+	#2	NADL+	#4	NADL+	#11	NADL+
Homologous	2.0	3.0	...	...	2.8	4.0	3.0	3.8
LA-6	0.4	3.0	0.8	3.4	2.2	3.2	2.2	3.2
PM 89	2.0	...	1.8	...	2.6	...	2.8	...
Cooper 15	1.4	...	...	...	3.0	...	2.0	...
Avg.	1.45		Avg. 2.65		Avg. 2.65		Avg. 2.5	

[a]Serum number refers to coded numbers of licensees.

Table 9 shows the NI of serum #7 against 4 viruses and the average NI of this serum for the 5 tests conducted with it (2 tests against Cooper 15 virus). While the titer of this serum is lower than the NADL+ serum, it does not seem to vary significantly with the different viruses tested.

Table 9. Neutralization index of serum from licensee #7

Viruses	Dates tested and serums 3-4 #7	3-4 NADL+	3-11 #7	Average[a] #7
Homologous	2.7	...	...	1.98
LA-6	1.8	3.6	...	...
PM 89	2.1	...	...	...
Cooper 15	1.6	...	1.7	...

[a]Average of all tests with serum #7.

Serum #10 (table 10) tested on 11-12 had a low NI for the LA-6 and Cooper 15 viruses. However, on retest the results

Table 10. Neutralization indexes of serum from licensee #10 tested with 4 viruses

Viruses	Dates tested and serums 11-12 #10	11-12 NADL+	1-19 #10	1-19 NADL+	2-4 #10	2-4 NADL+	Average[a] #10	Average[a] NADL+
Homologous	...	...	4.2	4.6	...	...	4.2	4.6
LA-6	1.2	2.2	...	3.2	2.4	3.0	1.8	2.8
PM 89	...	...	3.2	3.6	...	...	3.2	3.6
Cooper 15	1.8	...	...	4.2	2.4	...	2.1	4.2
						Avg.	2.8	3.8

[a]When more than 1 test has been conducted with the serum and virus, the average is shown. Otherwise the result of the single test is shown.

were quite near the average for all viruses. It should be pointed out that the virus titer of the LA-6 virus on this date was $10^{4.7}$.

Serum #8, as shown in table 11, does not seem to neutralize LA-6 or PM 89 viruses as well as it does the homologous virus and Cooper 15. The virus titer of the LA-6 and PM 89 viruses was low, $10^{4.6}$ on the same date. This would indicate that the low virus titer is not the only reason why the NI is low for these 2 viruses. The NI of the serum against the homologous virus was 2.0 while it was only 0.4 and 0.8 against the LA-6 and PM 89 viruses, respectively. The average NI of 2.35 for serum #8 against the homologous virus is 2 logs higher than the average against LA-6 virus.

Table 11. Neutralization indexes of serum from licensee #8 tested with 4 viruses

Viruses	Dates tested and serums				Average[a]	
	3-18		6-1			
	#8	NADL+	#8	NADL+	#8	NADL+
Homologous	2.7	...	2.0	...	2.35	...
LA-6	0.15	3.2	0.40	2.2	0.33	2.7
PM 89	...	...	0.8	...	...	...
Cooper 15	...	...	1.7	...	...	...

[a]Average of more than 1 test when conducted. Otherwise the result of the single test is shown.

Table 12 shows the NI of serum #5 with 7 viruses. The NI of serum #5 is practically the same for all the viruses except K-22. The NI against this virus was about 1 log lower than the average titer of the serum against all the viruses tested. The titer of this virus was $10^{4.5}$ on this date and the NI of the NADL+ against this virus was 2.2 on 11-12 which was the lowest titer recorded for this serum against any of the viruses with which it was tested.

Table 12. Neutralization indexes of serum from licensee #5 tested with 7 viruses

Viruses	Dates tested and serums				Average[a]	
	7-16		2-25			
	#5	NADL+	#5	NADL+	#5	NADL+
Homologous	3.8	4.2	...	...	3.8	4.2
LA-6	3.0	3.4	3.3	3.8	3.15	3.6
PM 89	3.4	...	...	...	3.4	...
Cooper 15	3.6	...	...	...	3.6	...
Ohio	...	...	3.2	...	3.2	...
Himstra-21	...	...	3.4	...	3.4	...
Cornell K-22	...	...	2.3	2.2	2.3	...
	Avg. 3.45		Avg. 3.05		Avg. 3.26	

[a]Average of more than 1 test when conducted. Otherwise the result of the single test is shown.

Serum #3 was tested against 6 viruses, table 13, and the titer did not seem to be significantly different with viruses

that it was tested against. The NI against the Dixon and Wennermuch viruses was somewhat lower but these viruses had low titers $10^{5.1}$ and $10^{4.3}$, respectively, which may partly account for lower titers against these viruses.

Table 13. Neutralization indexes of serum from licensee #3 tested with 6 viruses

Viruses	Dates tested and serums 6-11 #3	6-11 NADL+	10-14 #3	10-14 NADL+	Average[a] #3	Average[a] NADL+
Homologous	3.0	3.8	...	...	3.0	3.8
LA-6	2.0	3.0	2.2	4.2	2.1	3.6
PM 89	2.6	...	...	...	2.6	...
Cooper 15	2.8	...	...	...	2.8	...
Wennermuch	...	...	1.4	...	1.4	...
Dixon	...	...	1.6	...	1.6	...
	Avg. 2.6		Avg. 1.7		Avg. 2.25	

[a]Average refers to more than 1 test. Otherwise the result of the single test is shown.

Serum #9 was tested against 4 viruses as shown in table 14. The ability of this serum to neutralize the LA-6 and Cooper 15 viruses seems to be significantly less than its neutralizing ability for the homologous virus and the PM 89 virus. The average NI of 4 tests against LA-6 is 0.6 and the average NI of 4 tests against Cooper is <1.0. Two tests were not carried to the endpoint of this serum against Cooper 15

but these results are included to validate the conclusion that this serum had very little neutralizing ability for this virus. The average NI for 2 tests against the homologous virus and the PM 89 virus was 2.7 and 2.3, respectively. The low titer of this serum against these viruses cannot be attributed to low virus titers since the virus titers of LA-6 and Cooper 15 were higher than was the titer of the homologous virus.

Table 14. Neutralization indexes of serum from licensee #9 tested against 4 viruses

Viruses	Dates tested and serums								Average[a]	
	10-22		10-29		1-19	1-26		6-1		
	#9	NADL+	#9	NADL+	#9	#9	NADL+	#9	#9	NADL+
Homologous	2.2	...	...	...	3.2	...	...	...	2.7	...
LA-6	...	4.4	1.0	3.6	0.4	0.7	3.3	0.2	0.6	3.4
PM 89	2.0	...	...	...	2.6	...	...	...	2.3	...
Cooper 15	...	...	1.0	...	0.8	<1.7	...	<1.0	<1.0	...

[a]Average refers to more than 1 test of a serum with a virus. Otherwise the results of the single test is shown.

Serum #6 was tested against 11 different viruses as shown in tables 15 and 16. Serum was collected 14 and 21 days after vaccination by this licensee and the titer of the 14-day serum was lower than that collected 21 days after vaccination. The titers of serum #6 against Cornell I, K-22 and Himstra 6-79 viruses were slightly lower but these viruses also had low titers which may account for the lower NI against this virus.

The other variations in serum titers are considered to be well within the normally expected range of variation.

Table 15. Neutralization indexes of serum from licensee #6 tested against 11 viruses

Viruses	Dates tested and serums							
	6-4		8-6		11-5		2-25	
	#6[a]	NADL[+]	#6[b]	NADL[+]	#6	NADL[+]	#6	NADL[+]
Homologous	2.0	2.8	3.8	4.0	...	...	...	...
							2.0[b]	
LA-6	...	3.0	2.8	3.2	2.6	3.2	3.2	3.8
PM 89	2.8	...	3.0	...	...	...	...	...
Cooper 15	1.6	...	3.4	...	...	...	...	...
Dixon	...	...	...	...	2.6	3.2	...	...
Himstra 6-21	...	...	...	...	...	...	3.0	...
Himstra 6-79	...	...	...	...	2.2	3.4	...	...
Cornell K-22	...	...	...	...	...	...	2.3	...
Cornell I	...	...	...	...	2.4	3.2	...	...
ISU-239	...	...	...	...	3.0	4.4	...	...
Ohio	...	...	...	...	...	...	3.0	...
	Avg. 2.1		Avg. 3.25		Avg. 2.56		Avg. 2.87[c]	

[a]Collected 14 days after vaccination.

