The Effect of Dose, Route and Virulence of Bovine Herpesvirus 1 Vaccine on Experimental Respiratory Disease in Cattle

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ABSTRACT

Three experiments were conducted with calves in which, following intramuscular or intranasal vaccination with virulent or attenuated bovine herpesvirus 1, calves were protected against bovine herpesvirus 1 — *Pasteurella haemolytica* challenge. Calves receiving low doses of vaccine had lower levels of antibody and greater evidence of virus replication upon challenge than those receiving higher doses. In contrast 11/13 unvaccinated controls had fibrino-purulent pneumonia following challenge. The immune response developed later in younger calves and those given low doses of vaccine. Neutralizing antibodies to bovine herpesvirus 1 were not found in nasal secretions, but were present in serum seven days after vaccination. Bovine herpesvirus 1 was isolated before challenge from nasal secretions of calves vaccinated intranasally or intramuscularly with virulent virus but not those vaccinated intramuscularly with vaccine virus.

It was concluded that both routes of vaccination with either virulent or attenuated bovine herpesvirus 1 provided protection from challenge with homologous or heterologous bovine herpesvirus 1 and that live vaccines should contain at least 10^3 plaque forming units/dose for effective immunization.

RÉSUMÉ

Cette étude consistait à effectuer trois expériences avec des veaux qui s'avérèrent protégés contre une infection de défi par l'herpèsvirus bovin du type #1 et *Pasteurella haemolytica,* à la suite d'une vaccination intramusculaire ou intranasale avec une souche virulente ou atténuée de ce virus. Les veaux qui reçurent les doses de vaccin les plus faibles affichèrent aussi les taux d'anticorps les plus bas et démontrèrent une évidence plus grande de la réplication du virus, lors de l'infection de défi, comparativement à ceux qui avaient reçu les doses de vaccin les plus fortes. Par ailleurs, 11 des 13 témoins développèrent une pneumonie fibrino-purulente, à la suite de cette infection de défi. La réaction immunitaire se développa plus tard chez les veaux les plus jeunes et chez ceux qui avaient reçu les doses de vaccin les plus faibles. On ne détecta pas d'anticorps neutralisants spécifiques à cet herpèsvirus, dans les sécrétions nasales, mais on en décela dans le sérum, sept jours après la vaccination. Avant l'infection de défi, on isola l'herpèsvirus bovin du type #1 dans les sécrétions nasales des veaux vaccinés par la voie intranasale ou intramusculaire avec une souche virulente de ce virus, mais non dans celles des veaux vaccinés par la voie intramusculaire, avec une souche atténuée du même virus.

Les résultats de ces expériences amenèrent les auteurs à conclure que la vaccination par l'une ou l'autre route avec une souche virulente ou atténuée de l'herpèsvirus bovin du type #1 protège contre une infection de défi avec une souche homologue ou hétérologue de ce virus et que les vaccins vivants doivent contenir au moins 10^3 unités formatrices de plages/dose, pour conférer une immunité efficace.

INTRODUCTION

Routine production of respiratory disease by exposure to aerosols of bovine herpesvirus 1 (BHV1) and *Pasteurella haemolytica* (1) permits study of BHV1 vaccine efficacy and comparisons of intramuscular (IM) and intranasal (IN) vaccines. These routes were compared previously (2, 3, 4) by challenge with BHV1 only. Criteria for efficacy were: stimulation of antibodies to BHV1 in serum and nasal secretions, viral isolation from nasal passages (2, 4), interferon response and local and systemic cell mediated immune responses (3). In this study, the efficacy of BHV1 vaccination against challenge with virulent BHV1 and *P. haemolytica* was tested (5, 6). These experiments were designed to evaluate and compare: vaccine virus doses; virulent and attenuated virus; intranasal and intramuscular routes of

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vaccination; the serum (SNA) and nasal neutralizing antibody (NNA) response; and subsequent susceptibility to respiratory disease when challenged with BHV1 and *P. haemolytica*.

