Infectious Bovine Rhinotracheitis Virus Infection in Bulls, with Special Reference to Preputial Infection

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Received for publication 25 May 1973

In an experiment with infectious bovine rhinotracheitis (IBR) virus in two bulls, observed over a period of 122 weeks, the pattern of virus release was studied. Recurrent, unprovoked release of virus was demonstrated after one year in a nasal washing from one of the bulls and in preputial washings of both on 13 and 4 occasions, respectively, and finally in weeks 113 and 110, although clinical disease was not observed. During periods of recurrent virus release, concentrations of virus in the prepuce were generally much lower than during the period of primary infection; usually, however, they were not of negligible titer. The frequency of such periods and the virus titers observed strongly suggest that an IBR antibody carrier should always be considered as a potential source of infection to other animals. When virus was demonstrated in semen an almost equal amount was found in the preputial washing (50 ml). In week 120, virus replication in the respiratory tract and prepuce was induced in both bulls by prednisolone injections. It is concluded that antibody carriers will rarely attain a state of absolute immunity.

The first outbreak of infectious bovine rhinotracheitis (IBR) infection with preputial manifestations in a Danish artificial insemination (AI) center was diagnosed in 1969 (V. Bitsch, E. H. Autrup, and E. Blom, Proc. 11th Nordic Vet. Congr., Bergen, 1970, p. 256). Only three such outbreaks in AI centers were reported before this (2, 5, 10), with one additional outbreak having been reported since then (3).

The situation in Denmark called for measures to control IBR infection in bulls at AI centers. Since, however, nothing was published on the control of IBR virus infection in AI centers, it was felt there was a need for such studies.

This work deals with an infection experiment in two bulls with IBR virus which was started in March 1969 soon after the above-mentioned outbreak. Special interest was attached to the periodic excretion of virus from infected animals. Studdert et al. (11) reported recovery of virus from a bull 26 days after infection, whereas Snowdon (8), in a bull infected experimentally and examined over a period of 578 days, isolated virus on five occasions after the acute phase, the last time being 361 days after inoculation. In 1969, Kubin (6) reported the recovery of virus from the prepuce of a bull which had neutralizing antibodies in his serum. Virus was demonstrable in two periods 58 days apart and again 1 month later after treatment with prednisolone. In Denmark, Bitsch et al. (V. Bitsch, E. H. Autrup, and E. Blom, Proc. 11th Nordic Vet. Congr., Bergen, 1970, p. 256) demonstrated virus in one or more periods after the acute phase of infection in preputial washings collected from 11 naturally infected bulls, the last isolations being from two bulls more than 210 days after infection.

With the present experiment our intentions were, with special regard to the preputial infection, to examine the frequency of intermittent virus release and to measure the amounts of virus excreted in order to estimate the risk of virus transmission to other animals and the risk of obtaining virus-contaminated semen from antibody carriers. Our final purpose was to determine if the virus carrier state, with periodic release of virus, was a phenomenon that might be of life-long duration.

MATERIALS AND METHODS

Animals. Two bulls, bull A aged 3 years and bull B aged 2.5 years, of the Danish Red Dairy Breed, were used. During the whole examination period they were housed side by side in a separate room close enough to permit actual contact.
Virus. A preputial washing from a naturally diseased bull at the above-mentioned center (strain HBV1-DaB69), filtered through a Seitz EKS asbestos pad (virus p.), and the first passage of this strain (virus p.) in primary calf kidney cell cultures were used for inoculation.

Tissue cultures. Primary or secondary kidney cell cultures from newborn calves were used. The maintenance medium was Earle solution with 0.5% lactalbumin hydrolysate, 0.01% yeast extract, 100 IU of penicillin, 0.1 mg of streptomycin per ml, and 2% serum from a colostrum-deprived calf that was reared in isolation.

Inoculation. Bull A was infected intranasally with 2 x 10^4 mean tissue culture infective doses TCID_{50} of virus p. and by the prepuce with 2 x 10^4 TCID_{50} of virus p. Bull B was assumed to acquire a nasal infection by contact with bull A. On day 43 he was infected by the prepuce with 2 x 10^4 TCID_{50} of virus p. 

In week 119, on days 831, 833, and 834, both animals received intramuscular injections of 500 mg of prednisolone (prednisolonum, WHO).

