The anti-microbial activity of divinyl ether-maleic anhydride copolymer against *Hemophilus somnus* and infectious bovine rhinotracheitis virus

by

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INTRODUCTION

Bovine respiratory diseases are considered to be the major problem confronting the beef industry today. It has been estimated that the economic loss due to respiratory disease costs $10 to $20 for every calf in the feedlot (15).

The bovine respiratory disease complex (BRDC), as shown below (14, 16,47), lists what are thought to be the major factors and organisms involved in the BRDC. These agents may act alone or in combination.

Stress	+	Viral infection	+	Bacterial infection = BRDC
Heat		Infectious bovine rhinotracheitis		Hemophilus
Cold		Parainfluenza-3		Pasteurella
Chilling		Bovine viral diarrhea		Mycoplasmas
Dampness		Malignant catarrhal fever		Chlamydiae
Fatigue		Bovine Respiratory Syncytial		Other bacteria?
Dehydration		Enteroviruses		
Starvation		Rhinoviruses		
Crowding		Adenoviruses		
Anxiety		Reoviruses		
Fright		Other viruses?		

Several vaccines and bacterins are available and have been in use for some time. The most effective vaccines are the modified live virus vaccines but there are risks involved that may be more costly than the benefits. Attenuated vaccines administered to pregnant animals have been incriminated in abortions, fetal deformation and pathological lesions (22). Some animals may be highly susceptible to the attenuated virus and there

is the potential for reversion to the virulent virus. The control of BRDC is seldom accomplished by immunization due to the plurality of the agents involved.

Since the discovery of interferon (17), many researchers have hoped that it would be the answer to the control of viral diseases. Interferon can be induced by a number of viruses, synthetic nucleic acids and other synthetic anionic polymers. Because of the inherent danger of using live virus as inducers, a search for suitable synthetic inducers was implemented.

Among the polyanion inducers, pyran, a divinyl ether-malic anhydride copolymer (DIVEMA), has been shown to protect mice against several viruses (6,7,25,26,28,30,33,37) and some bacteria (27,35,41). In addition to interferon stimulation DIVEMA has been shown to produce a marked biphasic response in the functional activity of the reticuloendothelial system (RES) (32,39), enhance the humoral antibody response (3,4), alter drug metabolism (40), and retard the growth of induced tumor cells (18).

Although a number of successes with DIVEMA have been reported in laboratory animal systems, little work has been done in the applied field. DIVEMA, because of its broad-spectrum prophylaxis, appears to be a good candidate for further testing against the complexity of etiological agents of the BRDC.

This preliminary investigation evaluated the antimicrobial activity of DIVEMA in calves against two micro-organisms of the BRDC.

The first experiment was designed to test DIVEMA's ability to enhance the immune response to one or two doses of inactivated Infectious Bovine

Rhinotracheitis (IBR) vaccine. Also information was obtained on the effect of DIVEMA given alone 48 hours before challenge with IBR virus.

The second experiment tested the antibacterial effects of DIVEMA in calves infected with Hemophilus somnus.

LITERATURE REVIEW

Historically the anticoagulant effects of heparin in relation to its calcium binding was studied to evaluate its potential in retarding tumor growth. The use of anticoagulents or lipolytic agents produced alopecia, ulceration of the mucosa of the gastrointestinal tract and osteoporosis leading to pathological fractures (36).

Regelson's group then began a systematic search for heparnoids that have tumor inhibiting properties without side effects. They evaluated synthetic polymers derived from ethylene maleic anhydride. Two agents were selected for clinical testing. One of these agents was the poly-anionic pyran copolymer of carboxylic divinyl ether maleic anhydride also designated MA/DVE (26), DVE/MA (4), and DIVEMA[1].

Antiviral Activity

Regelson and Foltyn (38) reported that the synthetic polycarboxylate pyran produced an average of 65% inhibition of Friend Leukemia virus (FLV) induced splenomegaly in mice when given intraperitoneally (IP) daily for 6 days beginning 24 hours after viral infection. When they gave pyran 6 days prior to FLV inoculation there was a 78% inhibition of splenomegaly. Regression of established FLV tumors was not seen.

In continued studies with FLV Regelson (37) observed that pyrans were capable of antiviral activity as well as antitumor activity. This was consistent with the findings of other workers that had shown that

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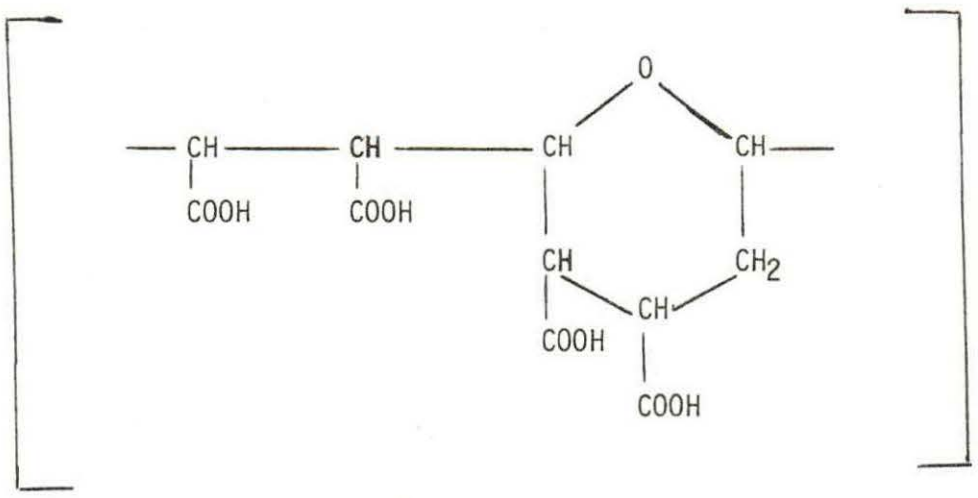
[1]Hercules Inc., Wilmington, Del.

polyanions possessed both antitumor activity and antiviral activity. Regelson attempted to determine a mechanism that would explain the inhibition obtained with pyran administration following FLV infection. Two phenomena were observed; one was the appearance of interferon in the serum of mice inoculated with pyran alone and the other was direct in vitro neutralization of FLV by pyran.

Merigan (26) became interested in the synthetic copolymers of maleic acid anhydride after Regelson suggested the antiviral activity might be due to interferon. Merigan confirmed Regelson's findings and characterized the antiviral activity, identified it as interferon and determined structural requirements of the copolymers for inducing interferon.

The required structure for the polyanions was found to be a saturated linear polymer with carboxylate groups on two out of every four carbons in either alternate or adjacent positions. Pyran copolymers were found to be the most potent inducers of interferon. Pyran is a copolymer of maleic anhydride and divinyl ether with a high density of carboxylate groups and a six-membered ring structure built into the polymeric backbone. Pyran has been synthesized in a variety of molecular weights ranging from 17,000 to 540,000. A molecular weight of 17,000 was required for activity while high molecular weight polymers were found to be less active. High molecular weight pyrans were very toxic in clinical trials in man (37).

The appearance of interferon may occur through two mechanisms. One involves the release of preformed interferon and the other the synthesis of new interferon. Vilcek (49) suggests that 'viral' types such as



Pyran

Newcastle Disease virus (NDV) and statolon (a fungal virus preparation) activate both mechanisms while non-viral stimulators, polynucleotides and endotoxin, only release preformed interferon.