MATERIALS AND METHODS

ANIMALS, IMMUNIZATION AND CHALLENGE

Hereford and Hereford-Angus cross three to four month old (experiment I) and eight to 10 month old (experiment II and III) calves were used and derived from a herd without history of respiratory disease (5). Groups of calves were kept in isolated pens removed from other livestock. On the day of viral challenge all groups of each experiment were mixed in one pen.

Day of vaccination was designated as day 0. Calves were challenged with virulent BHV1 (isolate 108) on days 27, 36 and 42 in experiments I, II and III respectively. The intervals between viral challenge and *P. haemolytica* exposure and *P. haemolytica* exposure and slaughter were four days in each experiment. On day 0 all calves were BHV1 seronegative and at the time of *P. haemolytica* exposure sera of only six calves (three of each experiment II and III) had complement fixing antibodies (1/8 to 1/16) to this organism. The details of the three experiments were as follows:

Experiment I — On day 0, 16 calves were weaned and divided into four groups, three of which were vaccinated intranasally (IN), (at least 9 cm into the nostrils). One group (n=4) was inoculated with 10^4 plaque forming units (PFU)/-animal of BHV1 in commercial vaccine[1] including parainfluenza-3 virus (1 mL into each nostril), a second (n=4) with 10^6 PFU/animal of BHV1 grown from this attenuated vaccine[1] (2.5 mL into each nostril) and a third (n=4) with 10^6 PFU/animal of virulent BHV1 (2.5 mL into each nostril). The control group (n=4) was not vaccinated (Table I).

Experiment II — Eighteen animals, which had been weaned 14 wk earlier were divided into three groups on day 0. One group (n=7) received a vaccinating inoculum of 2×10^6 PFU/animal of virulent BHV1 administered IN (1 mL into each nostril), the second (n=7) was given the same dose of virus IM. The third group (n=4) served as unvaccinated controls (Table II).

Experiment III — Twenty-seven calves which had been weaned 7 wk earlier were divided into six groups (Table III). One (n=5) was inoculated with 60 PFU/animal (low dose) IN, a second (n=5) with 6×10^3 PFU/animal (medium dose) IN, a third (n=5) with 2×10^6 PFU/animal (high dose) IN, a fourth (n=3) with 6×10^3 PFU/animal (medium dose) IM and the fifth group (n=4) with 2×10^6 (medium dose) IM of attenuated BHV1 cultured from a commercial vaccine[1] (Table III). Each inoculum was 2 mL and the IN vaccinates received 1 mL into each nostril. The sixth group (n=5) served as controls.

Each animal was challenged by exposure for 5 min to an aerosol generated from a suspension of virulent BHV1 using a Henderson apparatus as described previously

TABLE 1. BHV1 Inoculations and Serum Neutralizing Antibody Titers of Calves in Experiment I

BHV1 vaccination			No. of animals	Antibody measured on days postvaccination				
Type	Route	Dose[a]		3	7	14	21	27[b]
Attenuated[c]	IN[d]	10^4	4	<2[e]	0-2(3)[f]	0-32(3)	0-64(3)	0-64(3)
Attenuated	IN	10^6	4	<2	0-4(1)	0-64(3)	0-128(3)	0-64(3)
Virulent	IN	10^6	4	4	0-4(3)	24-128	32—128	32-128
Controls	—	—	4	0	0	0	0	0

[a]Plaque forming units per animal
[b]Virulent BHV1 challenge
[c]Commercial vaccine also contained parainfluenza-3 virus
[d]Intranasal
[e]Titers are reciprocals of serum dilution given as range where applicable
[f]Number of calves with antibody in parenthesis

TABLE II. BHV1 Inoculations and Serum Neutralizing Antibody of Calves in Experiment II

BHV1 vaccination			No. of animals	Antibody measured on days postvaccination				
Type	Route	Dose[a]		7	9	23	35[b]	40
Virulent	IN[c]	2×10^6	7	0	4-16[d]	32-256	16-128	8-128
Virulent	IM[c]	2×10^6	7	0	0-16(6)[f]	8-32	16-128	8-128
Controls	—	—	4	0	0	0	0	0

[a]Plaque forming units per animal
[b]Virulent BHV1 challenge
[c]Intranasal
[d]Titers are reciprocals of serum dilution given as range
[e]Intramuscular
[f]Number of calves with antibody in parenthesis

[1]Contravac, Connaught Laboratories, Willowdale, Ontario.

