Comparative Studies of the Etiological Agents of Infectious Bovine Rhinotracheitis and Infectious Pustular Vulvovaginitis

by D. G. McKercher, O. C. Straub, J. K. Saito and E. M. Wada

A condition has been observed in New York state for a number of years which was believed to be similar to, if not identical with, coital vesicular exanthema as described in the European literature. (1) (2). In 1958 Kendrick et al. (3) isolated a virus from cattle affected with this condition with which they could regularly reproduce the clinical syndrome on experimental inoculation. Because of the pathological features of the disease, they considered the term "infectious pustular vulvovaginitis" (IPV) to be more appropriate than the one coined by the German workers. On the basis of clinical and immunological observations in cattle, Gillespie was later led to believe that the virus causing IPV was related to the virus of infectious bovine rhinotracheitis (IBR). Accordingly, he carried out studies which verified this observation. (4)

The study reported herein was undertaken in an area where IPV has not been encountered, in order to obtain further confirmation of the etiological relationship of the two clinical conditions. It included comparative clinical and histopathological studies of the two syndromes, homologous and reciprocal cross protection tests in cattle, and a study of the rate of development in cattle of serum antibodies to each virus. It included also homologous and reciprocal cross serum virus neutralization tests, and the tissue host range of each strain of virus in vitro.

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STUDIES IN CATTLE

Procedure

A strain of IBR virus isolated from nasal secretions obtained by the senior author from clinical cases of IBR in Colorado, and one (K-22) of the three strains of virus recovered by Kendrick et al. (3) from clinical cases of IPV in New York state, were employed in this study. Each strain was propagated in roller tube cultures of fetal bovine kidney cells prepared, with slight modifications, according to the method of Madin et al. (5) (6) For the inoculation of cattle, the IBR virus was used at the fifth passage level and the IPV strain between the fifth and tenth passage levels.

Yearling Hereford heifers, uniform as to age, size, and background, were used as the experimental subjects. Four animals, comprising a somewhat less homogeneous group, were used as controls. Over the several-month period that the test animals were held on the premises prior to inoculation, they were examined periodically for clinical evidence of disease. Nasal and vaginal secretions of each were cultured at the time of inoculation for cytopathogenic agents which might be present and which would tend to confuse subsequent experimental findings. Blood samples were also collected at this time for serological study.

Each strain of virus was injected into four animals, two receiving 10 ml. of a freshly harvested culture by the vulvovaginal route, and two the same amount of virus given intranasally. Each strain of virus was inoculated at
a different time in order to preclude the possibility of cross infection, while multiple route exposure to the same strain was avoided, insofar as possible, by holding the intranasally exposed cattle in a pen widely separated from the one in which the vulvovaginally inoculated cattle were placed. As a further precaution in this regard, the animals were held in stanchions throughout the experiment, and great care was taken in the cleaning and feeding operations that the virus was not carried from the rear area of the pen to the front section, and vice versa. Temperatures of the cattle were recorded twice daily for several days prior to inoculation and for 12 to 14 days thereafter, and detailed clinical observations were made. Nasal and vaginal secretions were collected from each

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GRAPH 1

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Response to IBR Virus by 1) Nasal and 2) Vulvovaginal Route and Response to Challenge with the Homologous and Heterologous Virus Strain.
animal at predetermined intervals after inoculation and tested for the presence of virus. Biopsy material was obtained in each case from one of the two heifers exposed by the vulvovaginal route to each strain of virus. Blood was drawn at the times specified under the section on “Serological Studies”.

Challenge inoculations were carried out in exactly the same manner as was the initial exposure to virus. The intranasally exposed cattle were challenged by the vulvovaginal route, and those exposed initially by vulvovaginal inoculation were challenged intranasally. This pattern of challenge exposure was resorted to in order to circumvent the possible influence of any localized tissue immunity that might have developed as a result of the initial exposure to virus. Two control animals were inoculated with each virus by each of the two routes employed in this study, and all the cattle were held in stanchions throughout the remainder of the experiment. Temperatures were recorded twice daily for several days prior to challenge, and for 12 to 14 days following. Materials for biopsy were obtained at the times specified under the section entitled “Pathological Studies”.