[b]Collected 21 days after vaccination.

[c]Average of 21 day serum only.

Table 16 which summarizes the testing of all of the serums against all of the homologous viruses and viruses LA-6, PM 89,

Table 16. Summary of neutralization indexes[a] for all serums tested against the homologous and 3 selected viruses

Viruses	Titer	NADL+ serum	Coded no. of serums from licensees												Average[d]
			1	2	3	4	5	6[b]	6[c]	7	8	9	10	11	
Homologous	...	...	3.2[e]	2.0	3.0[e]	2.8	3.8[e]	2.0	3.8	2.7	2.3	2.7	4.2	3.0[e]	...
LA-6	5.3	3.3	3.4	0.6	2.1	2.2	3.2	2.0	2.9	1.8	0.3	0.6	1.6	2.2	2.4
PM 89	5.6	3.3	2.8	2.0	2.6	2.6	3.4	2.8	3.0	2.1	0.8	2.3	3.2	2.8	2.8
Cooper 15	6.2	3.8	4.0	1.4	2.8	3.0	3.6	1.6	3.4	1.6	1.7	<1.0	2.1	2.0	2.6

[a]When tested more than once, the average is reported.

[b]14-day bleeding.

[c]21-day bleeding.

[d]Average neutralization indexes except NADL+ and significantly lower serums against the 3 viruses.

[e]Virus used by company for SN testing--not vaccine virus.

and Cooper 15 again brings out the apparent significant antigenic differences detected. Attention is directed to serum #2 against LA-6 virus, serum #8 against LA-6 and PM 89 viruses, and serum #9 against the LA-6 and Cooper 15 viruses. If the NI of each of these serums against these viruses is compared to the average NI of all the other serums against these viruses, it would appear that the neutralizing ability of these serums is significantly less for these viruses than for the other viruses tested. For example, the NI of serums #2, #8, and #9 against LA-6 was 0.6, 0.3, and 0.6, respectively, whereas the average NI of all the other serums against this virus was 2.4. Likewise, the NI of serum #8 was 0.8 against PM 89 as compared to the average NI of all the other serums against this virus of 2.8. Also, the NI of serum #9 against Cooper 15 virus was less than 1.0 but the average NI of all other serums against this virus was 2.6. In many instances the serums were tested against these viruses more than once. This supports the significance of these results. In the case of serum #9, for example, the NI reported for LA-6 and Cooper 15 viruses was based on the average of 4 tests.

The variability of the NI of #2, #8, and #9 serums cannot be explained on the basis of lower virus titers. The titers of these viruses, LA-6, PM 89, and Cooper 15 were not generally low and were not unusually low on the dates when the lower neutralizing indexes were observed.

A review of table 16 will also reveal that none of the other serums demonstrated this degree of variability in regard to their ability to neutralize the viruses tested. These 3 serums, #2, #8, and #9 did not vary this much in their ability to neutralize other viruses.

Table 17 summarizes the results of testing several serums received from licensees and the NADL+ serum against viruses isolated from field cases of typical IBR, abortions, con-

Table 17. Summary of neutralization indexes[a] for the serums tested against IBRV isolates

Viruses	Titer	NADL+ serum	Coded no. of serums from licensees			
			6[b]	1	3	5
Wennermuch	4.3	3.2	...	3.5	1.4	...
Dixon	5.1	3.1	2.6	3.6	1.6	...
Ohio	5.8	2.4	3.0	...	...	3.2
Himstra 6-21	5.9	3.8	3.0	...	...	3.4
Himstra 6-79	6.1	3.4	2.2	...	...	...
Cornell I	4.5	3.2	2.4	...	...	...
Cornell K-22	4.6	2.2	2.3	...	...	2.3
Keo 2	5.9	3.4	...	...	...	...
ISU 239	6.7	4.4	3.0	...	...	...

[a]When tested more than once, the average is reported.

[b]21-day bleeding.

junctivitis, and IPV. Serum #3 has a low titer for 2 viruses but the titer of this serum for all viruses was not high (see

table 13). It is doubtful that the slightly lower NI for these 2 viruses is significant. All of the other neutralization indexes seem to be within the range of normal variation.

Discussion

An examination of the results of the comparative SN testing of the serums with the viruses which are recorded in tables 7-15 reveals some antigenic differences that are believed to be significant. These most significant differences were noted in only 2 of the 12 serums, #8 and #9, when they were tested against 3 laboratory viruses. Other less significant differences were noted.

In table 8 the NI for serum #2 is low against LA-6 virus, but this serum had a low titer against all viruses. The lower NI against LA-6 may be partly accounted for by the low virus titer but the NI against another virus, PM 89, was higher even though the virus titer was slightly lower. Therefore, the low NI, 1 to 1-1/2 logs below the titer for other viruses, confirmed by 2 tests of serum #2 against LA-6 is believed to be of at least some significance.

Serum #10 had a low NI for LA-6 and Cooper 15 viruses on the first test but on a subsequent test the NI was more nearly the average for this serum. The low virus titer for LA-6 on the date of the first test may be reflected in the lower serum titer. Furthermore, the lowest NI for other serums against this virus were recorded when the virus had a low

titer, therefore, less significance is attached to this low titer of #10 serum

The low NI for serum #8 against LA-6 as shown in table 11 is considered to be a significant difference. Especially since the average NI for 2 tests of this serum was 0.33 against the LA-6 virus which is 2 logs lower than the average NI against the homologous virus. Serum #8 was tested once against PM 89 virus, and it had an NI of 0.8 and this may be significantly lower than the NI against the homologous virus. But since it was not repeated, it is more difficult to assess its significance.

The slightly lower NI of serum #5 against the Cornell K-22 virus as shown in table 12 is believed to be of little significance. The titer of this virus was low on this date and the NI of the $NADL^{+}$ serum was also low on this date against this virus. Since the $NADL^{+}$ serum usually had a higher NI but was as low as the serum #5 on this date, this slight difference in serum #5 can probably be accounted for by the low virus titer of Cornell K-22.

Serum #9 is believed to have a significantly lower titer against the LA-6 and Cooper 15 viruses. The reasons are given in the discussion accompanying table 14. The low NI for this serum with these viruses cannot be satisfactorily explained in terms of virus titers or other recognized variables. The lower NI is considered more significant because it is an average of 4 tests and is consistently almost 2 logs below the

titer of this serum for the homologous virus.

It is interesting to note that the viruses that were regarded as field isolates were not found to have significant antigenic differences (see table 17). The significant antigenic differences were noted in the "laboratory" viruses LA-6, Cooper 15 and possibly PM 89. LA-6 was originally isolated from a herd undergoing IBR in California and has been passaged only 6 times. The Cooper 15 was originally isolated from an IBR outbreak in Colorado and had been passaged 15 times in tissue culture. The PM 89 was originally a Colorado isolate and has been passaged about 89 times in tissue culture.

In checking the source of viruses used by licensees for vaccine production, it appears that all may have been isolated originally from cattle in Colorado. The way this occurred was that certain laboratories supplied the commercial companies with viruses and these laboratories probably all received their virus originally from Colorado.

If all of the vaccine viruses were originally from 1 source one would not expect to detect antigenic differences unless they had been modified by passage. On the other hand, since the LA-6 virus was originally isolated from cattle in California and the Cooper 15 virus from cattle in Colorado, there is the possibility that these viruses could be antigenically different. If this is the case, the difference should be consistently apparent when serums are tested against these viruses, but this was not shown.

As shown in table 16, the antigenic differences noted that are considered most significant are with serums #2, #8 and #9 and with LA-6, PM 89, Cooper 15 viruses. It is difficult to evaluate the significance of these apparent differences. But where the serum has been tested against the same virus repeatedly, as has serum #9 against LA-6 and Cooper 15, and there is a consistent 2 logs difference in the titer as compared this serum against the homologous virus, it would be difficult to dismiss it as insignificant. However, the reason why this serum is not able to neutralize these 2 viruses as effectively as others cannot be explained on the basis of the origin of the virus. This difference may result from the modification of the virus during the development of the vaccine.

Since LA-6 and Cooper 15 are relatively low passage-level viruses, this could account for their apparent antigenic difference. However, if this is the explanation, it would be difficult to explain why serum #2 which had a low NI for LA-6 did not have a low titer for Cooper 15 too. Also, serum #8 which had a low titer for LA-6 had a low titer for PM 89, a vaccine virus, but not for Cooper 15.