(1). The viral suspensions contained 10^7 $TCID_{50}$/mL in experiments I and II and 10^8 $TCID_{50}$/mL in experiment III. Four days after viral challenge, each calf was exposed to an aerosol of *P. haemolytica*. The bacterial aerosol was delivered directly from the Collison nebulizer into the face mask as described previously (6). The bacterial suspensions from which the aerosol was produced contained 3.3×10^7 organisms/mL in experiment I, and $2.5\text{-}3.6 \times 10^5$ organisms/mL in experiment II and III. For each calf 1.6 ± 0.2 mL of viral and bacterial suspensions were aerosolized. Four days after *P. haemolytica* challenge, calves were killed and their respiratory tracts were examined for lesions and the presence of virus or bacteria.

Differences such as age of calves, date of weaning, dose and type of vaccine virus, timing of viral challenge, and viral and bacterial challenge doses, prevented comparison of the results between experiments.

SAMPLE COLLECTION

In experiments I and II, nasal secretions were collected by aspiration of nasal secretion (1 mL) from the anterior nasal passage into glass vials with a soft rubber tube under 1.76 kg cm^2 vacuum. These samples were used for the determination of NNA to BHV1 and for viral isolation. Secretions and serum for neutralizing antibody determinations were collected on nine days throughout experiment I. In experiment II secretions were collected on seven days between day 0 and 16 and serum on 13 days throughout the experiment. In experiment III serum only was collected on eight days throughout the experiment.

VIRAL AND BACTERIAL EXAMINATIONS

In experiment I, nasal swabs were not taken but bronchial lymph nodes were examined for the presence of BHV1. In experiment II nasal swabs were examined for BHV1 on five days between day 17 and the end of the experiment, and for *P. haemolytica* on days 0, 7 and 40. In experiment III, nasal swabs were examined for BHV1 on eight days up to and including the day of viral challenge, and for *P. haemolytica* on days 0, 42 and 50. In experiments II and III, samples of trachea and lung were assayed for the presence of virus and bacteria as described previously (1).

VIRUS CULTURE AND TESTS

Georgia bovine kidney (GBK) cells were cultured in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum (FBS). Each litre was supplemented with 2 mM glutamine,[2] 25 mg of gentamycin,[3] 2.5 g of sodium bicarbonate and nonessential amino acids.[4]

An attenuated bovine herpesvirus 1 (BHV1) from a commercial vaccine[1] and a virulent BHV1 (isolate 108) were cultured as described previously (7, 8).

Two methods for detecting neutralizing antibodies were employed. The method used in experiment I consisted of mixing 200 PFU of BHV1 with twofold dilutions of heat inactivated serum or nasal secretions as described previously (8). The virus-antibody mixture was incubated for one hour at 37°C before it was added to duplicate wells in a Microtiter plate[5] containing GBK cells. After a one hour absorption period, the unabsorbed virus was removed and replaced with MEM + 2% fetal bovine serum and anti-BHV1 serum. Forty-eight hours later cultures were stained and the antibody titer was determined (9). This method was used to test for SNA and NNA in experiment I. A second method described previously (7) was used for NNA in experiment I and II and for SNA in experiments II and III. Thus both methods were used to test for NNA in experiment I.

ANTIBODY DEPENDENT CELL-MEDIATED CYTOTOXICITY TEST

Assays for antibody dependent cell-mediated cytotoxicity (ADCC) assays were performed only for experiment I, as described previously (10). Serial dilutions of serum were added in 20 μL volumes

TABLE III. BHV1 Inoculations and Serum Neutralizing Antibody Titers of Calves in Experiment III