Pyran resembles statolon in that the interferon it induces is of 70,000 MW (25). Pyran also resembles statolon in production of long lasting protection. Schmidt et al. (45) compared the duration of protection of mice from encephalomyocarditis virus challenge. The longest significant protection provided by each inducer was endotoxin, 5 days; poly I/C, 30 days and statolon or pyran, 55 days. Peak levels of interferon appeared several hours (24-48) after administration of pyran (26) or statolon (50), whereas peak levels stimulated by endotoxin (50) or poly I/C (9) were seen at 2-3 hours.

Pyran differs from statolon and NDV in ability to stimulate interferon in vitro. Statolon and other viral inducers are able to induce

interferon in a variety of cell types whereas pyran only induced interferon in the less fastidious cells such as mouse peritoneal macrophages and human fibroblasts (11). In vivo studies (13) in mice show the spleen to be a major source of interferon synthesis. If the spleen has been removed there is a marked reduction (87%) in interferon titers following NDV. When pyran was injected 14 days after spleen removal there was a 62% reduction in interferon levels; however, if pyran was injected 30 days after spleen removal there was no reduction in interferon titers. Pyran also differed from the viral inducers in that interferon production induced by pyran was significantly more resistant to Actinomycin D (12).

Pearson et al. (33) extended studies of the antiviral effects of pyran in mice to Rauscher leukemia virus (RLV), murine sarcoma virus (Moloney Plasma Variant) MSV (MPV) and a first generation transplant induced by the Moloney leukemia virus (MLV-T), as well as FLV. Four parameters were used to measure pyran's effectiveness: viral-induced spleen foci (SF), splenomegaly (Sp), survival time (ST) and virus reduction. The model systems showed a marked decrease in SF, Sp, and viral replication, and an increase in ST following pyran administration. Surviving mice appeared to be free of the disease. Interferon was hypothesized to be the mechanism of action against the viruses.

In all of the studies that reported prolonged viral protection, the model systems used were intraperitoneal injections of mice with both pyran and virus (26,28,29,37). DeClercq and Merigan (7) explored other routes of injection of pyran and virus. Pyran was administered either intranasally or intraperitoneally to mice and the animals were challenged intranasally with vesicular stomatitis virus (VSV). Either route of

treatment significantly protected against virus infection; however, the long term protection seen in other studies was not obtained. Intranasal instillation of pyran was only effective if applied 3-4 hours before infection. Intraperitoneal injection of pyran provided protection for only a few days. The findings suggested protection was due to interferon production.

Richmond (42) conducted experiments in mice to test pyran's ability to protect against Foot-and-Mouth Disease virus (FMDV). He found a biphasic pattern of protection. Peak levels of resistance to infection were found at 4 hours, which was of short duration, and at 48-72 hours, gradually subsiding around the 7th or 8th day. Serum levels of interferon were demonstrated in the samples taken on days 1 and 2 after pyran administration. Richmond hypothesized that the protection seen at 4 hours was the release of preformed interferon and the protection seen 48-72 hours later was newly synthesized interferon.

Richmond and Campbell (43) continued their experiments to see if they could determine a mechanism of protection afforded by pyran against FMDV infection. They had ruled out the possibility of direct neutralization in a previous study (42). They examined the possibility that pyran affects the adsorption of FMDV onto susceptible cells. Virus adsorption was tested in various minced target organs from pyran treated mice and non-treated mice. They found that the pyran treated tissues adsorbed virus to the same degree as the non-treated tissues. It was noted, however, that the pyran treated tissues did not support <u>in vitro</u> replication of the virus. It was not determined if the repressed multiplication was

due to interferon because protection had occurred beyond the time interferon could be detected in the tissues.

In addition to interferon production pyran is capable of direct neutralization of virus. Regelson (37) reported that when FLV was incubated with pyran at room temperature for 30 minutes prior to inoculation there was little or no infective virus remaining. Other researchers confirmed this direct polyelectrolyte interaction with Mengo virus, VSV, Semliki Forest virus, Echovirus 9, and Vaccinia (7,28). Richmond (42) found that preincubation of pyran and FMDV did not alter the infectivity of that virus.

Since interferon has only been demonstrated in the serum and tissues for 7 days after pyran treatment and protection has been shown to last up to 2 months in mice against several viruses, mechanisms other than interferon have been proposed for the antiviral activity of polyanions. Regelson (36) reviewed the work of others and hypothesized that polyanions may activate lipases and esterases which could in turn inactivate the lipid-containing viruses. Moreover, lipid fractions would stimulate the reticuloendothelial system (RES).

Billiau et al. (1) also proposed that stimulation of the RES cells is a logical hypothesis for the prolonged antiviral action of polycarboxylates. He points out that polycarboxylates have been shown to be ingested by macrophages and that these compounds are not readily biodegradable. He suggested that the RES cells are continually being 'activated' and this may result in increased uptake and destruction of virus.

Antibacterial Activity

Antibacterial effects of known interferon inducers were investigated by Pindak (35). If pyran, statolon or endotoxin were given to mice 24 hours before infection with Klebsiella pneumonia 80-100% of the mice survived the lethal challenge, whereas in the untreated controls the survival rate was 25%. The surviving mice were rechallenged 21 days later. The survival rate for all mice on rechallenge was nearly 100% indicating that the mice pretreated with interferon inducers were able to develop immunity of the same order as that produced in a natural infection. Mice challenged for the first time at 21 days following treatment with the inducers were no more resistant than the untreated controls.

In a second investigation Pindak (34) used the same protocol as described in the preceding paragraph except that on day 6 the survivors were inoculated with encephalomyocarditis virus (strain MM). The mice were protected from the virus indicating that the intervening bacterial challenge did not diminish the effects of the interferon inducers.

Regelson and Munson (39) compared the effects of pyran and poly I/C in mice against bacterial (pneumoccocus) and fungal (Cryptococcus neuformans) infections. They found complete protection with both treatments if the interferon inducers were given 2 days preceding the challenge.

Remington and Merigan (41) were interested in determining if the long term resistance produced against viral challenge by pyran could also be produced against intracellular bacterial infections. They found that when pyran was administered to mice intraperitoneally 18 hours before Listeria monocytogenes challenge by the same route no resistance was conferred. In

fact the treated mice died earlier than the controls. However, further experiments showed that if Listeria was given during the period of 4 days to 2 months after pyran administration mortality was decreased. Remington and Merigan also noted that more protection was conferred if pyran was given subcutaneously. They presumed that this route lessened the toxicity that had been seen when pyran had been administered close to the bacterial challenge. Efforts to find a mechanism of action revealed that macrophages from pyran treated mice were resistant to challenge with listeria in vitro. They hypothesized that resistance to intracellular bacterial infection may be related to the inducer's ability to enhance cell mediated defense mechanisms against foreign antigens.

Adjuvant disease in rats is believed to be a form of delayed hypersensitivity to mycobacterial antigens in Freunds complete adjuvant. The first symptoms (polyarthritis, periostitis, and tendinitis) are apparent at 10-14 days. When Kapusta and Mendelson (19) gave pyran during the period of 1 day before until 7 days after injection of adjuvant, the extent of the hypersensitive reactions was significantly decreased. They proposed that pyran had induced an antiinflammatory or an antiimmune effect.

Reticuloendothelial Stimulation

A biphasic response in the phagocytic activity of the RES is produced by pyran. Regelson et al. (40) measured intravascular clearance of colloidal carbon and organ uptake of I^{131} lipid emulsion and Cr^{51} sRBC in mice. The first 2 days following injection of pyran there was a marked

depression of phagocytic activity but at days 7 and 9 the activity was markedly stimulated. During the depressed phagocytic stage there was also a depression of hepatic and splenic uptake of the lipid emulsion and sRBC whereas in the lung and thymus there was an increased uptake of sRBC but no increase was noted for the lipid emulsion. During the stimulated phagocytic stage there was increased uptake of sRBC in the liver and spleen but lipid emulsion uptake was increased only in the spleen. The degree of depression or stimulation appeared to be dependent upon the test agent. SRBC showed a greater depression as well as greater stimulation of RES function.