There are no references in the literature which note specific antigenic differences between IBR viruses but some authors make reference to possible differences. McKercher and Straub (64) suspected that a virus isolated from a range cow differed in certain antigenic details from a known IBRV. Straub et al. (94) concluded that the different but character-

istic mobilities detected by electrophoretic studies of virus indicated that they were not identical but sub-types of the same virus group.

Most of the previous SN comparisons of IBR viruses and antiserums have been conducted with the Beta method of constant virus and decreasing serum. Bovine viruses have been compared with the Alpha method of constant serum and decreasing virus (50,60,28). The Alpha method is recognized (57) as more precise in detecting differences in serum titers and was therefore used in these tests.

Also, some SN testing reported in the literature was done utilizing hyperimmune rabbit antiserum. Hyperimmune serum is not considered to be as specific as antiserum resulting from the first inoculation of an antigen. All of these serums from licensees were produced as the result of 1 inoculation of the antigen or vaccine into the calf. These serums are therefore considered to be more specific than would be hyperimmune serums. Since the NADL^{+} is a hyperimmune serum, it may not reflect minor antigenic differences as quickly as do the serums from calves that were inoculated only once.

In summary, it appears that there are some significant antigenic differences in the viruses tested which cannot be satisfactorily explained. However, based upon the earlier results of SN testing at NADL by Dr. Phillips it was anticipated that there

would be some antigenic differences detected. The statistical significance of these differences cannot be determined since the number of tests conducted with the serums and viruses disclosing the antigenic differences are not great enough to permit statistical analysis. However, the average 2 log difference in the NI of these serums for these viruses as compared to their titers against other viruses would indicate that these are real antigenic differences. Finally, the varying neutralizing ability of these serums was not demonstrated for other serums against any of the viruses they were tested against.

Conclusion and Summary

Two of the 20 viruses tested were found to have antigenic differences considered to be significant by testing them against serums from calves inoculated with vaccines by 11 licensed producers of IBR vaccines.

Two of the 12 serums tested were found to have significantly different neutralizing ability for certain viruses. One other serum showed a variability in neutralizing ability, but not as marked as the other 2. The serums, collected from the vaccinated calves by the licensees, were all tested against the homologous and 3 selected viruses. A known positive hyperimmune serum and selected serums from licensees were tested against the vaccine viruses and viruses isolated from field cases of typical IBR, conjunctivitis, abortion, and IPV. No

antigenic differences were noted in the "field" viruses isolated from the diseases herds.

There was a close correlation between the NI of the hyperimmune serum and virus titers when tested repeatedly against one virus and with the 20 viruses it was tested against. The correlation coefficients between the serum NI and virus titers are 0.80 and 0.75, respectively.

PART III. INTERFERON PRODUCTION AND SUSCEPTIBILITY

Introduction

Interferon (IF) was first recognized and identified by investigators who were studying virus interference, the phenomenon long observed in which 1 virus, once it has infected cells, is able to prevent other viruses from invading or infecting these same cells. Later it was observed by several workers that IF production was a reaction of cells from many species of vertebrates to infection with a number of viruses. It was further demonstrated that the interfering activity produced as a result of the reaction between the cells and viruses was released into the fluid menstrum. Also, that the active principle, freed of any residual virus, elicited resistance to cells against other viruses. Since these first discoveries IF has been the subject of extensive investigation.

Interferon has been characterized as a non-dialyzable protein of non-viral origin. Apparently, its mode of action is the inhibition of some phase of virus replication in the cells.

The discovery that less virulent strains of virus induced the production of more IF, led to the suggestion that this might be a general characteristic of the less virulent strains of virus. This hypothesis was confirmed for several viruses and suggested the studies reported in this part.

It is a common practice to attempt to develop a vaccine for a disease by modifying a virus so as to reduce its virulence. The vaccine utilized for IBR prevention was developed by the rapid serial passage of a virulent virus. It was possible to reduce the virulence of the virus, after many passages in EBK cells, so that it does not cause an untoward reaction when inoculated into susceptible calves. This raised the question: Had the modification of the IBR virus altered its ability to produce interferon or its sensitivity to interferon? If it could be shown that this virus was modified in either of these ways, it might partially explain what occurs when virus virulence is reduced in the development of a vaccine.

This part is a report of the experiments conducted in an effort to show that the IF production or sensitivity was involved in the modification of IBR virus.

Review of Literature

Interferon (IF) was first detected by Isaacs and Lindemann (45) while investigating virus interference. They observed that IF was produced when fragments of chorio-allantoic membrane from chicken embryos were exposed to inactivated influenza virus. The IF was able to block infection of normal cells by myxoviruses and also a pox virus. Subsequently, it was shown that IF is also produced in response to live virus (10).

Ho and Enders (40) and Henle *et al.* (38) found that IF was produced in cultures of cells chronically infected with virus *in vitro*. It appeared that IF was responsible for the cellular resistance to virus destruction in these cultures. Glasgow and Habel (31) found that if the IF was allowed to accumulate in the cultures they could recover completely from the virus infection.

Very young chick embryos (CE) are known to be more sensitive to the lethal action of a number of viruses than are older CE. CE do not produce antibodies to virus but Baron and Isaacs (7) reported that the time of the development of resistance to the virus infection corresponded closely to time of the development of the IF mechanism.

Several investigators have studied the biological and biochemical characteristics of IF. Hilleman (39) and Ho (41) have reviewed these studies and agree that IF is sensitive to some proteolytic enzymes, e.g. trypsin, chymotrypsin, pepsin, and papain and that it is relatively heat stable and stable at high and low pH. The molecular weight seems to vary with the species from which it is elaborated but is probably between 25,000 and 70,000. It is produced by cells, does not directly inactivate virus, inhibits replication of virus and infectious nucleic acid intracellularly, is more effective in species of cells from which it is produced, is not inactivated by antibodies to virus and is relatively non-antigenic.

Lwoff and Lwoff (54) have shown that the course of a

virus infection can be greatly influenced in vitro and in vivo by a small temperature rise. They suggested that fever may play a part in recovery from virus infection.

Dubes and Wenner (22) and Bedson and Dumbell (8) have shown that strains of polio virus and pox viruses, respectively, that are able to grow at higher temperatures are often more virulent than viruses unable to do so. This raised the question of the possible relation of virus virulence and temperature to interferon production or sensitivity.

Ruiz-Gomez and Isaacs (78) investigated the growth and virulence of viruses in CE over a range of temperatures from 25-42° C. and the sensitivity of these viruses to IF. They found a close correspondence between the optimal temperature for growth and the sensitivity of the virus to interferon, i.e., the higher the optimal temperature, the less sensitive the virus was to IF. In the virulence studies they took into account the relation of the age of the CE to virus susceptibility. They found that when 10-day and 12-day-old CE were used, the virulence of 8 of 10 viruses was related to their optimal temperature and their sensitivity to interferon.

Enders (23) commented on the higher yield of IF from cells infected with an avirulent strain of measles than from cells infected with a virulent strain. He suggested that this relationship might be a more general one which could yield an interesting clue to the nature of virus virulence. DeMaeyer and Enders (21) found that 5 strains of polio virus of low

virulence induced production of IF while 4 virulent strains did not produce detectable interferon. Ruiz-Gomez and Isaacs (77) found that strains of Newcastle Disease Virus (NDV) which were most virulent for the CE produced less IF than strains of lesser virulence. Glasgow and Habel (31) observed that mouse cells that showed lesser susceptibility to vaccinia virus produced more IF than cells that were more susceptible to the same virus. Ruiz-Gomez and Isaacs (77) noted the NDV which grew well and produced plaques in CE cells produced low yields of IF. The same virus grew poorly in human amnion cells but produced large yields of IF. Wagner (104) studied the production of IF in L cells by mutants of vesicular stomatitis virus (VSV) of differing virulence for mice. The more virulent virus produced less IF and was less sensitive to the antiviral action of IF in vitro than the less virulent virus.

Thiry (99) working with "red" mutants of NDV of differing virulence for mice and CE found that the lower the virulence the higher the yield of IF induced. Sellers (87) found that strains of foot-and-mouth disease virus of differing virulence show a corresponding variation in sensitivity and production of interferon.