Period of examination. The inoculation of bull A took place on March 3, 1969 (day 1), and the bulls were observed up to July 1, 1971 (day 851, week 122) when they were slaughtered.

Samples. Nasal and preputial washings, blood samples, and semen ejaculates were collected. After 2 months, except in weeks 52 to 53 and 120 to 122, nasal swabs were taken instead of washings. To collect nasal washings, approximately 10 ml of maintenance medium was used. A medium with 3% Thiol Broth (Difco) was used for preputial washings, in quantities of about 50 ml. After 1 year the washing medium was changed to phosphate-buffered saline (PBS) with 2% calf serum. Semen was collected in an artificial vagina, usually two separate ejaculates being obtained from each animal.

Samples, apart from semen ejaculates, were collected daily or every second day during the acute phase, and then once a week. After about 6 months, however, preputial washings were taken at least twice a week. Semen samples were taken at irregular intervals over the whole period, except during the first 2 weeks, when such samples were taken approximately every second day.

Body temperatures were recorded daily during the whole period.

Virological examination. Each of three tubes of calf kidney cells was inoculated with 0.2 to 0.5 ml of each sample. After 1 h of incubation at 37 C, the inoculum was discarded and fresh medium was added to the cell cultures. When semen was examined, however, inoculum was discarded after 30 min at room temperature.

Until week 63, all samples were examined in two serial passages, but after that time no second passage was made.

In cases of virus isolation, a titration was made on the original material (washings) which had been stored at −55 C. After preparation of serial 10-fold dilutions, each of two tubes, or for preputial washings preferably three tubes, was inoculated with 0.1 ml per dilution.

Identification of virus isolates was carried out in a neutralization test with rabbit immune serum.

VNA. Serum samples were stored at −20 C and inactivated at 56 C for 30 min. The seventh passage of the strain HBV1-DaB69 and twofold dilutions of serum samples were used in the examination for virus-neutralizing antibodies (VNA). Each of two tissue culture tubes was inoculated with 100 TCID of virus and 0.1 ml serum dilution, the virus-serum mixture having been preincubated for 1 h at 37 C. To determine serum titers below 1, an additional examination was performed with 10 TCID of virus and undiluted serum (1). Titers were calculated as 50% end points.

RESULTS

Clinical findings. Both animals developed a distinct rhinitis with hyperemia of the nasal mucosa, excessive nasal discharge, and marked salivation. Symptoms appeared 1 to 2 days later in bull B than in bull A, and bull A showed an appreciable loss of appetite on days 7 to 9. Bull A developed a unilateral conjunctivitis, but virus isolation was not attempted.

In bull A, congestion of the mucosa of the penis and prepuce was noted on days 3 and 5. In addition many small areas showed marked hyperemia. On day 8 the mucosa was covered with numerous pustules. On days 9 and 10 the bull refused to mount, and up to day 12 handling of the prepuce seemed very painful to him. On day 13 a few ulcers, 10 to 13 mm in diameter and with raised edges, were seen at the mucosal reflection. Even as late as day 23, many small hyperemic areas were seen in the mucosa.

After inoculation of virus into the prepuce of bull B on day 43 there was no rise in temperature, and hyperemia of the preputial mucosa was the only change noted.

Bull A showed a rise in body temperature up to 39.0 to 40.8 C on days 4 to 9, with a maximum on day 6. Bull B had temperatures from 39.0 to 39.6 C on days 5 to 8, with a maximum on day 8.

In week 120, after treatment with prednisolone (see Fig. 4) a slight rise in temperature, 0.3 to 0.6 C, was noted in both bulls and was accompanied by some nasal discharge. In bull A growth of virus in the prepuce was associated with diffuse congestion and focal hemorrhages of the mucosa, but no pustules were observed.

During periods of unprovoked virus release there was no rise in temperature (bull B, weeks 52–53, see below), and no pathological changes in the prepuce were noted.