Munson et al. (32) found the primary immune response as measured by the Jerne plaque technique to be closely related to the biphasic response of phagocytic activity. During the depressed RES phase there was a delay in production of antibody-forming cells. When the antigenic stimulus was given during the enhanced RES phase the antibody response appeared to be due to an increase in the number of spleen cells responding rather than an increase in production of antibody by competent cells.

Kapila et al. (18) noted that phagocytic activity was stimulated by pyran and applied this finding to studies in mice that had methylcholanthrene induced tumors. Mice were injected daily with pyran for 3 days and challenged with a suspension of tumor cells. Pyran delayed the onset of the tumors by 4 weeks but did not decrease the incidence of tumors. The average weight of the tumors was significantly reduced in the pyran treated mice.

Antibody Formation

Since polynucleotides and endotoxin were known to stimulate antibody formation, Braun et al. (3) evaluated the effects of pyran on antibody-forming cells in mice. Pyran was injected IP at the same time sRBC were injected intravenously (IV). Hemolysin-forming spleen cells were assayed by the Jerne plaque method. Pyran enhanced the early response to sRBC; however, by 72 hours there was no enhancement. Histological sections of mouse spleen showed that within 24 hours after injection of pyran the small cells of the germinal centers disappear and the spleen becomes filled with large pyroninophilic reticulum cells of the kind associated with enhanced phagocytosis.

Campbell and Richmond (4) investigated the ability of pyran to enhance the immune response to FMDV vaccine. Mice given vaccine and pyran simultaneously were significantly more resistant to challenge than those receiving the vaccine or pyran alone. Peak levels of resistance occurred at days 2 and 3, but began to wane at days 7 and 8. Neutralization tests were performed to determine if the greater resistance of mice given both pyran and vaccine was due to enhancement of antibody production. They found no significant differences in antibody levels in the mice given both the inducer and vaccine or the vaccine alone.

Deleterious Effects

Merigan and Regelson (29) reported that in patients given pyran for solid tumor therapy, thrombocytopenia was a major side effect in all patients whose dosage was 8 mg per kg or higher. A few days after the drug

was discontinued platelet levels returned to normal and no serious hemorrhages occurred. The only other side effect seen in these patients was fever over 101 F which subsided within 48 hours after the drug was administered.

After an occurrence of a hemolytic-uremic-like syndrome in a patient undergoing treatment with pyran, Leavitt et al. (20) studied the effects of pyran on intravascular coagulation in three patients that were being treated with the copolymer. Within several hours after pyran administration patients exhibited a prolongation of the partial thromboplastin time and a rapid drop in factor V activity. This was consistent with in vitro studies with pyran added to normal plasma which produced prolongation of both partial thromboplastin time and thrombin time and decreased factor V. Leavitt and co-workers also reported that pyran administration consistently produced a fever and in most cases was accompanied by a morbiliform rash and leucocytosis.

Munson and Regelson (31) observed that mice given pyran 24 hours before intravenous injection of endotoxin were more sensitive to the lethal effects of the toxin. This sensitivity occurred during both the depressed and stimulated phases of RES activity.

In an attempt to obtain protection of pigs against FMDV, McVicar et al. (23) gave the animals high doses of pyran. The pigs developed elevated temperatures, were extremely lethargic, had signs of abdominal pain and refused to eat. On the second day after pyran treatment the pig receiving the highest dose was found dead, A necropsy revealed a severe

peritonitis. The condition of the other pigs was so poor challenge with FMDV was not done.

Pyran is taken up by the reticuloendothelial system and remains there for prolonged periods of time. This may be a therapeutic disadvantage. The effects of the prolonged presence of the copolymer in the RES are not known (36).

MATERIALS AND METHODS

Experiment I

DIVEMA

Solutions of DIVEMA[1] (XA146-85-2) were prepared in 0.15 M NaCl. Addition of 20% NaOH helped to speed dissolution. When the recommended pH of 7.2 was reached, adjustment to volume was made. The final concentration of DIVEMA was 100 mg/ml. The DIVEMA solution was sterilized by filtration through 0.22 μ Nalgene disposable filter[2] and refrigerated (4 C) until used.

Cell cultures

Either Madin-Darby bovine kidney[3] (MDBK) or Georgia bovine kidney[4] (GBK) cells were utilized for the cell work throughout this study. Cell cultures were grown in Dulbecco's modification of Eagles minimum essential medium (MEM)[5] supplemented with 10% fetal calf serum (FCS)[5] that had been heat inactivated at 56 C for 1 hour. The buffers used in 100 ml of medium were 3 ml of 7.5% sodium bicarbonate and a combination of organic buffers[6] N,N'-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES),

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[1]Hercules, Inc., Wilmington, Del.

[2]Nalgene Labware Division, Rochester, N. Y.

[3]American Type Culture Collection

[4]R. F. Solarzono, University of Missouri, Columbia, Mo.

[5]Grand Island Biological Corp., Grand Island, N. Y.

[6]Nutritional Biochemical Corp., Cleveland, Ohio.

N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid (HEPES), and N-2-hydroxyethyl-piperazine propane sulfonic acid (HEPPS) in final concentration of 10, 15, and 10mM, respectively (11). Antibiotics used in all media were penicillin G[1] (100 I.U./ml) and streptomycin sulfate (100 mg/ml). Cells were grown in 250 ml plastic flasks[2] at 37 C in the presence of 5% CO_2.

Cells were subcultured as needed for viral work or at 4 or 5 day intervals to maintain cell viability. Cells to be subcultured were washed three times with 3 ml of Rinaldini enzyme solution (R-saline) (44). Two ml of a trypsin solution (0.1% trypsin[3], 0.02% versene[4], and R-saline) were dispensed to the cell surface and then removed after 2 minutes. Flasks were incubated at 37 C for a few minutes to loosen the cells completely. Cells were suspended in MEM and dispensed into new flasks or test plates. All tissue culture work was done under a positive pressure plexiglass hood.

IBR vaccine

The Cooper strain of IBR virus[5] was propagated in GBK cells. Virus was freed from the cellular debris by centrifugation for 10 minutes at 2,500 rpm. The virus had a titer of $10^{8.5}$ $TCID_{50}$/ml and was concentrated

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[1]Eli Lilly and Co., Phillipsburg, N. J.

[2]Falcon Plastics, Oxnard, Calif.

[3]Difco Laboratories, Detroit, Mich.

[4]J. T. Baker Chemical Co., Phillipsburg, N. J.

[5]Veterinary Biologics Division U.S.D.A., Ames, Ia.

one additional log through an Amicon ultrafiltration system[1]. Virus was checked for sterility, inactivated by a 1:1000 dilution of beta-propriolactone and placed in a Wheaton vaccine bottle. The vaccine was kept at -70 C until used.

Serum neutralization test

Serum neutralization (SN) tests were performed in Linbro[2] micro-titer tissue culture plates. Test serums were heat inactivated at 56 C for 30 minutes. Two-fold serial dilutions of the test serum (0.05 ml) were made in micro-titer plates using MEM as the diluent. Twenty to forty $TCID_{50}$ of virus in 0.05 ml of GBK cells suspended in MEM with 10% FCS, containing approximately 10,000 cells, were added to each well. Plates were incubated at 37 C in a 5% incubator for 2 days. An inverted microscope[3] was used to examine the plates for cytopathogenic effect (CPE). Neutralization titers were calculated by the method of Spearman and Karber and recorded as the reciprocal of the dilution that produced a 50% endpoint (21).