Isaacs (77) concludes in regard to the work conducted with Ruiz-Gomez that it seems clear that the virulent strains with high optimal temperature for virus growth, give very poor yields of IF, whereas, the avirulent strains give much better yields. He points out that avirulent strains, which

grow less well at 37° C. and higher, give their best yields of IF at the higher temperatures and is in support of the postulation of Lwoff and Lwoff (54) that fever may have a beneficial affect on recovery from virus infection by favoring the production of IF. This author further suggests that when a virus particle enters a cell it is either stimulated to produce IF which prevents production of new virus or it allows the virus to multiply.

Furthermore, that an avirulent virus may be one in which a high proportion of its population is made up of virus particles that stimulate cells to make IF and that such a virus may also be very sensitive to the antiviral action of IF.

Ho (41) in discussing the "induction" of IF summarizes his hypothesis by stating that infective virus may either inhibit or enhance interferon formation and that intracellular IF can inhibit viral infectivity or inhibit the cell-disruptive influence of infective virus and thereby increase IF formation.

Tamm and Eggers (98) in discussing the kenetics and mechanism of IF production and action point out that it has been shown that IF becomes detectable only after virus multiplication has reached a peak, and that it is most effective when added to cells some hours before the virus but can inhibit virus multiplication when added at the same time or even hours after the virus inoculation. Its effect in animals has been demonstrated in rabbits by: (1) protecting against intradermal infection with vaccinia virus, and (2) reducing the effect of

corneal inoculations of vaccinia virus by prior application of IF to the eyes. The same treatment did not affect the course of herpes simplex virus infection. Interferon has shown a slight but significant protective effect in mice inoculated with an encephalitis (Bunyamwera) virus. These authors also list as IF producing viruses members of 4 major groups: picorna-arbo-myxo- and pox viruses. Sensitivity to interferon has been demonstrated for all except papova and herpes viruses. They state that there are marked quantitative differences in the production and IF sensitivity of viruses.

Materials and Methods

Interferon production

Two IBR viruses were used. One was a virulent strain identified as C6 which had been obtained from Dr. McKercher (see Part I - Source of viruses) as the 4th passage of the Cooper strain and was passaged 2 more times in EBK cells. The other was a commercially produced vaccine virus designated as 6A. The swine influenza virus (SIV) was received from Dr. J. B. Gratzek at Iowa State University and was originally isolated from a field case.

Eighty ml. of an embryonic bovine kidney cell suspension prepared as described in Part I were dispensed into 1-liter Blake flasks. When the cells were confluent, they were inoculated with 1 ml. of a virus dilution containing about 50,000 $TCID_{50}$. Except in 2 trials where temperatures were varied,

the inoculated cells were incubated at 37° C. In 1 trial the cells were incubated at 39° C. and in another they were maintained at 25° C. for 14 days and then at 37° C. for 5 days.

The inoculated Blake flasks were removed from the incubator 48-72 hours after inoculation when the CPE was approximately 75-90% complete. The cells and medium were frozen and thawed twice. In the first trials the fluid containing the virus was centrifuged for 2 hours at 30,000 r.p.m. or 76,000 g. to remove the virus. Since this did not eliminate all the virus, in later trials the virus was inactivated without prior centrifugation. The SIV was removed by hemadsorption with chicken red blood cells before inactivation. The viruses were inactivated chemically by reducing the pH of the media to pH 2.0 - 2.5. In the first trials this was accomplished by dialysis against Sorenson's glycine buffer for 24 hours. Later the pH was reduced by adding 0.3N HCl directly to the medium and holding at 4° C. for 24 hours. In the first trials the fluid was restored to pH 7.4 by dialysis against Sorenson's phosphate buffer pH 7.4. The fluid treated in this way was toxic to the cells and, therefore, the pH was restored by the addition of 0.3N NaOH and by dialysis against Earle's balanced salt solution for 2 24-hour periods at 4°C. The fluid was filtered through a 0.45 μ millipore filter and tested for bacterial sterility by inoculating thioglycolate broth and for residual virus by inoculating EBK cells.

Interferon assays

The IBR viruses used in the plaque and tube assays were virulent, low passage Cooper strains (See Part I - Source of viruses). The vesicular stomatitis viruses (VSV) were both New Jersey type. One was furnished by Dr. E. W. Jenney of the National Animal Disease Laboratory and the other was supplied by Dr. J. B. Gratzek of Iowa State University, originally from Wisconsin and is known as the Jackson strain. The ISU-1 was also supplied by Dr. Gratzek and has been identified as an IBR virus.

Two methods were utilized for IF assays. They were: the inhibition of plaque formation and the inhibition of cytopathic effect (CPE). Embryonic bovine kidney (EBK) cells were used in 60 ml. Petri dishes for the plaque experiment and 16 x 150-ml. tissue culture tubes for the CPE observations. However, 1 plaque assay was conducted in embryonic bovine testicular (EBT) cells. Usually, 5 Petri dishes or tubes were used for each virus dilution and virus-IF combination. The tissue culture cells were prepared as described in Part I. Eight ml. of the medium containing the suspension of cells were placed in each Petri dish. The cells were observed for growth and when confluent, usually in 48-72 hours, the medium was removed. The cells were washed with PBS and then 0.5 ml. of the harvested fluid (HF) believed to contain IF was placed on the cell sheet. The cells treated with HF were kept at room temperature for 1/2 hour and then 5 ml. of Earle's medium with 4%

calf serum was added to each dish. They were then incubated for 24 hours at 37° C. at which time the medium was poured off. The cells were then inoculated with 0.1 ml. of the appropriate virus dilutions. After allowing 1 hour for virus absorption, the cells were overlaid with 5 ml. of a mixture of equal parts of double strength Eagles' medium with 5% calf or lamb serum and 2% Noble agar adjusted to a pH of 7.4. The overlaid cells were incubated at 37° C. in a 2-4% CO_2 atmosphere for a period of 48-96 hours. After the period of incubation, 0.3 ml. of 1% neutral red or 5 ml. of 1-10,000 dilution of neutral red in distilled water was added to each plate. Plaque counts were made 3-4 hours after staining.

Certain minor deviations of these procedures will be noted in the tables included in the results.

The assays for inhibition of CPE were conducted in tubes containing EBK cells prepared as described in Part I. When the cells were confluent, the medium was removed. The cell sheet was washed twice with 1 ml. of PBS and 0.5 ml. of fluid being assayed for IF was placed on the cells in each tube. After allowing 30-60 minutes for absorption, 1 ml. of Earle's maintenance medium with 4% lamb serum was added to the cells and they were placed in a 37° C. incubator for 24 hours. After the incubation period, the medium was removed from the cells and they were inoculated with 0.2 ml. of the appropriate virus dilutions. Following the 1 hour allowed for virus absorption, 1.8 ml. of either Earle's or Eagles' maintenance medium with

4% calf or lamb serum was added. The cells were observed for CPE beginning 24-48 hours after virus inoculation. Minor exceptions to the above are noted in the tables in the Results section.

Results

The harvested fluid (HF) designated as IBRV-HF was the fluid harvested from the EBK cells inoculated with IBRV and treated to preserve IF as described in Materials and Methods. That designated as SIV-HF was the result of the SIV inoculations.

Minor exceptions to the procedures described in the Materials and Methods section are noted in the titles and footnotes to the following tables which report the results of the trials conducted.

Table 18. Test for interferon in cells treated with IBRV-HF and inoculated with virus at the same time

IBRV and dilution	Average no. plaques per plate	
	HF + virus	Virus alone
Cooper 13 10^{-5}	60	54.5
10^{-6}	4	5
10^{-7}	0.2	0.2

Table 19. Assay for interferon in EBT cells treated with IBRV-HF 30 minutes before the virus inoculation

Virus and dilution		Average no. plaques per plate[a]	
		HF + virus	Virus alone
NADL-VS	10^{-4}	.67	3
	10^{-5}	0	.67
ISU-1 IBR	10^{-5}	5	8
	10^{-6}	1	.67
Cooper 13 IBR	10^{-5}	TNTC[b]	TNTC
	10^{-6}	140	83

[a]Six plates for each virus dilution.

[b]Too numerous to count.

Table 20. Assays for IF in HF from 6A and C6 IBR viruses

Virus and dilution		Average no. plaques per plate		
		6AHF + virus	C6HF + virus	Virus alone
Cooper 13 IBR	10^{-6}	12.5	19	12.5
	10^{-7}	2.0	1.8	Contam.
ISU-VS	10^{-2}	ND[a]	ND	TNTC
	10^{-3}	18.1	19	0[b]
	10^{-4}	1.4	1.5	1

[a]Not done.