Results

Preinoculation temperatures remained well within the normal range, and the nasal secretions of all animals were found to be negative for the presence of cytopathogenic agents. The two heifers inoculated intranasally with the IBR virus responded with a more marked febrile response than did the two inoculated by the vulvovaginal route, although the nature of the temperature curve in each case was identical, as can be seen from Graph I. Lesions that were grossly identical with those described in IPV infection developed in the vulvar and vaginal mucosa of the cattle exposed vulvovaginally to the IBR virus. However, no genital

<table>
<thead>
<tr>
<th>Number of Cattle</th>
<th>Inoculum and Route</th>
<th>Vulvovaginal Lesions</th>
<th>Virus Recovered Postinoculation on Day</th>
<th>3</th>
<th>5</th>
<th>8</th>
<th>11</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>N.S.*</td>
<td>V.S.*</td>
<td>N.S.</td>
<td>V.S.</td>
<td>N.S.</td>
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<tr>
<td>2 IBR virus Nasal</td>
<td>0/2*</td>
<td>2/2</td>
<td>0/2</td>
<td>N.T.</td>
<td>N.T.</td>
<td>0/2</td>
<td>1/2</td>
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<tr>
<td>2 IBR virus Vulvovaginal</td>
<td>2/2</td>
<td>2/2</td>
<td>N.T.</td>
<td>N.T.</td>
<td>0/2</td>
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<td>2 IPV virus Vulvovaginal</td>
<td>2/2</td>
<td>N.T.</td>
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<td>2/2</td>
<td>N.T.</td>
<td>0/2</td>
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</tr>
<tr>
<td>2 IPV virus Nasal</td>
<td>1/2**</td>
<td>N.T.</td>
<td>2/2</td>
<td>0/2</td>
<td>N.T.</td>
<td>N.T.</td>
<td>0/2</td>
</tr>
</tbody>
</table>

*N.S. — Nasal secretions
*V.S. — Vaginal secretions
*N.T. — Not tested
* — Number of cattle showing lesions or yielding virus/Number of cattle tested.
** — Atypical lesions
lesions were observed in the nasally exposed cattle. Virus was recovered on the days after inoculation that are shown in Table I.

Recovery of virus as late as the 11th day after inoculation is rather unusual although it has been accomplished on several occasions in previous studies of IBR. The fact that the vaginal secretions were negative on the eighth day after inoculation might indicate that the presence of virus therein on the 11th day resulted from inadvertent contact exposure to virus from the nasal passages. Again, since both isolations were made from the same animal, blood transfer might be suspected. However, efforts in the past to recover the IBR virus from the blood during the course of IBR infection have always failed.

Of the cattle inoculated with the IPV strain of virus, a slightly more marked febrile response occurred in the ones exposed vulvovaginally than in those exposed by the nasal route. As compared with the response of those inoculated with the IBR virus, the febrile reaction was slower in developing and attained a much lower peak (Graph II). Differences in the degree of response to the two might be due to the fact that the IPV strain was used at a higher passage level in the study than was the IBR virus. A few atypical lesions were observed in the vulvar mucosa of one of the intranasally exposed animals, but virus was not recovered from the vaginal secretions of this animal. Typical lesions were observed in the case of those exposed vulvovaginally. As shown in Table I, virus was recovered on the fifth day after inoculation from the nasal secretions of the nasally exposed animals and from the vaginal secretions of those exposed by the vulvovaginal route. All were negative for virus on the 11th day after inoculation.

All animals were found to be refractory to either homologous or heterologous challenge, as shown in Graph I, while the control animals displayed the usual signs of infection. However, the more severe symptoms occurred in the controls inoculated with the IBR virus.

Despite quantitative differences in response to the original inoculation with the two virus strains, and the lack of a consistent pattern of spread in the case of each from the site of inoculation to the alternative site of localization (IBR was recovered from both the nasal and vaginal secretions but the IPV strain was isolated only from the area of inoculation), the results of the reciprocal cross challenge inoculations were considered to provide sufficient evidence on which to base the conclusion that, if not identical, the two strains of virus are so closely related as to be considered as such.

**PATHOLOGICAL STUDIES**

Vulvovaginal inoculation of the IBR and the IPV strains of virus produced lesions in the vulvar mucosa that were similar to the IPV lesions reported previously by Kendrick et al. (8). Small white pustules that tended to form over the lymphatic follicles reached maximum development on the second or third day after inoculation. At this time there was a slight purulent discharge from the vagina, while the mucosa was reddened and manipulation resulted in evidence of pain.

Biopsy of the vulvar mucosa from one of the two heifers exposed to each virus was made on either the second or third day after inoculation. The tissues were fixed in Bouin's solution, embedded in paraffin and sections six microns in thickness, were cut. These were stained with hematoxylin-and-eosin.