BHV1 vaccination			No. of	Antibody measured on days postvaccination				
Type	Route	Dose[a]	animals	7	11	18	42[b]	50
Attenuated	IN[c]	60	5	0	0	4-64[d]	8-16	64-128
Attenuated	IN	6×10^3	5	0	0-4(1)[e]	4-64	4-128	128
Attenuated	IN	2×10^6	5	0	0-8(4)	32-128	8-64	128
Attenuated	IM[f]	6×10^3	3	0	4	16-32	8-16	128
Attenuated	IM	2×10^6	4	0	8-32	4-128	16-32	128
Controls	—	—	5	0-4(1)	0	0-8(1)	0	0-32(4)

[a]Plaque forming units per animal
[b]Virulent BHV1 challenge
[c]Intranasal
[d]Titers are reciprocals of serum dilution given as range where applicable
[e]Number of calves with antibody in parenthesis
[f]Intramuscular

[2]Gibco, No. 503, Grand Island, New York.
[3]Schering Diagnostics, Pointe Claire, Quebec.
[4]Gibco, No. 114, Grand Island, New York.
[5]No 3040 Falcon Plastics, Becton, Dickinson & Co., Cockeysville, Maryland.

to quadruplicate Microtiter wells containing 100 μL of ^{51}Cr-labelled BHV1 infected GBK cells and 100 μL of bovine mammary polymorphonuclear neutrophils (PMN). The PMN were obtained by stimulation of the mammary gland with 5 μg of *Escherichia coli* lipopolysaccharide (0128.B12)[6] as described previously (11). The PMN:target cell ratio was 50:1. Controls included wells from which antibody or PMN were omitted. Results were expressed as the highest dilution of serum that sensitized target cells and thus allowed specific release of ^{51}Cr in a 12-hour assay.

TABLE IV. Frequency of Viral-bacterial Pneumonia and Extent of Pneumonic Changes in Calves Challenged with BHV1 and *P. haemolytica*

Experiment	BHV1 vaccination			No. of calves with pneumonia	Percent of pneumonic tissue
	Type	Route	Dose[a]		
I	Attenuated[b]	IN[c]	10^4	3/4	<1
	Attenuated	IN	10^6	3/4	0-1
	Virulent	IN	10^6	0/4	0
	Controls	—	—	3/4	0-50
II	Virulent	IN	2×10^6	0/7	0
	Virulent	IM[d]	2×10^6	0/7	0
	Controls	—	—	4/4	5-35
III	Attenuated	IN	60	1/5	2
	Attenuated	IN	6×10^3	0/5	0
	Attenuated	IN	2×10^6	0/5	0
	Attenuated	IM	6×10^3	0/3	0
	Attenuated	IM	2×10^6	0/4	0
	Controls	—	—	3/5	0-15

[a]Plaque forming units per animal
[b]Commercial vaccine also contained parainfluenza-3 virus
[c]Intranasal
[d]Intramuscular

RESULTS

CLINICAL RESPONSE

On the fourth day after viral challenge, the day on which rectal temperatures peak in susceptible calves in response to BHV1 exposure (7), temperatures were normal in all groups vaccinated with at least 10^4 PFU/animal of virulent or attenuated virus. On the same day average group temperatures were elevated to 40 and 40.3°C in calves vaccinated with 10^3 PFU/animal or less in experiment III and in control groups these temperatures were 40, 41.0 and 41.1°C in experiment I, II and III respectively.

PATHOLOGICAL FINDINGS

Nine of the 13 controls in the three experiments had necrotic and purulent tonsillitis. Four of these also had necrotic and purulent tracheitis and 10/13 had purulent lobar (viral-bacterial) pneumonia, some with fibrinous exudate. Two of five controls in experiment III were without such lesions. The extent of pneumonic lesions was estimated to range from 1-50% of total lung volume (Table IV). One control calf died two and another four days after the *P. haemolytica* aerosol.

None of the 18 calves vaccinated with virulent BHV1 in experiments I and II developed fibrinopurulent respiratory disease. Only a few 1-3 cm^2 surface area foci of atelectasis were seen in lungs of 5/14 of these animals (2 IM, 3 IN) in experiment II and in 3/4 in experiment I.