Plaque reduction SN test

Test serums were reheat inactivated at 56 C for 10 minutes. Two-fold serial dilutions were made in tubes using MEM without serum as the diluent. An equal volume of IBR virus calculated to contain 50 plaque-forming units (PFU) was added to each tube and the serum-virus mixtures were incubated at 37 C for 1 hour. Linbro 6-well tissue culture trays containing 48 hour monolayers of MDBK cells were used to titrate the virus. The medium was

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[1]Amicon Corp., Lexington, Mass.

[2]Linbro Chemical Co., New Haven, Conn.

[3]Unitron Instrument Co., Newton Highlands, Mass.

aspirated from the cultures and 0.5 ml of each virus-serum mixture were inoculated into two wells and incubated for 60 minutes at 37 C to allow the virus to adsorb to the cells. The inoculum was aspirated from the cells and the cells were overlaid with 0.5% Agarose[1] and MEM with 2% FCS. Plates were incubated for 72 hours at 37 C. Cells were fixed in 10% formalin, the agar was removed and the cells stained with 1% crystal violet. The number of plaques were counted and titers were expressed as the reciprocal of the dilution that produced a 50% reduction in the number of plaques as compared to the number in the control cultures.

Interferon assay

Viral plaque reduction was used to measure interferon levels. Medium was aspirated from MDBK cell monolayers that had been grown in Linbro 6-well tissue culture trays. Serial two-fold dilution of non-heat inactivated serum were made in MEM and duplicate samples of each serum dilution (0.5 ml) were applied to the cell monolayer and incubated overnight. Control cultures were treated with negative serum. At the end of incubation, the serums were aspirated and the plates washed three times with R-saline. Vesicular stomatis virus (VSV) Indiana strain, calculated to contain 50 PFU, was added to each well. Virus was adsorbed at 37 C for 1 hour, excess viral fluids were aspirated, and the cultures were overlaid with 0.5% Agarose in MEM containing 2% FCS. Plates were incubated for 48 hours and then fixed with 10% formalin. The agar was removed and the cells were stained with 1% crystal violet. Titers were calculated by the method stated in the plaque reduction test.

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[1]Marine Colloids, Inc., Rockland, Me.

Characterization of the bovine interferon

Six criteria were used to ascertain if the anti-viral inhibitor was interferon. Pools of serums from each of two calves that had high inhibitory titers were used for the characterization. After each treatment serums were compared in an assay with non-heat inactivated serum.

1. Serums were heat inactivated at 56 C for 1 hour.
2. Serums were placed in 3/8 inch dialysis tubing and dialyzed against Sorensen's (48) pH 7.4 for 24 hours at 4 C with two changes of buffer. The volume ratio of serum to buffer was 1:200.
3. VSV and serum was mixed in equal volumes and allowed to neutralize at room temperature for 1 hour.
4. Serum was centrifuged in a Beckman L2-65B ultracentrifuge at 100,000 g for 1 hour.
5. Serums were adjusted to pH 2 with sterile 1 N HCl, held at that pH for 1 hour and then brought back to pH 7.4 with sterile 1 N NaOH. Volumes of serum, HCl and NaOH were carefully measured and the serum was adjusted with MEM to a 1:2 dilution.
6. Serums were applied to cell monolayers and incubated for 4 hours and compared with serums that had been applied to cell monolayers and incubated over night.

Animal inoculation schedule

Fifteen bull calves ranging in age from 6 weeks to 5 months were purchased from three local dairies and the Veterinary Medical Research Institute hereford herd. Calves were allowed to graze on pasture land

and were fed Calf Krunch[1]. Three of the youngest calves were given a commercial milk replacer.

Group	Day	Vaccine	DIVEMA
Group A			
433	1	4 ml IM	-
436	1	4 ml IM	-
Group B			
435	1	4 ml IM	-
	14	2 ml IM	-
6902	1	4 ml IM	-
	14	2 ml IM	-
Group C			
434	1	4 ml IM	10/kg IV
439	1	4 ml IM	10/kg IV
Group D			
416	1	4 ml IM	10 mg/kg IV
	14	2 ml IM	10 mg/kg IV
426	1	4 ml IM	10 mg/kg IV
	14	2 ml IM	10 mg/kg IV
Group E			
413	1	-	10 mg/kg IV
428	1	-	10 mg/kg IV
	14	2 ml IM	10 mg/kg IV
438	14	-	10 mg/kg IV

[1]Calf Krunch-6101-27, Allied Mills, Chicago, Ill.

Group F			
445	28	-	10 mg/kg IV
447	28	-	10 mg/kg IV
Group G			
444	-	-	-
No number	-	-	-

On day 30 all calves received 4 ml of the Cooper challenge strain of IBR[1] virus intranasally (IN) with a gas-powered atomizer using the method of Sinclair and Tamoglia (46).

Temperature and blood samples were taken daily for 5 days after each vaccination and weekly thereafter. Temperatures and blood samples were also collected daily for 5 days after challenge and on post-challenge (PC) days 7, 10 and 12. Blood samples were collected for serum and for total and differential white blood cell (WBC) counts.

Temperatures of 102.7 F to 103.0 F were considered in the high-normal range. Those temperatures of 103.0 or higher were considered above normal. Normal values for WBC counts range from 5,000 to 12,000

Experiment II

DIVEMA

Solutions of DIVEMA were prepared the same as described in Experiment I.

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[1]Veterinary Biologics Division, U.S.D.A., Ames, Ia.

Modified complement fixation test

Microtiter adaptation of the complement fixation test as outlined by Casey (5) was used. Fresh bovine serum was added to the test system as described by Boulanger and Bannister (2).

Complement fixation antigen

An aliquot from the H. somnus challenge preparation was diluted 1:500 in phosphate buffered saline (PBS) (0.015 M) pH 7.2. One tenth ml of the 1:500 dilution was plated onto each of two chocolate agar plates (10). Plates were incubated at 37 C in a 5% CO_2 incubator for 24 hours. Colonies were harvested by adding 4-5 ml PBS and gently scraping the colonies off the agar surface with a bent glass rod. This suspension was used to inoculate 30 plates. Inoculum, growth, and harvesting were the same as for the first two plates. The harvest was sedimented in an International centrifuge at 2,500 rpm for 15 minutes. The supernatant was decanted and the H. somnus resuspended in 50 ml of PBS. The bacteria were disrupted by sonic oscillation for 10 minutes in a Raytheon sonic oscillator, model DF 101[1] to prepare antigen.

Challenge agent

H. somnus strain 1229[2] was passed two times in 7 day old embryonated eggs. The criterion for harvesting the yolk material was that the embryo die within 24 hours. The second passage of the micro-organisms was

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[1]Raytheon Industrial Operation, So. Norwalk, Conn.

[2]R. E. Dierks, Veterinary Medical Research Institute, Iowa State University, Ames, Ia.

harvested, dispensed in 2 ml volumes and kept frozen at -70 C until used as challenge agent 1 week later. The inoculum contained 10^8 organisms per ml.

Animal inoculation schedule

Six calves were selected from animals which had been used in Experiment I. Serums from the calves were tested by complement fixation and found negative for antibodies to H. somnus. Four of the calves (Groups 1 and 2) had no previous exposure to DIVEMA and the calves in Group 3 had been given DIVEMA 80 days prior to this experiment.