The lesions observed were similar to those previously reported for IPV. (3) There was a focal necrosis of the epithelium which was followed by infiltration of neutrophiles into the necrotic area, and of lymphocytes into the underlying connective tissue. The epithelial cells on the periphery of the lesions were undergoing degeneration and the nuclear inclusions, characteristic of those associated with IPV infection, were ob-
erved. In other areas of the epithelium, the necrotic process was in the early stages and was evidenced only by the presence of the intranuclear inclusion bodies.

Duplicate coverslip preparations of each virus in cultures of bovine embryo kidney cells were fixed in Bouin's solution when early cytopathogenic changes were noted, and stained with hematoxylin-and-eosin. The degenerating cells in both preparations exhibited intranuclear

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**GRAPH II**

- ——— IPV virus intranasally
- ——— IPV virus introvaginally
- ——— IPV immune-IPV challenge
- ——— IPV immune-IBR challenge

**Response to IPV Strain of Virus by 1) Nasal and 2) Vulvovaginal Route, and Response to Challenge with the Homologous and Heterologous Virus Strain.**
inclusions which in turn were similar to those previously reported for IPV (3) and IBR. (7)

**HOST CELL RANGE OF THE VIRUS STAINS STUDIED**

**Procedure**

Serial monolayer line cultures of dog, sheep (fetal), rabbit, pig, and horse kidney; normal bovine lymph node and lymph node from a cow affected with lymphomatosis; and bovine (fetal) lung, were tested for their susceptibility to each strain of virus. Dilutions of $10^{-2}$, $10^{-3}$, $10^{-4}$, and $10^{-5}$ were inoculated to a series of tube cultures of each of the cell types listed above. The usual procedure of inoculating one ml. of virus dilution to each tube, after removing the nutrient medium, was followed. The inoculated tubes were incubated in stationary racks, and readings were made at 72 and 120 hours after inoculation. Any degree of cytopathogenic activity was regarded as a positive reaction.

**Results**

With the exception of the dog kidney, all cultures supported growth of each strain of virus as indicated by the development of variable degrees of cytopathogenic effects by 72 hours after inoculation. Final readings were made at 120 hours at which time the dog kidney cell cultures were still unchanged. The virus titer varied somewhat in each of the remaining tissues tested although it was essentially identical for each strain of virus.

Since marked differences exist in the susceptible host cell range between many of the animal viruses, the fact that the two strains studied herein possess the identical host cell range is strong evidence that they represent one and the same virus.

**SEROLOGICAL STUDIES**

**Procedure**

The experimental cattle were bled prior to inoculation and again on the 5th, 11th, 15th, and 26th day following exposure. Some of the animals were bled again on the 12th day after challenge, that is, on the 42nd day following the initial exposure to virus.

The blood was held at room temperature until it had clotted, and after overnight refrigeration at 4°C., the serum was separated by centrifugation. It was then dispensed in small ampoules and stored in the frozen state. The serum was not inactivated prior to testing. Antibody was measured by means of the serum-virus neutralization test in tubes of primary or low passage level monolayer cultures of fetal bovine kidney cells. The strains of virus that were used for the inoculation of cattle were also used for the serological testing, but at a higher passage level for the latter purpose. Since these virus strains were found to be very stable when held under dry ice refrigeration, it was possible to use the same batch of seed virus for all the serological tests. Accordingly, a large pool of each strain of virus was distributed in small ampoules, shell frozen, and stored under dry ice refrigeration. The contents of several vials were pooled and titrated, and the dilution containing the amount of virus used in the test (100 TCID$_{50}$ per ml.) was calculated. The virus was retitrated each time a test was made.

Serial two-fold dilutions of serum in tissue culture medium, beginning with the 1-5 dilution, were mixed with an equal volume of virus containing 100 TCID$_{50}$ of virus per ml. Thus, each ml. of serum-virus mixture consisted of serial two-fold dilutions of serum in the presence of 50 TCID$_{50}$ of virus. After holding the mixtures at room temperature for 90 minutes, each series was added in a volume of 1.0 ml to tubes of fetal bovine kidney cells from which the medium had just been withdrawn. A minimum of five tubes was used for each dilution of serum and virus. The serum of each animal was tested with both the homologous and heterologous virus antigen. Readings
Fig. 1.

Comparative Rate of Development in Cattle of Antibody to IBR and IPV Virus Strains, as Measured by the Homologous and Heterologous Virus Strain.
were made at daily intervals, beginning 24 hours after inoculation, and were repeated daily for the next five days. Any degree of cytopathogenic activity was considered to be a positive reaction.