Following vaccination with attenuated virus single or multiple 2-5 cm^2 foci of atelectasis were seen in 3/15 IN and 5/7 IM vaccinates in experiment III and in 6/8 IN vaccinates in experiment I. None of these vaccinates had purulent (bacterial) reactions in the upper respiratory tract. However, purulent pneumonia (25% of the left posterior cranial lobe) was evident in one IN vaccinate given low-dose attenuated virus in experiment III. Six of eight calves vaccinated with medium or high doses of attenuated virus in experiment I, showed traces of purulent pneumonia involving up to 1% of lung tissue only. Macroscopically recognizable focal atelectatic changes occurred in both IN and IM vaccinates.

EFFECT OF VIRAL DOSE AND ROUTE OF ADMINISTRATION ON ANTIBODY RESPONSE

In all groups a broad range of SNA titers was observed in vaccinated calves (Tables I-III). Despite intranasal inoculation with 10^4 or 10^6 PFU of attenuated BHV1 vaccine virus, two animals in experiment I did not develop any detectable antibody as measured by ADCC (Fig. 1) or by viral neutralization. The earliest antibody responses were observed between day 7 and day 11 regardless of whether neutralization assays (Tables I-III) or more sensitive ADCC assays were used (Fig. 1). In general, animals receiving high doses of virus developed high levels of antibody at an earlier time after vaccination than those receiving low and medium doses of virus. The onset of this response did not appear to be influenced by the route of vaccination, but rather by the dose of virus administered. Thus, SNA were present by day 11 in 17/18 calves given high doses of virulent virus and in 11/13 calves given a high dose of attenuated virus, whereas following administration of low or medium doses of vaccine only 8/17 vaccinates had a neutralizing titer by this time.

In experiment II, significant differences in SNA responses were not observed between groups given high dose virulent BHV1 IN or IM (Table II). Similarly in experiment III in the two groups given high dose attenuated virus IN or IM, SNA responses were detected by day 11 and reached comparable mean titer levels in both groups by day 18 (Table III). When animals

[6]Difco, Detroit, Michigan.

in this experiment were challenged on day 42, all produced a strong anamnestic response even if the initial titer was relatively low [as in the case of animals immunized with low levels of virus (Table III)].

Nasal neutralizing antibody was tested only in experiment I and II, and none was found.