Calves were held in isolation units throughout the experiment and fed Wayne Calf Krunch and alfalfa hay.

Group 1 (calves 433 and 436) each received 10 mg/kg of DIVEMA IV 48 hours before challenge with H. somnus.

Group 2 (calves 435 and 6902) were given DIVEMA IV 8 hours after infection which was at the first sign of a clinical illness. Both calves had temperatures of 104.0 F.

Group 3 (calves 434 and 439) were control calves and received only the challenge agent.

All six calves were injected intratracheally with 2 ml of H. somnus organisms. Daily blood samples were collected for serum and for total and differential WBC counts. Rectal temperatures were taken at 8 hours post-challenge and then every 24 hours. Calves were necropsied at death or 9 days after challenge.

RESULTS

Experiment I

Calves were inoculated according to the schedule as described in Materials and Methods. All of the calves were challenged on day 30 with a low passage Cooper strain of IBR virus. They were inoculated intranasally using a gas-powered atomizer. All calves, except those in Group F, developed clinical signs of illness including fevers of 104.0 F or higher, accelerated respiration rates, coughing, malaise, and conjunctivitis. One control calf (calf 444) died on post-challenge (PC) day 2. The necropsy showed that the calf had a mild pneumonia and a small cervical abscess. The abscess was apparently due to infection in a wound suffered when the animal was caught on the fence.

Temperatures

Temperatures of the calves in Group A are shown in Graph 1. The temperature of calf 433 remained normal throughout the vaccination period. Calf 436 had a rise in temperature on days 1 and 2 after vaccination, possibly a reaction to the vaccine. It also had a temperature rise on day 25 that is unexplained. Both calves in this group had fevers from the IBR infection, beginning 48 hours after challenge and remaining elevated for 5 to 6 days.

Temperatures of the calves in Group B are shown in Graph 2. The temperature of calf 434 was above normal on day 1. Calf 439 had a fever lasting from day 1 through day 3. The fever likely was a response to the DIVEMA or the vaccine. Calf 439 had unexplained above normal temperatures

from day 5 to day 12 and again on day 25. After challenge both animals had fevers, lasting from PC day 2 to PC day 4 and 5, as a result of the IBR infections.

Temperatures of the calves in Group C are shown in Graph 3. The temperature of calf 435 remained normal throughout the pre-challenge period except for an elevation above normal on day 16 following the second dose of vaccine. Calf 6902 had a fever on day 2 and day 16 which may have been a reaction resulting from the vaccine. It had an unexplained fever on days 11 and 12 and also temperatures in the high normal range during the period following the second vaccination until 24 hours after challenge. Both calves developed post-challenge fevers as a result of the IBR infection.

Temperatures of calves in Group D are shown in Graph 4. Calf 416 had a fever for 5 days after receiving the vaccine and DIVEMA. This calf's pre-challenge reaction was the most severe of all of the animals. It also had an increase in temperature on day 17, which was 3 days after the second inoculation and again on day 25. The temperature of calf 426 remained normal throughout the pre-challenge period except on day 25. Both calves again developed fevers after challenge with IBR virus.

Temperatures of calves in Group E are shown in Graph 5. Calf 428 exhibited a temperature rise on day 14 and 15. The high-normal temperature on day 14 may have been caused by the calf being chased on a hot day. The fever on day 15 probably was due to the vaccine plus DIVEMA. Total WBC counts and differential staining tend to support this explanation. Calf 428 also had an unexplained fever on day 25. Calf 413 had a rise in

temperature above normal on days 1 and 2 after DIVEMA administration. On day 29 its temperature started to increase again and was high-normal on days 29 and 30. This rise in temperature is not explained. Its temperature rose to 103.6 F on PC day 1 and was above normal through PC day 6. Temperature of calf 438 remained normal through the pre-challenge period. All three calves developed fevers as a result of the IBR infection.

Temperatures of calves in Group F are shown in Graph 6. Both calves in this group had temperatures that remained normal throughout the entire experiment.

Group G temperatures are shown in Graph 7. Calf 444's temperature was normal both before and after challenge but the animal was found dead on PC day 2. The other control calf, no number, had a fever on day 29 but his temperature was high-normal on the day of challenge. No number had a fever from the IBR infection.

Hematology

WBC counts and differentials are shown in Tables 1-7. Almost all values are in the normal range, however, trends can be seen. Rises in WBCs occurred in every treatment group, but not every calf, at the beginning of the vaccination period. This was presumably a response to the vaccine, DIVEMA, or both. An increase in WBCs also was observed during the middle of the pre-challenge period for at least one calf out of every group and may have been a response to the second dose of vaccine or DIVEMA. For those groups not receiving a second dose, as well as those that did, elevated WBCs may have been the result of the stress caused by moving the calves together into a single pen. The calves had been put together on day 12.

During the post-challenge period a slight leucopenia occurred for calves of all groups on PC day 1 followed by a return to slightly higher values on day 2.

Serological studies

Micro-titer serum neutralization tests were performed on all serums and the results are shown in Table 8. Calves in Groups A, B and D did not develop any detectable antibodies to IBR vaccine. Calves in Group D developed low transient titers. Calves in all groups except Group F developed post-challenge titers as though it was a primary response.

To ascertain if the sensitivity of the neutralization tests could be improved plaque-reduction tests were done. It was felt that low titers might be detected more easily with this method and this procedure may also disaffirm or confirm the transient titers seen in Group C.

The results of the plaque-reduction tests are shown in Table 9. Not all of the serums were retested. The differences found are noted in Table 8. The only significant changes are found in Group F. Possible explanations will be discussed later.

Interferon assays

The results of the interferon assay are shown in Table 10. The responses to the vaccine and DIVEMA were varied. Interferon was not detected in the sera of calves in Group A after vaccination. A low level of interferon was detected for both calves in Group B after inoculation with vaccine and DIVEMA. For both calves in Group C detectable low levels of interferon occurred 2 and 3 days after the second dose of vaccine but not after the first dose. Calf 416 (Group D) had interferon levels in the serum throughout the experiment but had slightly higher levels after each

dose of vaccine and DIVEMA. Calf 426 (Group D) had low levels of interferon on and following both vaccination days. For calf 428 (Group E) interferon production was detected with DIVEMA injection on days 4 and 5 and also after vaccine and DIVEMA was given on days 16 and 18. Calf 413 (Group E) had low level interferon titers throughout the experiment with no significant peaks during the pre-challenge period. Calf 438 (Group E) produced the highest pre-challenge titers of all the calves. On the third day after DIVEMA administration it developed a high serum level of interferon which was still relatively high on the 7th and 11th days after the interferon first appeared.

During the post-challenge period all calves produced interferon. Groups A, B, C, D and E peak levels of interferon are seen on PC days 3, 4 and 5. Peak levels of interferon appeared on PC day 2 for Group F calves which had been given DIVEMA 48 hours before challenge. The serum levels of one control calf (no number) are not consistent with what one might expect to find in view of the findings of Group A through E.

A summary of the characteristics of the bovine interferon measured in this assay can be found in Table 11.

Experiment II

Test animals were closely observed after administration of DIVEMA and after challenge with H. somnus. Calves in Group 1 had been given DIVEMA 48 hours before challenge. Calves in Group 2 were treated with DIVEMA 8 hours after challenge when their temperatures had reached 104.0 F. Group 3, the control group, received only the challenge agent.