[b]Absence of plaques at this dilution unexplained. Perhaps virus inoculation was omitted. Plaques at 10^{-2} and 10^{-4} support the validity of the assay.

Table 21. Assays for interferon in HF from C6 and 6A IBR viruses by CPE inhibition

$TCID_{50}$ of IBRV in inoculum	Hours post-inoculation	% of tubes[a] showing CPE		
		C6HF + virus	6AHF + virus	Virus alone
30	24	70	70	70
158	24	80	80	80
30	48	90	100	100
158	48	100	100	100
50	48	70	70	90
150	48	80	100	100
50	60	100	90	100
150	60	100	100	100

[a]Ten tubes for each inoculation and virus dilution.

In an effort to stimulate IF production by varying the incubation temperatures, EBK cells in Blake bottles were inoculated with 1.0 ml. of an undiluted harvest of media containing 6A IBR virus and a 10^{-2} dilution of the harvest of a previous passage and incubated for 14 days at room temperature approximately 25° C. Since there was no evidence of CPE after 14 days at 25° C. the bottles were transferred to a 37° C. incubator. The CPE was complete after 5 days at 37° C. This harvest was treated in the usual manner. This HF was assayed for IF in the manner previously described (see table 22).

Table 22. Assays for IF in IBRV-HF after incubating at 25° C. for 14 days and 37° C. for 5 days by CPE inhibition

$TCID_{50}$ of Cooper IBRV	Hours post-inoculation	% of tubes[a] showing CPE		
		Undiluted virus HF	10^{-2} virus dilution HF	Virus alone
30	24	20	40	ND
150	24	100	80	80
30	48	80	60	ND
150	48	100	100	100

[a]Ten tubes per dilution.

Table 23. Assays for IF in HF from 6A and C6 IBR viruses

Cooper IBRV dilution		Average no. of plaques per plate		
		C6HF + virus	6AHF + virus	Virus alone
Trial 1	10^{-5}	62	ND	54.5
	10^{-6}	4	ND	5
	10^{-7}	1	ND	1
Trial 2	10^{-5}	TNTC	ND	TNTC
	10^{-6}	67	75	69
	10^{-7}	6.1	10.5	5.6

Table 24. Assays for IF in HF from SIV in EBK cells with VSV

VSV, dilution and quantity inoculated			Average no. plaques per plate	
			Virus + HF	Virus alone
Trial 1	NADL 10^{-2}	.5 ml.	83	TNTC
	10^{-3}	.2 ml.	8.4	73
Trial 2[a]	NADL 10^{-2}	.5 ml.[b]	0	23
	10^{-3}	0.5 ml.	0	2
	ISU 10^{-5}	0.5 ml.	0	1.4

[a]Three plates for each assay.

[b]See illustration 1.

Table 25. Assay for IF in HF from SIV in EBK with IBRV

Dilution and quantity of Cooper IBR virus inoculated			Average no. plaques per plate	
			Virus + HF	Virus alone
Trial 1	10^{-5}	0.5 ml.	40	45
	10^{-5}	0.25 ml.[a]	18	18
Trial 2	10^{-6}	0.2 ml.	4.3	4.1
	10^{-7}	0.2 ml.	0.6	0.6
Trial 3	10^{-5}	0.3 ml.	15	15[b]
	10^{-6}	0.5 ml.[b]	1	1
Trial 4	10^{-5}	0.5 ml.	27.2	33.2
	10^{-6}	0.5 ml.	2.2	2.75

[a]Three plates for each assay.

[b]Four plates for each assay.

Illustration 1. Vesicular stomatitis virus plaques and inhibition by interferon. Right row with interferon --left without.

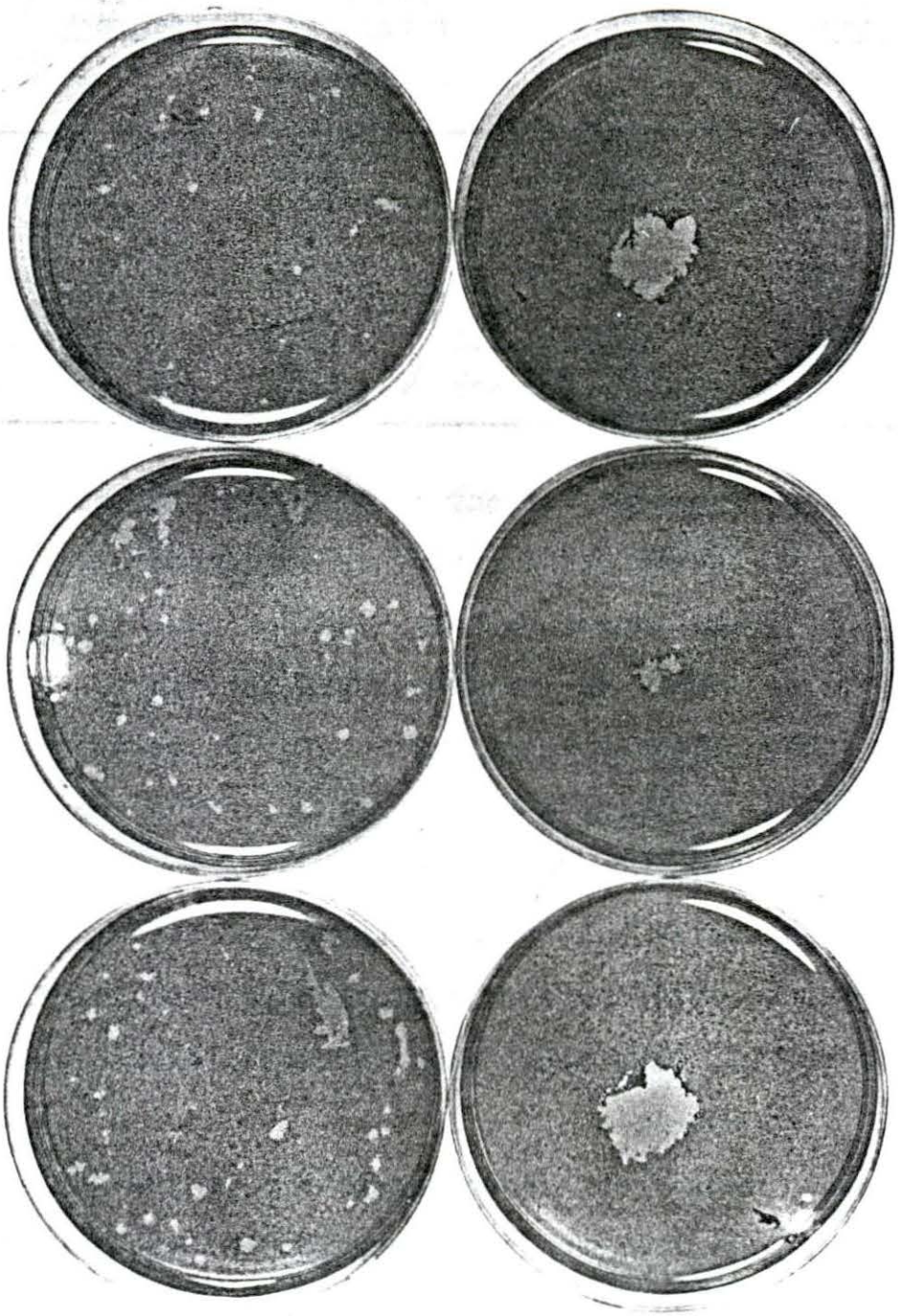


Table 26. Assay for IF in IBRV-HF in EBK cells

Virus	Virus dilution and quantity inoculated		Average no. plaques per plate: Virus + HF	Average no. plaques per plate: Virus alone
NADL-VS	10^{-1}	0.5 ml.	114	76
	10^{-2}	0.25 ml.	19	14
ISU-VS	10^{-4}	0.25 ml.	36	15
Cooper IBR	10^{-5}	0.25 ml.	7	7
	10^{-5}	0.5 ml.	10	10

There is a report in the literature (78) that higher than optimal temperatures caused certain viruses to produce more IF. Therefore EBK cells inoculated with IBRV 6A were incubated at 39-40° C. for 72 hours. The virus was inactivated in one-half of the harvested fluid by heat (56° C. for 22 minutes) and the other half by the addition of 0.3 N HCl to reduce the pH to 2. The pH of one-half of the IF fluid in which the virus was inactivated chemically was restored by the addition of 0.3 N NaOH and in the other half by dialysis against Earle's balanced salt solution. The fluid that was not dialyzed was not detrimental to the cells.