Virological findings. After inoculation, virus was demonstrated in nasal washings of bull A for 12 days and also on day 17 (March 19) as
recorded in Fig. 1. In bull B, which became infected by contact with bull A, virus was not demonstrated until day 6 (March 8), and excretion lasted for 10 days (Fig. 1). Whereas, in the case of bull A, virus was not recovered again from nasal washings or swabs until week 120, virus was demonstrated once (week 53) in a nasal washing of bull B. In week 52 the body temperature of this bull suddenly rose to 40 C and remained between 39 and 40 C for the following 9 days, and a slight tympanitis developed. Treatment with antibiotics did not influence this condition. During the febrile period nasal washings were taken every second day, and virus was demonstrated in a washing from the sixth day, i.e., 1 year after the start of the experiment. On reisolation the titer was found to be just 1 TCID\textsubscript{50} per 0.1 ml.

From preputial washings of bull A virus was isolated regularly for 30 days after inoculation (Fig. 1), and virus multiplication in the prepuce was demonstrated in 13 later periods before week 120 (Fig. 2).

No virus was found in preputial washings of bull B taken before day 43 (April 14; Fig. 1). After inoculation on day 43, virus was present in washings for 7 days, and up to week 120 an additional 4 periods of virus excretion in the prepuce were recorded (Fig. 3).

In week 120, after the prednisolone injections, virus was demonstrated in the upper respiratory tract and the prepuce of both animals and in relatively high concentrations except in the prepuce of bull B (Fig. 4).

In summary, virus was demonstrated in 43 preputial washings out of 230 from bull A and in
17 out of 237 from bull B, i.e., in 60 out of 467 samples. The longest period between preputial virus isolations was 163 days (weeks 53–75) in the case of bull A and 279 days (weeks 70–190) in the case of bull B, and the shortest time between periods with fairly good virus replication was, respectively, 12 days (weeks 31–32) and 72 days (weeks 8–17).
Virus was recovered from only seven of the semen samples collected from bull A and from one sample from bull B (Table 1). Virus isolates examined in neutralization tests were all fully neutralized. Not all isolates were tested when virus was recovered from a series of samples; in such cases, however, at least one isolate was examined.

VNA. The VNA titers of all serum samples are recorded in Fig. 2 and 3. In Fig. 1 and 4 relevant titers are repeated. Extremely high antibody levels were obtained in both animals after the provoked virus growth in week 120, whereas unprovoked virus replication caused a very small or, in most cases, no rise in VNA titer.

**DISCUSSION**

**Nasal infection.** The rhinitis, the virus excretion, and, in bull B, the rise in temperature are in accordance with what is described elsewhere as being characteristic of uncomplicated nasal infections (7). Thus, virus spread from an infected AI center should be considered capable of producing clinical respiratory disease.

The recurrent presence of virus in nasally infected cattle was demonstrated by Snowdon.
In the present study it was found to occur in week 53 in bull B with no other symptoms than a febrile reaction lasting several days. No certain conclusions, however, can be drawn concerning the relationship between virus isolation and the presence of fever.

The excretion of virus in week 120 (Fig. 4)—in high titers, accompanied by nasal discharge, and followed by a rise in antibody titers—was simultaneous in the two animals, which would seem to exclude the possibility that in this case one bull had in fact been infected by contact with the other. Thus, the prednisolone treatment served to prove that virus was still present in the respiratory tract 120 weeks after primary infection.

Preputial infection. The clinical picture in bull A during the primary phase of infection showed no deviation from that of bulls at the infected center or from descriptions in the literature (4, 8, 11). Excretion of virus continued for about 1 month as against the usual 8 to 10 days (9, 11). The sudden fall in VNA titer in week 4 (Fig. 2), which coincided with renewed virus growth and which was followed by another rise in VNA titer, might be explained by the unusually long primary phase of infection (lasting about 3 weeks) followed by an early period of recurrent virus multiplication.

When bull B was infected by the prepuce on day 43, he had a serum antibody titer of 2.8 but was not refractory to infection.

Previously it was pointed out that, during periods of recurrent virus excretion, the concentration of virus in the prepuce is considerably lower than during the acute phase of infection (V. Bitsch, E. H. Autrup, and E. Blom, Proc. 11th Nordic Vet. Congr., Bergen, 1970). This has been confirmed in the present study. A maximum titer of $10^4$ TCID$_{50}$ per 0.1 ml was demonstrated in a preputial washing from week 2, while thereafter, apart from the replication caused by prednisolone, the titer was in no case found to be higher than $10^3$, i.e., about 30 times lower. Titors of $10^2$ and $10^3$ TCID$_{50}$ per 0.1 ml have been found in 2 naturally infected, but as yet serologically negative, bulls. However, in 25 virus-positive preputial washings from naturally infected antibody carriers, titers were in no case higher than $10^2$, and usually lower, the average being just below $10^2$ TCID$_{50}$ per 0.1 ml (V. Bitsch, unpublished). During several periods these lower virus titers were nevertheless of a magnitude to suggest that, without certain precautions, antibody carriers in AI centers constitute a severe threat of infection to other bulls.