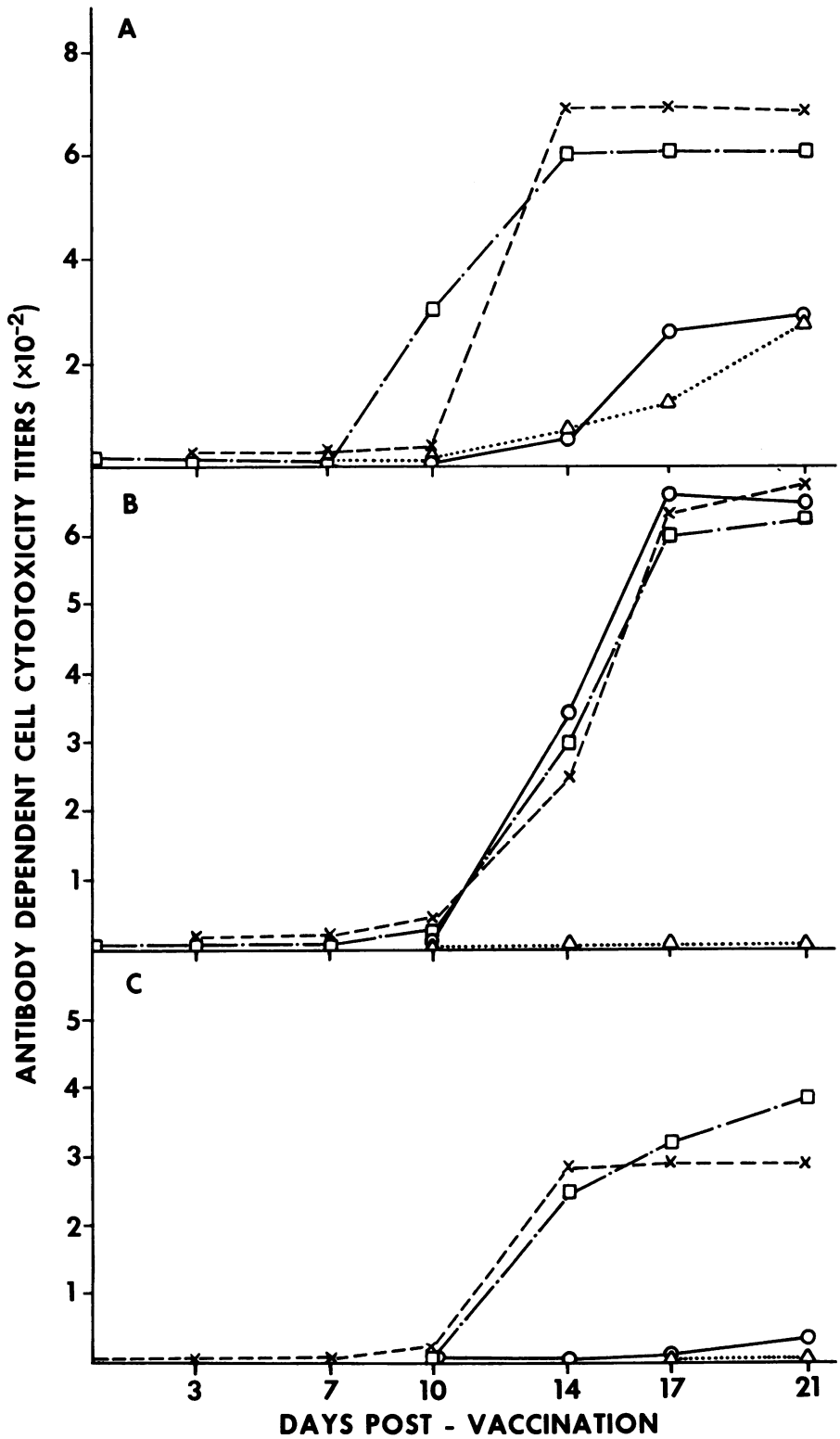

Fig. 1 Antibody dependent cell cytotoxicity titers in experiment I calves. Group A were given 10^6 PFU of virulent BHV1, group B 10^6 PFU of attenuated BHV1 and group C 10^4 PFU of attenuated BHV1. The results of each of the four calves per group are depicted.

RELATION OF SERUM NEUTRALIZING ANTIBODIES TO VIRAL-BACTERIAL PNEUMONIA

All control calves were SNA negative on the day of viral challenge and yet the extent of pneumonia ranged from 0 (2 calves) to 50%. Three vaccinated calves with SNA titers of 1/8, 1/32 and 1/64 on the day of viral challenge had <1, 1 and 2% of pneumonia respectively. In experiment I, two vaccinates remained SNA negative throughout the experiment, one of which had $<1\%$ and the other no pneumonia.

ISOLATION OF BHV1

In experiment I isolations were only attempted from bronchial lymph nodes with negative results.

In experiment II, BHV1 was isolated from nasal swabs of 4/7 IM vaccinates between days two and seven and from 2/7 calves on the fifth day after viral challenge. By contrast in IN vaccinates, BHV1 was isolated from 21/35 samples of nasal secretion taken between day 2 and 13 but only once thereafter. At slaughter on day 44 BHV1 was isolated from the lungs of 4/4 controls and 0/14 vaccinates; and the tracheas of 3/4 controls and 1/7 IM vaccinates.

In experiment III, BHV1 was isolated from 33/45 nasal swabs from the 15 IN vaccinates by day 11 but from 0/49 nasal swabs taken from seven IM vaccinates throughout the experiment. At slaughter on day 50 virus was isolated from both trachea and lung of 2/5 controls only.

P. HAEMOLYTICA ISOLATIONS

Pasteurella haemolytica was isolated from control calves only as follows: in experiment I from two bronchial lymph nodes, in experiment II from three tracheas and three lungs, and in experiment III from two nasal swabs (day of slaughter), three tracheas and two lungs.