There was no evidence of plaque inhibition of IBRV by any of the fluids harvested from the high temperature incubation trial.

Discussion

Under the conditions of these trials the IBRV strains utilized did not produce IF that was detectable with the assay procedures employed. In an effort to increase the production of IF the virus was propagated at higher and lower temperatures than those considered optimal for IBRV. These adjustments did not result in the production of detectable IF.

Failure to detect any IF production using IBRV for the assays led to the use of VSV which is known to be sensitive to IF. The IBRV-HF had no detectable inhibitory effect on VSV.

Interferon produced by inoculating EBK cells with SIV did have a marked inhibitory effect on VSV. Infectious bovine rhinotracheitis virus was not shown to be inhibited by the SIV-IF which inhibited VSV.

The IBRV is accepted as a member of the herpes group of viruses. Tamm and Eggers (98) do not list the herpes viruses as IF producers or as being sensitive to IF. Therefore, it is perhaps not surprising that IBRV was found to be inert as far as IF is concerned.

Fruitstone et al. (27) using the GCA 3 strain of herpes simplex virus (HSV) produced an interferon-like substance in CE that suppressed the activities of vaccinia, herpes, and influenza viruses. Since the authors were able to use the HSV for both the production and assay of IF, it is clear that HSV can be both stimulatory and sensitive in an IF system.

On the other hand, interferons have been shown to demonstrate more tissue or species specificity than viral specificity. Interferon produced in chick cells is more active when assayed in chick cells than when assayed in calf kidney cells (86). Some interferons are active only in the cells of the same species in which they were produced (103) or much more active in the homologous species (70,6). This species specificity is not absolute, however, and Sellers (86) in reviewing this subject suggests that these differences may reflect a restriction of the challenge virus in cells other than those in which it customarily reproduces thus enabling the IF to be more effective.

Because of this species specificity, EBK cells were used for IF production and assays. Since IBR is primarily a bovine disease, it would be most likely that IF would be produced and most easily detected in bovine cells. When one takes into account the tissue specificity of IF, which is generally recognized, and the system that was used, it would seem that IBRV is relatively inert insofar as IF production and sensitivity are concerned. It is interesting to note in this regard that the swine influenza virus did produce IF in bovine cells and that it was readily assayable in the bovine kidney cells.

It may be possible to produce IF with IBRV by using other systems. Perhaps in other cells or chicken embryos with other modifications of the system detectable IF can be produced.

However, with the systems used in these trials, no IF was detectable.

Conclusion and Summary

Studies were conducted to measure the interferon production and sensitivity of virulent and vaccine strains of IBRV. The inoculation of EBK cells with the IBRV strains used did not result in the production of sufficient IF to noticeably inhibit the CPE or plaque formation by IBRV or VSV. The assays for IF were conducted with 2 strains of IBRV and VSV in EBK cells. The inoculation of EBK cells with swine influenza virus did produce IF that inhibited VSV but did not inhibit the IBRV.

IBR virus is considered to be a herpes virus. The herpes viruses as a group are not considered to be IF producers or sensitive to IF. It is not surprising therefore that it was not possible to produce detectable amounts of IF with IBRV.

GENERAL SUMMARY

In an effort to find a smaller, less expensive animal for assaying the potency of IBR vaccines, goats were tested for susceptibility to IBRV. Goats were found to be refractory to intranasal, contact and intracerebral exposure. A calf inoculated at the same time as the goats in 1 trial reacted with a typical response. The resistance of the goats to IBRV reported in this work is in contrast to a report in the literature.

Apparently, significant antigenic differences were observed in 2 IBR viruses when tested against serums from calves vaccinated by licensed commercial vaccine producers. The neutralizing ability of a hyperimmune calf serum was not significantly different when tested against each of the 20 viruses. A correlation was shown between the NI of this serum and the titers of the viruses against which was tested. No significant antigenic differences were demonstrated among the different IBR viruses isolated from field cases of disease showing different clinical manifestations such as the respiratory form of IBR, conjunctivitis, abortion and IPV.

The vaccine for IBR was developed by attenuating a virulent virus by rapid serial passage in tissue culture. Avirulent strains of polio and measles viruses were shown to produce more interferon than virulent strains. Two strains of IBRV, 1 vaccine and 1 virulent strain, were tested for interferon

production and susceptibility. Neither strain could be shown to produce or to be sensitive to interferon but interferon produced by SIV in EBK cells inhibited the plaque formation of VSV. The same interferon had no detectable inhibitory effect on IBR viruses.

LITERATURE CITED

1. Abinanti, F. R. and Plumer, G. J. The isolation of infectious bovine rhinotracheitis virus from cattle affected with conjunctivitis--observations on the experimental infection. American Journal of Veterinary Research 22: 13-17. 1961.

2. Armstrong, J. A., Pereira, H. G., and Andrewes, C. H. Observations on the virus of IBR and its affinity with the herpesvirus group. Virology 14: 276-285. 1961.

3. Baker, James A., McEntee, Kenneth, and Gillespie, James H. Effects of infectious bovine rhinotracheitis--infectious pustular vulvovaginitis (IBR-IPV) virus on newborn calves. Cornell Veterinarian 50: 156-170. 1960.

4. Baker, James A., York, Charles J., Gillespie, James H., and Mitchell, Grayson B. Virus diarrhea in cattle. American Journal of Veterinary Research 15: 525-531. 1954.

5. Barenfus, Morris, Quadri, C. A. Delli, McIntyre, R. W., and Schroeder, R. J. Isolation of infectious bovine rhinotracheitis virus from calves with meningoencephalitis. American Veterinary Medical Association Journal 143: 725-728. 1963.

6. Baron, Samuel, Barban, Stanley, and Buckler, Charles E. Host cell specificity of mouse and chicken interferons. Science 145: 814. 1964.

7. Baron, S. and Isaacs, A. Mechanism of recovery from viral infection in the chick embryo. Nature 191: 97-98. 1961.

8. Bedson, J. S. and Dumbell, K. R. The effect of temperature on the growth of pox viruses in the chick embryo. Journal of Hygiene 59: 457-469. 1961.

9. Brown, W. W. and Chow, T. L. Field trials of infectious bovine rhinotracheitis vaccine. American Veterinary Medical Association Journal 134: 29-31. 1959.

10. Burke, D. C. and Isaacs, A. Some factors affecting the production of interferon. British Journal of Experimental Pathology 39: 452-458. 1958.

11. Cheatham, William J. and Crandell, Robert A. Occurrence of intranuclear inclusions in tissue cultures infected

with virus of infectious bovine rhinotracheitis. Society of Experimental Biology and Medicine Proceedings 96: 536-538. 1957.

12. Chow, T. L. and Davis, R. W. The susceptibility of mule deer to infectious bovine rhinotracheitis. American Journal of Veterinary Research 25: 518-519. 1964.

13. Chow, T. L., Deem, A. W., and Jensen, Rue. Infectious rhinotracheitis in cattle. U.S. Livestock Sanitary Proceedings 59: 168-172. 1955.

14. Chow, T. L., Molello, J. A., and Owen, N. V. Abortion experimentally induced in cattle by infectious bovine rhinotracheitis virus. American Veterinary Medical Association Journal 144: 1005-1007. 1964.

15. Crane, C. S. Infectious bovine rhinotracheitis abortion and its relationship to nutrition in California beef cattle. American Veterinary Medical Association Journal 147: 1308-1309. 1965.

16. Crane, C. S., Lukas, G. N., and Watkins, W. W. Infectious bovine rhinotracheitis abortion in California beef cattle. American Veterinary Medical Association Journal 144: 13-18. 1964.

17. Cruickshank, J. G. and Berry, D. M. Morphology of infectious bovine rhinotracheitis virus. Virology 25: 481-482. 1965.

18. Darbyshire, J. H. and Shanks, P. L. The isolation of infectious bovine rhinotracheitis virus in Scotland. Veterinary Record 75: 897-899. 1963.

19. Dawson, P. S. and Darbyshire, J. H. The occurrence and distribution of antibodies to parainfluenza 3 and infectious rhinotracheitis viruses in bovine sera. Veterinary Record 76: 111-115. 1964.

20. Dawson, P. S., Darbyshire, J. H., Loosmore, R. M., Paterson, A. B., and Faull, W. B. Infectious bovine rhinotracheitis (IBR) a clinical condition of cattle occurring in the United Kingdom. Veterinary Record 74: 1379-1383. 1962.