In regards to the natural occurrence of virus in semen, there seems to be no other reasonable explanation than that it is derived from the mucosa of the penis and prepuce. Regarding a relationship between amounts of virus in the prepuce and semen, Table 1 illustrates that, when expressed in TCID$_{50}$ per 0.1 ml, titers were of about the same level in preputial washings and semen of the two experimentally infected bulls, perhaps a little lower in semen. With low concentrations of virus in the prepuce, there was a small or nondemonstrable content of virus in the semen. Similarly, in the cases of the two naturally infected bulls mentioned above, and which had preputial virus titers of $10^2$ and $10^3$ TCID$_{50}$ per 0.1 ml, respectively, the corresponding titers in semen were $10^2$ and $10^3$ per 0.1 ml.

Virus was excreted while VNA was present in the serum. However, since in certain infections immunity is better correlated with the local antibody level than with serum antibodies (12), this is not contradictory. (In this respect it may be of interest that the average virus-neutralizing effect of semen, measured in more than 40 samples of each animal from more than 20 different weeks, was eight times lower in bull A than in bull B. In seven cases after the first 2 weeks of the examination period (Table 1), when semen and virus-containing preputial

### Table 1. Comparison of virus concentrations in semen and prepuce

| Bull | Week no. | Date       | Ejaculate no. | Virus titer: preputial washing |
|------|----------|------------|---------------|-------------------------------|
| A    | 1        | 5 Mar      | I 10^4        | 6/3: 10^4                    |
|      | 1        | 5 Mar      | II 10^4       | 8/3: 10^4                    |
|      | 2        | 10 Mar     | I 10^4        | 9/3: 10^4                    |
|      | 3        | 18 Mar     | I 10^1        | 11/3: 10^4                   |
|      | 29       | 19 Sept    | I 10^2        | 17/3: 10^4                   |
|      | 46       | 15 Jan     | I 10^-2       | 19/3: 10^2                   |
|      | 47       | 22 Jan     | I 10^-2       |                               |
|      | 52       | 26 Feb     | I 10^-2       |                               |
|      | 114      | 3 May      | I 10^1        |                               |
|      | 120      | 18 June    | I 10^1        |                               |
| B    | 7        | 18 Apr     | I 10^4        | 10^1                         |
|      |          |            | II 10^4       |                               |

*TCID$_{50}$ of virus per 0.1 ml of semen or washing.
* After treatment with prednisolone.
washings of bull A were collected simultaneously, there was a very low or nondemonstrable virus-neutralizing effect of semen. This phenomenon needs clarification. Examinations so far have indicated that the effect is not of definite IBR specificity.) Nevertheless, it was surprising to us that a serum VNA response was not regularly seen after formation of even greater amounts of virus.

Kubin (6) succeeded in provoking replication of virus in the prepuce of a naturally infected bull by injections of prednisolone. This was reproduced in the present work in which the prednisolone caused viral replication concurrently in the respiratory tract and the prepuce of both bulls.

The observations reported here, together with publications referred to above, give good reasons to expect life-long persistence of virus in infected animals. Yet it may be possible for some animals to reach a permanent immunological state which will prevent recurrent virus multiplication. Virus replication patterns of bulls A and B were highly different, and greater variations must be expected in field cases. It is not feasible, however, to demonstrate a possible absolute immunity, not even by the use of prednisolone, so any antibody carrier must be regarded as a potential threat of infection to uninfected animals.

Danish measures for control of the IBR infection (V. Bitsch, E. H. Autrup, and E. Blom, Proc. 11th Nordic Vet. Congr., Bergen, 1970, p. 257) are directed towards its eradication in AI centers, and the present study has strongly emphasized the importance of the rule that antibody carriers should be kept out of direct or indirect contact with uninfected animals and removed as soon as practicable.

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