DISCUSSION

Studies with the BHV1-*P. haemolytica* disease model have shown that vaccination with virulent or attenuated BHV1 will reduce colonization of the respiratory tract by *P. haemolytica* (5, 6). Nevertheless in the experiments reported herein some purulent pneumonia was produced in 6/8 four to five month old calves (experiment I) despite vaccination with low (60), medium (10^4), or high (10^6 PFU) doses of attenuated BHV1. This response may be expected in the field when using such doses of BHV1 vaccines although it is not known whether the experimental viral and bacterial challenge was more or less severe than occurs in the field. The experimental calves are unlikely to be more susceptible than commercial calves.

Bovine herpesvirus 1 was isolated from only 2/28 nasal swabs taken after viral challenge of calves vaccinated IN or IM with virulent virus in experiment II. A significant reduction of intranasal virus was also observed in calves challenged IN with $4 \times 10^{7.6}$ $TCID_{50}$ BHV1 following IM vaccination with killed vaccine (12). Similarly BHV1 was not isolated two days after viral aerosol challenge from nasal swabs of calves vaccinated by aerosol but virus was isolated from nonimmune calves for up to 12 days after aerosol exposure to virulent BHV1 (13). This agrees with results of this study using attenuated and virulent BHV1.

The mechanism of protection offered by viral vaccination against viral-bacterial challenge is presently unknown. In this study NNA were not detected in the animals immunized either by the IN or IM route, although it is generally believed that local antibody is produced after intranasal infection and has been detected in the case of BHV1 infections (4, 14). Lack of specific BHV1 antibody in nasal secretions could relate to the method of collection. Nasal secretions in which antibodies were found previously (3, 4, 14, 15), were collected after prolonged, physical irritation of the nasal passages whereas our method involved minimal irritation. Using a method similar to ours, Frank *et al* (2 and personal communication) also failed to find antibody in nasal secretions. The absence of NNA suggests that either very low levels of nasal antibody are produced following immunization with BHV1 or they cannot be detected in nasal secretions. Alternatively, all antibody present in nasal passage may be cell-bound. Curiously, NNA have been detected in calves double vaccinated IM with killed BHV1 vaccine, but not in calves given $4 \times 10^{7.6}$ $TCID_{50}$ of virulent BHV1 IN (12). In the absence of local antibody, cellular defense mechanisms should be considered.

A dose of 60 PFU of attenuated virus produced only a marginally slower development and lower level of SNA than higher doses of attenuated virus. Delayed onset and production of low levels of SNA have been reported previously in four month old calves vaccinated IN twice, one month apart, using 4 mL of a low dose (10^2 $TCID_{50}$/mL) BHV1 vaccine[1] (6). Two three to four month old calves in experiment I failed to develop ADCC (Fig. 1) or SNA following vaccination (Table II). In experiments II and III, eight to ten month-old calves all responded, regardless of the dose of vaccine virus. This suggested SNA responses following vaccination with BHV1 may depend on virus dosage as well as age of the animals vaccinated.

In two calves (three and one-half to four months old) of experiment I neither SNA against BHV1 nor antibodies against plasma membrane of BHV1 infected cells, as measured by ADCC, were present. This suggests that none of the effector mechanisms mediated by antibody were present and thus were not involved in preventing infection. One calf had received 10^4 and the other 10^6 PFU of attenuated virus. The calf receiving the high dose of vaccine had a temperature of 40.2°C on day 6 and a minimal amount of viral-bacterial pneumonia. Both calves had viral lobular lesions, which were more numerous in the latter.

An elevated rectal temperature response occurred following challenge in animals vaccinated with low or medium doses of virus but only those animals immunized with a low dose of virus developed purulent pneumonia (experiment III, Table IV), which suggests that the dose of live virus in vaccines should not be lower than 10^3 PFU/animal, if adequate protection is to be achieved.

There is no significant difference beween IN and IM vaccination in protection against BHV1 (4) or BHV1-*P. haemolytica* challenge as shown by this study. Intramuscular vaccination may have the desirable feature of minimizing shedding of vaccine virus from the nostrils although minimal nasal shedding of attenuated vaccine virus has been demonstrated in cattle following IM vaccination (4).

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