Graph 8 shows the numbers of days each animal survived after challenge with H. somnus. As can be seen in the graph, DIVEMA given either before or after challenge did not protect against the lethal effects of the bacterial infection. Calf 6902 died 48 hours post challenge. Four of the five remaining calves started showing signs of a clinical illness typical of H. somnus infection on PC day 2. On the morning of PC day 3 both calves in Group 1 and one control calf were dead. The condition of one of the two remaining calves, calf 435, progressively deteriorated and the animal was nearly dead when killed on PC day 9. Calf 434 recovered from the infection.

Temperatures

Graphs 9, 10 and 11 show temperature responses of each of the calves. Calves in all groups had temperatures of 104.0 F or higher 8 hours after challenge. This was presumably a reaction to endotoxin that was in the inoculum. Temperatures of five of the six calves quickly returned to normal. The temperature of calf 6902 did not return to normal but instead rose to 104.8 F at 24 hours post challenge. Four out of the five remaining calves had fevers by PC 2. Control calf 434's temperature remained normal throughout the course of its infection.

Hematological studies

Data collected from the hematological studies are shown in Tables 12, 13 and 14. Group 1 calves had WBC counts in the high-normal range 24 hours after administration of DIVEMA but the counts returned to pre-DIVEMA levels the day of challenge. The white count elevation was probably a response induced by DIVEMA.

A rise in WBCs occurred 8 hours after infection in calves of all three groups. The WBC counts ranged from 5,000 to 18,000 over the counts that had been obtained 8 hours earlier. In Group 1 calf 433's WBC count dropped at 24 hours post challenge and was increasing again when it died. The other calf in Group 1, calf 436, had a WBC count that remained above normal after challenge. For calves in Groups 2 and 3 there was a slight drop in WBCs at 24 hours but the numbers remained above normal.

The differentials (Tables 12, 13 and 14) show that all calves had a PC left shift from approximately 80% lymphocytes down to 20%-50% lymphocytes. The calves in Group 1 had a 30% increase in neutrophil count 24 hours after DIVEMA administration which had returned to normal by the time the animals were challenged.

No distinctions based on the hematologic tests could be made between the groups that received DIVEMA and the controls during the post-challenge period.

Necropsy reports

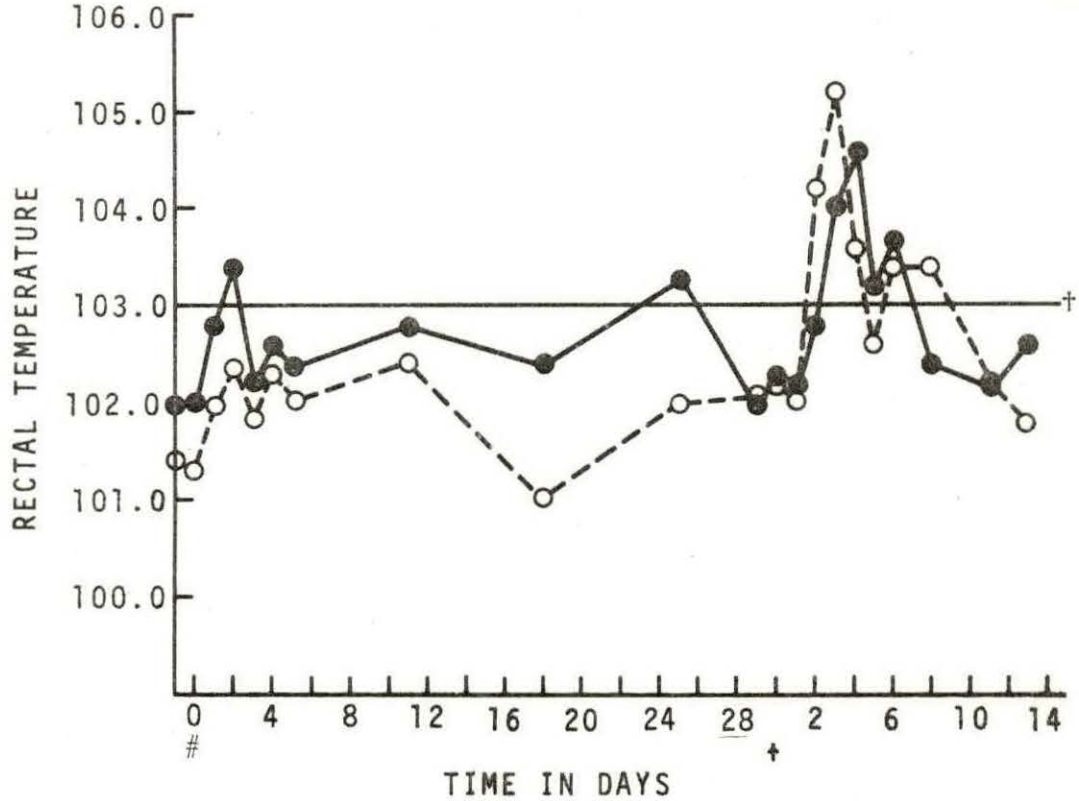
The two calves in Group 1 were found dead on the morning of PC 3. Both calves had a mild congestion in the dependent portion of the lung, petechial hemorrhage on the serosal surface of the heart, excess peritoneal fluid, and a small abscess at the tracheal injection site. Calf 436, in addition, showed mild adhesions in the mesentery and an enteritis in the jejuneum.

Calf 435 in Group 2 was nearly dead when he was killed for the post mortem examination. This calf was blind. The blindness was thought to be a recrudescence of the herpes infection. The necropsy revealed that it had pneumonia, petechial hemorrhage in the brain stem, mild adhesions in

the pericardium. Calf 6902 from Group 2 died on PC day 2. It had pneumonia in the dependent portion of the lung, meningeal congestion and a small abscess at the tracheal injection site.

One control calf (calf 439) in Group 3 was found dead on PC day 3. The necropsy examination showed he had a small abscess at the injection site, moderate consolidation in the bottom half of the lung, petechial hemorrhage of the serosal surface of the heart, hemorrhage in the trachea and cervical lymph nodes, and an enteritis. The other control calf (calf 434) had recovered from his infection when he was killed for the post mortem examination. The examination revealed a moderate pneumonia in the dependent cardiac lobe, adhesions in the pericardium, and moderate fibrin tags in the mesentery.

There could be no distinctions made between those animals receiving DIVEMA and the control animals on the basis of the post mortem examination.



---- Calf 433

____ Calf 436

†Temperatures above this line considered above normal range.

#Vaccine administered.

†Challenged.

Graph 1. Temperatures of calves in Group A[a].