21. DeMaeyer, E., Enders, J. F. An interferon appearing in cell cultures infected with measles virus. Society of Experimental Biology and Medicine Proceedings 107: 573-578. 1961.

22. Dubes, G. R. and Wenner, H. A. Virulence of polio viruses in relation to variant characteristics distinguishable on cells in vitro. Virology 4: 275-296. 1957.

23. Enders, J. F. A consideration of the mechanism of resistance to viral infection based on recent studies of the agents of measles and poliomyelitis. Philadelphia, Pa., Philadelphia College of Physicians Transactions and Studies 28: 68-79. 1960.

24. Fastier, L. B. and Smith, F. B. Infectious bovine rhinotracheitis-infectious pustular vaginitis--virus isolation and vaccination studies. New Zealand Veterinary Journal 10: 11-17. 1962.

25. French, E. L. Relationship between infectious bovine rhinotracheitis (IBR) virus and a virus isolated from calves with encephalitis. Australian Veterinary Journal 38: 555-556. 1962.

26. French, E. L. A specific virus encephalitis in calves: Isolation and characterization of the causal agent. Australian Veterinary Journal 38: 216-221. 1962.

27. Fruitstone, M. J., Waddell, G. H., and Sigel, M. M. An interferon produced in response to infection by Herpes simplex virus. Society of Experimental Biology and Medicine Proceedings 117: 804-807. 1964.

28. Gillespie, J., Coggins, L., Thompson, J., and Baker, J. Comparison by neutralization tests of strains of virus isolated from virus diarrhea and mucosal disease. Cornell Veterinarian 51: 155-159. 1961.

29. Gillespie, J. H., Lee, K. M., and Baker, J. A. Infectious bovine rhinotracheitis. American Journal of Veterinary Research 18: 530-535. 1957.

30. Gillespie, James, H., McEntee, Kenneth, Kendrik, John W., and Wagner, William C. Comparison of infectious pustular vulvovaginitis virus with infectious bovine rhinotracheitis virus. Cornell Veterinarian 49: 288-297. 1959.

31. Glasgow, L. A. and Habel, K. The role of interferon in vaccinia virus infection of mouse embryo tissue culture. Journal of Experimental Medicine 115: 503. 1962.

32. Greig, A. S. The detection of antibody to infectious bovine rhinotracheitis virus in Ontario cattle. Canadian

Journal of Comparative Medicine and Veterinary Science 25: 31-34. 1961.

33. Greig, A. S. and Bannister, G. L. Infection of the bovine udder with bovine herpesvirus. Canadian Journal of Comparative Medicine and Veterinary Science 29: 57-62. 1965.

34. Griffin, T. P., Howell, W. V., Crandell, R. A., and Maurer, F. D. Stability of the virus of infectious bovine rhinotracheitis. American Journal of Veterinary Research 19: 990-992. 1958.

35. Grinyer, I., Savan, M., and Studdert, M. J. An electron microscopic comparison of bovine infectious pustular vaginitis virus and infectious bovine rhinotracheitis virus in tissue culture. Canadian Journal of Microbiology 8: 373-378. 1962.

36. Grunder, H. D., Reuleaux, I. R., and Liess, B. Feststellung der virusbedingten rhinotracheitis infectiosa des Rindes (English summary). Herkunft und Isolierung des virus. Deutsche tierärztliche Wochenschrift 67: 514-519. 1960.

37. Hahnefeld, H. and Hahnefeld, E. Nucleic acids of bovine infectious rhinotracheitis virus and cortal vesicular exanthema virus. Archiv. für Experimentelle Veterinarmedizin 18: 417-425. 1964.

38. Henle, Werner, Henle, G., Deinhardt, F., and Bergs, V. V. Studies on persistent infections of tissue culture. III. Evidence for the production of interferon in MCN cells by myxovirus. Journal of Experimental Medicine 110: 525. 1959.

39. Hilleman, M. R. Interferon in prospect and perspective. Journal of Cellular and Comparative Physiology 60: 337-353. 1963.

40. Ho, M. and Enders, J. F. An inhibitor of viral activity appearing in infected cell cultures. National Academy of Science Proceedings 45: 385-389. 1959.

41. Ho, Monto. Identification and "induction" of interferon. Bacteriological Reviews 28: 362-381. 1964.

42. Hughes, John P., Olander, Harvey J., Wada, Midori. Keratoconjunctivities associated with infectious bovine rhinotracheitis. American Veterinary Medical Association Journal 145: 32-39. 1964.

43. Hutyra, F., Marek, J., and Manninger, R. Special pathology and therapeutics of the diseases of domestic animals. Vol. 2. 4th ed. Chicago, Illinois, Alexander Eger. 1938.

44. Isaacs, A. Antiviral action of interferon. British Medical Journal 5301: 353-355. 1962.

45. Isaacs, A. and Lindemann, J. Virus interference. I. Interferon. Royal Society of London Proceedings Series B, 147: 258-267. 1957.

46. Johnston, L. A. Y., Simmons, G. C., and McGavin, M. D. A viral meningo-encephalitis in calves. Australian Veterinary Journal 38: 207-215. 1962.

47. Kendrik, K. W., York, C. J., and McKercher, D. G. A controlled field trial of a vaccine for infectious bovine rhinotracheitis. U.S. Livestock Sanitary Association Proceedings 60: 155-158. 1956.

48. Kendrik, J. W., Gillespie, James H., and McEntee, Kenneth. Infectious pustular vulvovaginitis of cattle. Cornell Veterinarian 48: 458-495. 1958.

49. Kennedy, Peter C. and Richards, W. P. C. The pathology of abortion caused by the virus of infectious bovine rhinotracheitis. Pathologia Veterinaria 1: 7-17. 1964.

50. Langer, P. H. and McEntee, Kenneth. Characterization of virus isolates from cattle. U.S. Livestock Sanitary Association Proceedings 65: 389-396. 1961.

51. Liess, B., Knocke, K. W., and Schimmelpfennig, H. Feststellung der virusbedingten rhinotracheitis infectiosa des Rindes. II. Deutsche tierärztliche Wochenschrift 67: 551-558. 1960.

52. Liess, B., Reuleaux, I. R., and Grunder, H. D. Feststellung der virusbedingten rhinotracheitis infectiosa des Rindes. III. Deutsche tierärztliche Wochenschrift 67: 605-614. 1960.

53. Lukas, G. N., Weidentach, S. J., Palmer, K. G., Dickie, C. W., Duncan, R. F., and Berrera, J. A bovine fetal viral isolate neutralized by IBR immune serum as a cause of abortion in cattle. U.S. Livestock Sanitary Association Proceedings 67: 108-128. 1963.

54. Lwoff, A. and Lwoff, M. The factors of viral development and their role in the evolution of infection. Pasteur Institute Annals 98: 173-203. 1960.

55. Madin, S. H., York, C. J., and McKercher, D. G. Isolation of the infectious bovine rhinotracheitis virus. Science 124: 721-722. 1956.

56. Maré, J. and van Rensburg, S. J. The isolation of viruses associated with infertility in cattle: A preliminary report. Journal of the South African Veterinary Medical Association 32: 201-210. 1961.

57. Mascoli, Carmine C. and Burrell, Robert G. Experimental virology. Minneapolis, Minnesota, Burgess Publishing Company. 1965.

58. McIntyre, R. W. Experimental studies of acute upper respiratory infection in calves. American Veterinary Medical Association Journal 125: 473-474. 1954.

59. McKercher, D. G. The infectious bovine rhinotracheitis virus as a cause of abortion in cattle. California Veterinarian 16: 24. 1962.

60. McKercher, D. G. Studies of the etiologic agents of infectious bovine rhinotracheitis and Blaschenausschlag (coital vesicular exanthema). American Journal of Veterinary Research 24: 501-509. 1963.

61. McKercher, D. G., Moulton, J. E., and Jaspar, D. E. Virus and virus-like cattle diseases entities new to California. U.S. Livestock Sanitary Association Proceedings 58: 260-269. 1954.

62. McKercher, D. G., Moulton, J. E., Kendrik, J. W., and Saito, J. Recent developments on upper respiratory disease of cattle. U.S. Livestock Sanitary Association Proceedings 59: 151-167. 1955.

63. McKercher, D. G., Saito, J. K., Wada, E. M., and Straub, Otto. Current status of the newer virus diseases of cattle. U.S. Livestock Sanitary Association Proceedings 62: 136-158. 1958.