**Results**

The results of the serological testing are presented in Figure I.

The most significant findings were that essentially complete reciprocal cross neutralization reactions occurred, and that the antibody response developed at a comparable rate in the case of each virus strain. Of significance also was the finding that the antibody response was more marked in the case of each strain when the inoculations were made intranasally. It was interesting to note also that in the case of the cattle receiving, the IBR virus, the homologous and heterologous challenge alike resulted in a sharp elevation of the antibody curve, as measured by both strains of virus.

Inasmuch as the serological findings correlated closely with the results of the cross protection tests in cattle, further critical evidence of the relationship of the two virus strains was provided.

**DISCUSSION**

The studies described in this report fully confirm the findings of Gillespie et al. (4) that the virus of IBR and the one causing IPV are one and the same agent. 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. In an endeavour to detect possible minor antigenic differences, attempts were made to produce antibody in roosters to each virus strain so that critical antigenic studies could be conducted. It was found that, unfortunately, the birds gave no antibody response to either strain of virus.

The finding that the IBR virus produces two distinct clinical syndromes in cattle is of considerable significance from the standpoint of epizootiology as it undoubtedly provides an explanation for certain findings made in the past which could not be accounted for on the basis of the information then available. It accounts, for example, for the presence of IBR antibody in the serum of cattle in New York state in 1957, and also in serum obtained 16 years earlier from cattle in New Jersey — in the absence of clinical signs of IBR in the eastern part of the United States at any time. It now appears that these antibodies resulted from infection of the genital tract with the IBR virus which occurred as a condition now referred to as infectious pustular vulvovaginitis.

It is not surprising to the authors that the IBR virus did not produce clinical manifestations of respiratory infection in cattle in the eastern sections of the United States comparable to that which it causes in cattle in the western part of the country. Based on their observations that the respiratory infection is more severe in beef breed animals than indairy cattle, and that it occurs in its most serious form in large concentrations of cattle, they have held the view that IBR is primarily a disease peculiar to large feedlot operations such as those located in the western part of the country. Under conditions existing therein the virus has an excellent opportunity to pass rapidly from animal to animal. In so doing it undoubtedly acquires enhanced virulence which, together with the greater susceptibility of beef animals to respiratory infection with this virus enables it to produce a severe form of respiratory disease.

Undoubtedly the virus produced genital tract infection of cattle in the eastern part of the United States for a long period of time. However, while the studies reported herein suggest that the agent might be transmitted more
readily from the genital tract to the respiratory passages than in the reverse direction, it is visualized that, in the absence of circumstances conducive to extensive and widespread respiratory system exposure in herds of dairy cattle in the east, the infection remained localized in the genital tract, with little opportunity for the virus to spread from one herd to another. Possibly, too, the influence of stresses associated with breeding might also result in enhanced susceptibility of the genital tract of dairy cattle to infection with this virus.

The studies reported herein indicate that the virus might occasionally reach the genital tract from the respiratory passages of cattle, suggesting that IPV can occur in feedlots. However, this possibility has not, thus far, been investigated. While, as suggested above, susceptibility to vaginal infection might be conditioned by physiological factors associated with reproduction, these factors would be largely absent in feedlot heifers, which are essentially reproducitively inactive. Also, the rapid spread of the virus by respiratory tract transmission would tend to result in the rapid development of immunity in feedlot cattle. It would appear, therefore, that there is less likelihood for the virus to produce genital infection in feedlot cattle than infection of the respiratory tract.

Should, as clinical and histopathological studies suggest, IPV be the same disease as coital vesicular exanthema as it occurs in Europe, another fascinating example is provided of the versatility of viruses in modifying their biological behaviour and disease producing potential. After 75 years, and possibly more, of causing a genital infection of cattle, the virus suddenly manifested itself under an entirely different clinical and epizootiological guise. As we appreciate more fully the role played by

the unusual and the unexpected in the activities of viruses, might it not be advisable, at least in the case of some of the new diseases that have been reported recently, to look into the possibility that they might not be new diseases in the true sense of the word but, rather, new manifestations of disease caused by viruses that have been recognized over a long period of time.

**SUMMARY**

The authors discuss some of the more apparent epizootiological features of the two clinical conditions produced by the IBR virus, and speculate as to the factors which resulted in the agent assuming a dual pathogenic role in infection of cattle.

**ACKNOWLEDGMENTS**

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**REFERENCES**