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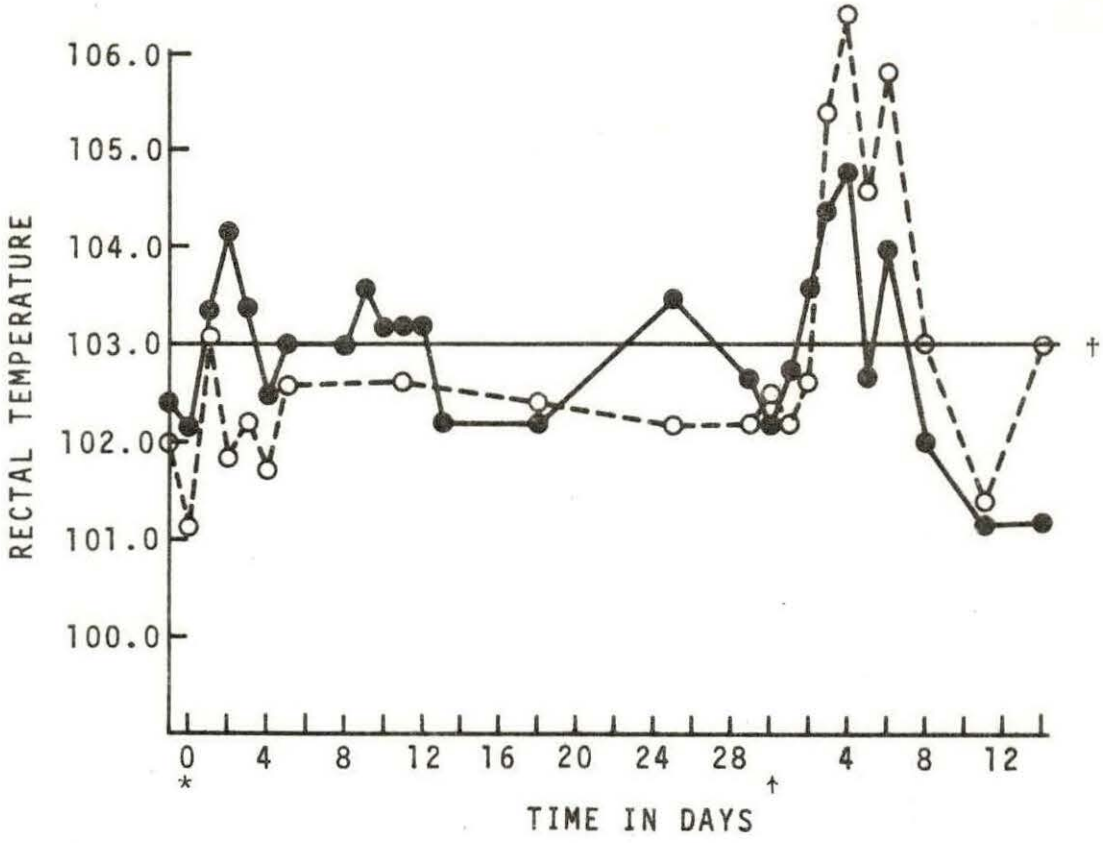
[a]Received one dose of vaccine IM.



---- Calf 434

____ Calf 439

†Temperatures above this line considered above normal range.

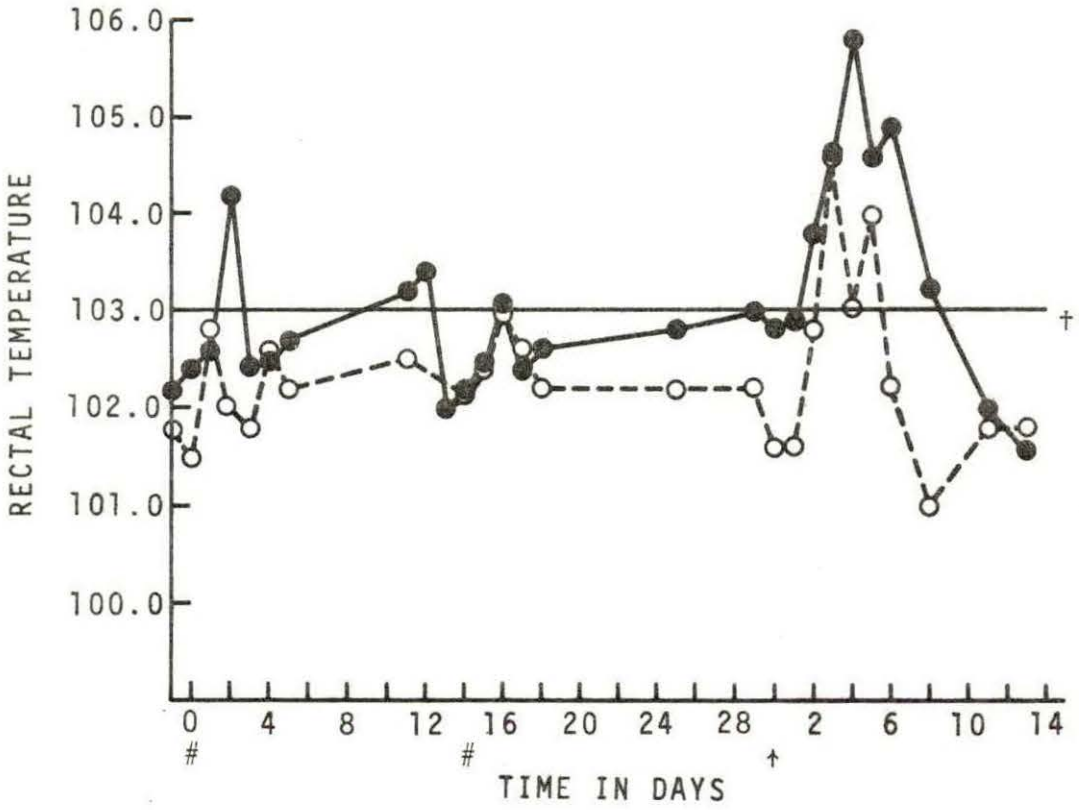
*DIVEMA and vaccine administered.

†Challenged.

Graph 2. Temperatures of calves in Group B[a].

---

[a]Received one dose vaccine IM plus one dose DIVEMA IV.



---- Calf 435

____ Calf 6902

†Temperatures above this line considered above normal range.

#Vaccine administered.

†Challenged.

Graph 3. Temperatures of calves in Group C[a].

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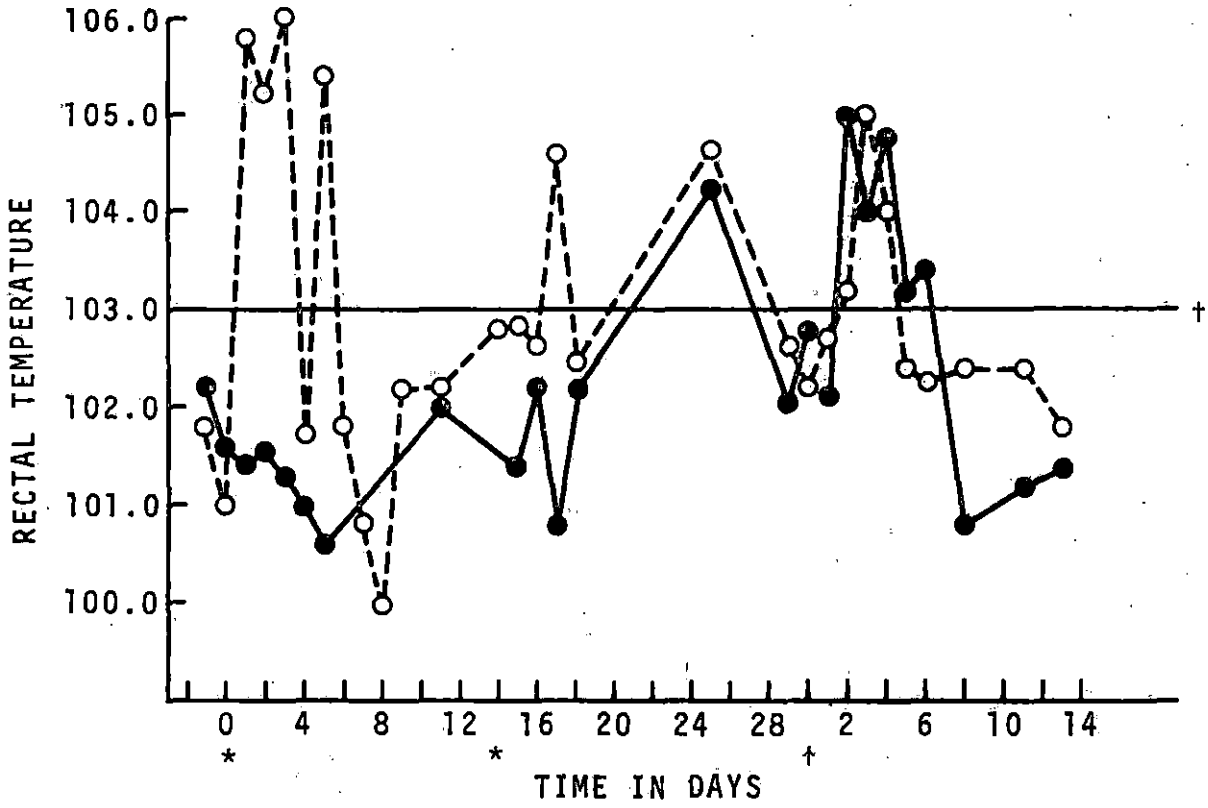
[a]Received two doses vaccine IM.