64. McKercher, D. G. and Straub, O. C. Isolation of the virus of infectious bovine rhinotracheitis from range cattle. American Veterinary Medical Association Journal 137: 661-664. 1960.

65. McKercher, D. G., Straub, O. C., Saito, J. K., and Wada, C. M. Comparative studies of the etiological agents of infectious bovine rhinotracheitis and infectious pustular vulvovaginitis. Canadian Journal of Comparative Medicine and Veterinary Science 23: 320-328. 1959.

66. McKercher, D. G. and Wada, E. M. The virus of infectious bovine rhinotracheitis as a cause of abortion in cattle. American Veterinary Medical Association Journal 144: 136-142. 1964.

67. McKercher, D. G., Wada, E. M., and Straub, O. C. Distribution and persistence of infectious bovine rhinotracheitis virus in experimentally infected cattle. American Journal of Veterinary Research 24: 510-514. 1963.

68. McKercher, P. D. A comparison of the viruses of infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), and rinderpest. Canadian Journal of Comparative Medicine and Veterinary Science 28: 77-88. 1964.

69. McKercher, P. D. A comparison of the viruses of infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), and rinderpest. Part II. Plaque assay. Canadian Journal of Comparative Medicine and Veterinary Science 28: 113-120. 1964.

70. Merigan, Thomas C. Purified interferons: physical properties and species specificity. Science 145: 811-813. 1964.

71. Miller, N. J. Infectious necrotic rhinotracheitis of cattle. American Veterinary Medical Association Journal 126: 463-467. 1955.

72. Niilo, L., Avery, R. J., Darcel, D. le Q., and Greig, A. S. Serum antibodies to padlock and IBR viruses in Alberta. Canadian Journal of Comparative Medicine and Veterinary Science 26: 39-41. 1962.

73. Orsi, E. V. and Cabasso, V. J. Infectious bovine rhinotracheitis. IV. Cytological changes in infected bovine kidney and HeLa cultures. Society for Experimental Biology and Medicine Proceedings 98: 637-639. 1958.

74. Owen, N. V., Chow, T. L., and Molell, J. A. Bovine fetal lesions experimentally produced by infectious bovine rhinotracheitis virus. American Journal of Veterinary Research 25: 1617-1626. 1964.

75. Plummer, G. Serological comparisons of the herpes viruses. British Journal of Experimental Pathology 45: 135-141. 1964.

76. Reed, L. J. and Muench, H. A simple method of estimating 50% endpoints. American Journal of Hygiene 27: 493-497. 1938.

77. Ruiz-Gomez, J. and Isaacs, A. Interferon production by different viruses. Virology 19: 8-12. 1963.

78. Ruiz-Gomez, J. and Isaacs, A. Optimal temperature for growth and sensitivity to interferon among different viruses. Virology 19: 1-7. 1963.

79. Sabina, Leslie, R. and Parker, Raymond C. Studies of infectious bovine rhinotracheitis virus. I. Plaque assay and some characteristics in bovine kidney cells. Canadian Journal of Microbiology 9: 367-376. 1963.

80. Sattar, S. A., Bohl, E. H., Senturk, M. Viral causes of bovine abortion in Ohio. American Veterinary Medical Association Journal 147: 1207-1210. 1965.

81. Schimmelpfennig, H., and Liess, B. Entwicklung und histochemie der kernein-schlusskorperchen in gewebekulturen aus rinderhodenzellen bei der rhinotracheitis infectiosa des rindes. Zentralblatt für Veterinärmedizin 8: 461-472. 1961.

82. Schroeder, Robert J. and Moys, M. D. An acute upper respiratory infection of dairy cattle. American Veterinary Medical Association Journal 125: 471-472. 1954.

83. Schulze, P., Hahnefeld, H., Hantschel, H., and Hahnefeld, E. Fine structure of the viruses of bovine rhinotracheitis and bovine cortal vesicular exanthema. Archiv. für Experimentelle Veterinarmedizin 18: 417-425. 1964.

84. Schwarz, Anton, J. F., York, Charles J., Zirbel, Lowell W., and Estela, Lois A. Modification of infectious bovine rhinotracheitis virus in tissue culture and development of a vaccine. Society for Experimental Biology and Medicine Proceedings 96: 453-458. 1957.

85. Segre, Diego. Report to the NC-34 Technical Committee on Mucosal Diseases of Cattle. Unpublished paper presented at Brookings, South Dakota, October 10-11, 1960. Urbana, Illinois, Illinois Agricultural Experiment Station. 1960.

86. Sellers, R. F. Interferon specificity. Royal Society of Medicine Proceedings 55: 725-732. 1962.

87. Sellers, R. F. Multiplication, interferon production and sensitivity of virulent and attenuated strains of the virus of foot-and-mouth disease. Nature 198: 1228-1229. 1963.

88. Smith, R. E., Clark, G. W., Reynolds, I. M., and Damon, R. A. Serologic survey for infectious bovine rhinotracheitis and bovine virus diarrhea antibodies. American Veterinary Medical Association Journal 145: 40-41. 1964.

89. Stevens, J. G. and Chow, T. L. Effects of some fixatives on inclusion bodies of infectious bovine rhinotracheitis. Society for Experimental Biology and Medicine Proceedings 100: 856-859. 1959.

90. Stevens, Jack G. and Groman, Neal B. Infectious bovine rhinotracheitis virus replication, cytopathology, and plaque formation in the presence of an absence of nucleic acid analogues. Journal of Bacteriology 87: 446-453. 1964.

91. Stevens, Jack G. and Groman, N. B. Properties of infectious bovine rhinotracheitis virus in quantitated virus-cell culture system. American Journal of Veterinary Research 24: 1158-1163. 1963.

92. Straub, O. C. and Bohm, H. O. Experimental infection of the central nervous system by bovine rhinotracheitis virus. Deutsche tierärztliche Wochenschrift 72: 124-128. 1965.

93. Straub, O. C. and Böhm, H. O. Two outbreaks of bovine pustular vulvovaginitis in South Germany. Deutsche tierärztliche Wochenschrift 69: 616-667. 1962.

94. Straub, O. C., Matheka, H. D., and Strohmaier, K. Differentiation between viruses of bovine infectious rhinotracheitis and infectious pustular vaginitis by carrier-free virus zone electrophoresis in a glucose density gradient. Zentralblatt fur Veterinarmedizin Reihe B, 11: 565-571. 1964.

95. Studdert, M. J., Barker, C. A. V., and Savan, Milton. Infectious pustular vaginitis virus infection of bulls. American Journal of Veterinary Research 25: 303-314.

96. Studdert, M. J., Radostits, O. M., and Savan, M. An outbreak of infectious bovine rhinotracheitis in Ontario. Canadian Veterinary Journal 2: 201-206. 1961.

97. Studdert, M. J., Wada, E. M., Kortmum, W. M., and Groverman, F. A. Bovine infectious pustular vaginitis in the western United States. American Veterinary Medical Association Journal 144: 615-619. 1964.

98. Tamm, I. and Eggers, Hans J. Selective inhibition of viral reproduction. In Viral and rickettsial infections of man. 4th ed. pp. 305-338. Philadelphia, Pennsylvania, J. B. Lippincott Co. 1965.

99. Thiry, L. A mutant of Newcastle disease virus which makes negative plaques. Abstracts of the VIIIth International Congress for Microbiology. p. 85. Montreal, Quebec, Canada (address and place of availability not known). 1962.

100. Tousimis, A. J., Howells, W. V., Griffin, T. P., Porter, R. P., Cheatham, W. J., and Maurer, F. D. Biophysical characterization of infectious bovine rhinotracheitis in virus. Society of Experimental Biology and Medicine Proceedings 99: 614-619. 1958.

101. Tyrrell, D. A. J. Interferon produced by cultures of calf kidney cells. Nature 184: 452-453. 1959.

102. Van Kruiningen, H. J. and Bartholomew, R. C. IBR diagnosed by lesions in a calf. American Veterinary Medical Association Journal 144: 1008-1012. 1964.

103. Wagner, R. R. Biological studies of interferon. I. Suppression of cellular infection with Eastern equine encephalomyelitis virus. Virology 13: 323-337. 1961.

104. Wagner, R. R. Discussion. Cold Spring Harbor Symposia on Quantitative Biology 27: 349. 1962.

105. York, S. J. and Schwarz, A. J. F. Immunological studies on infectious bovine rhinotracheitis. U.S. Livestock Sanitary Association Proceedings 60: 149-154. 1956.

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