---- Calf 416

____ Calf 426

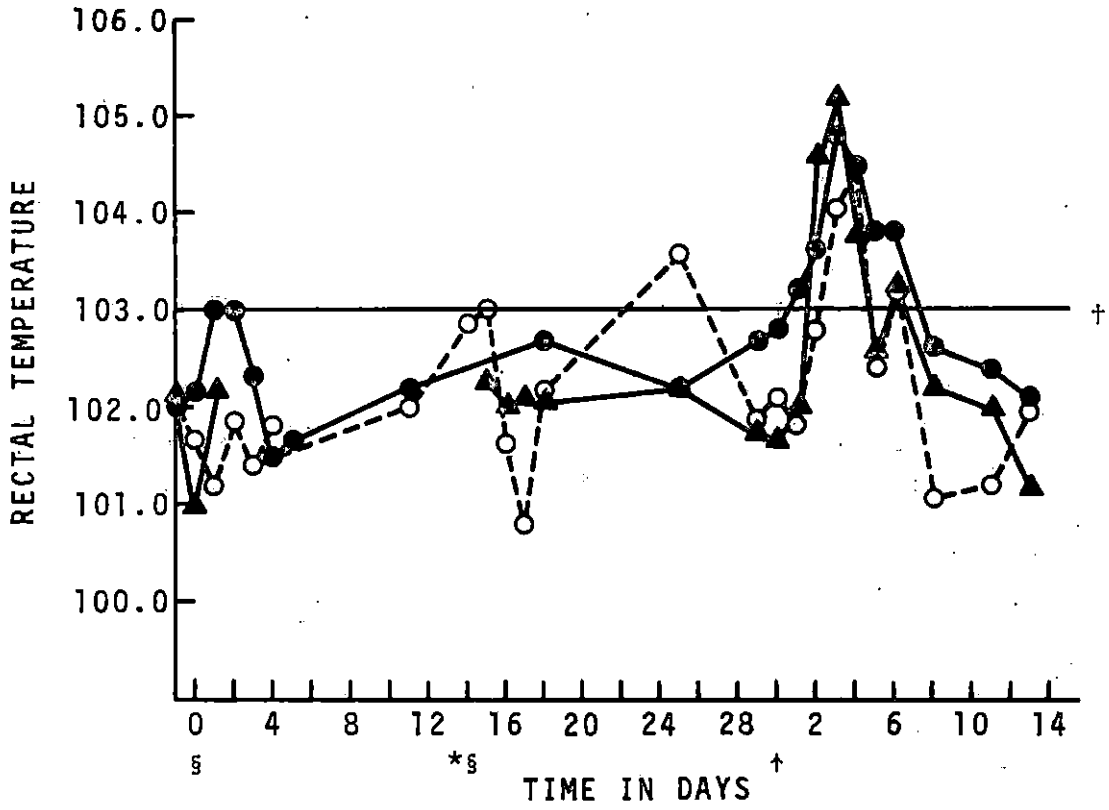
†Temperatures above this line considered above normal range.

*DIVEMA and vaccine administered.

†Challenged.

Graph 4. Temperatures of calves in Group D[a].

[a]Received two doses vaccine IM and two doses DIVEMA IV.



---- Calf 428

●——● Calf 413

▲——▲ Calf 438

†Temperatures above this line considered above the normal range.

§DIVEMA administered.

*DIVEMA and vaccine administered.

†Challenged.

Graph 5. Temperatures of calves in Group E[a].

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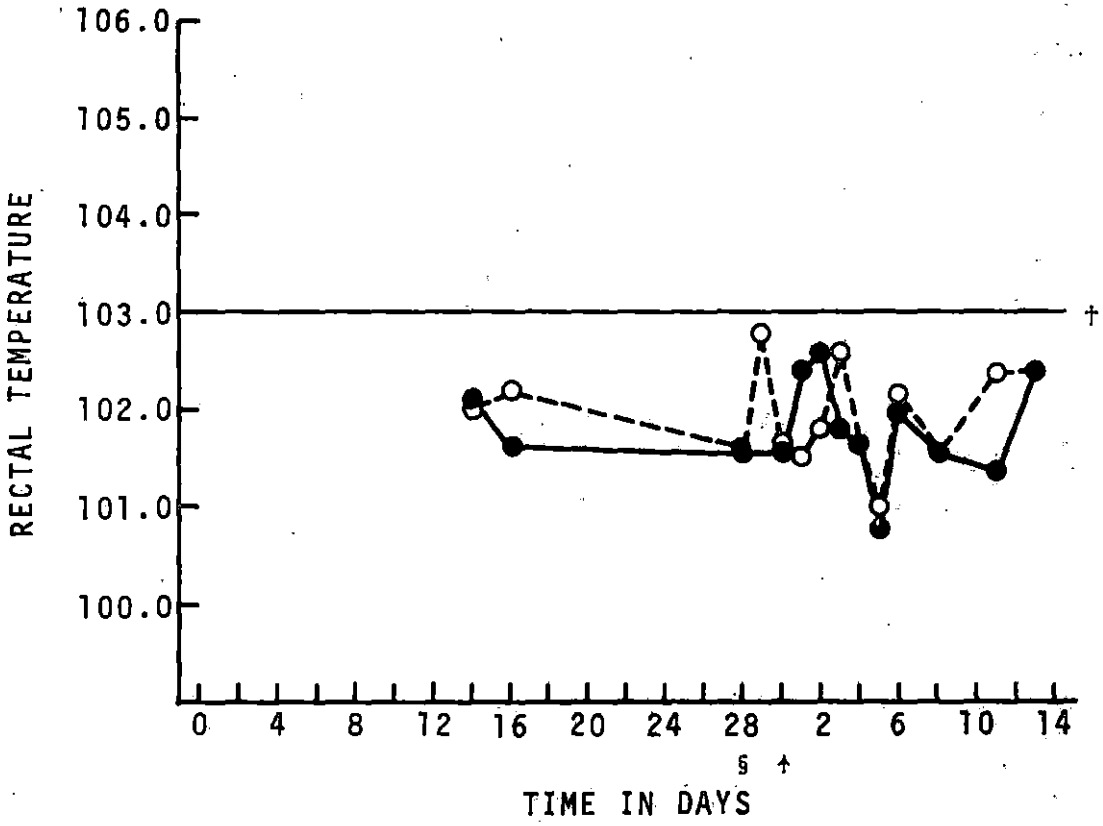
[a]Calf 428 and 413 one dose DIVEMA day 1; calf 438 one dose DIVEMA day 15; and calf 428 one dose vaccine and DIVEMA day 14.



---- Calf 445

____ Calf 447

†Temperatures above this line considered above the normal range.

§DIVEMA administered.

†Challenged.

Graph 6. Temperatures of calves in Group F[a].

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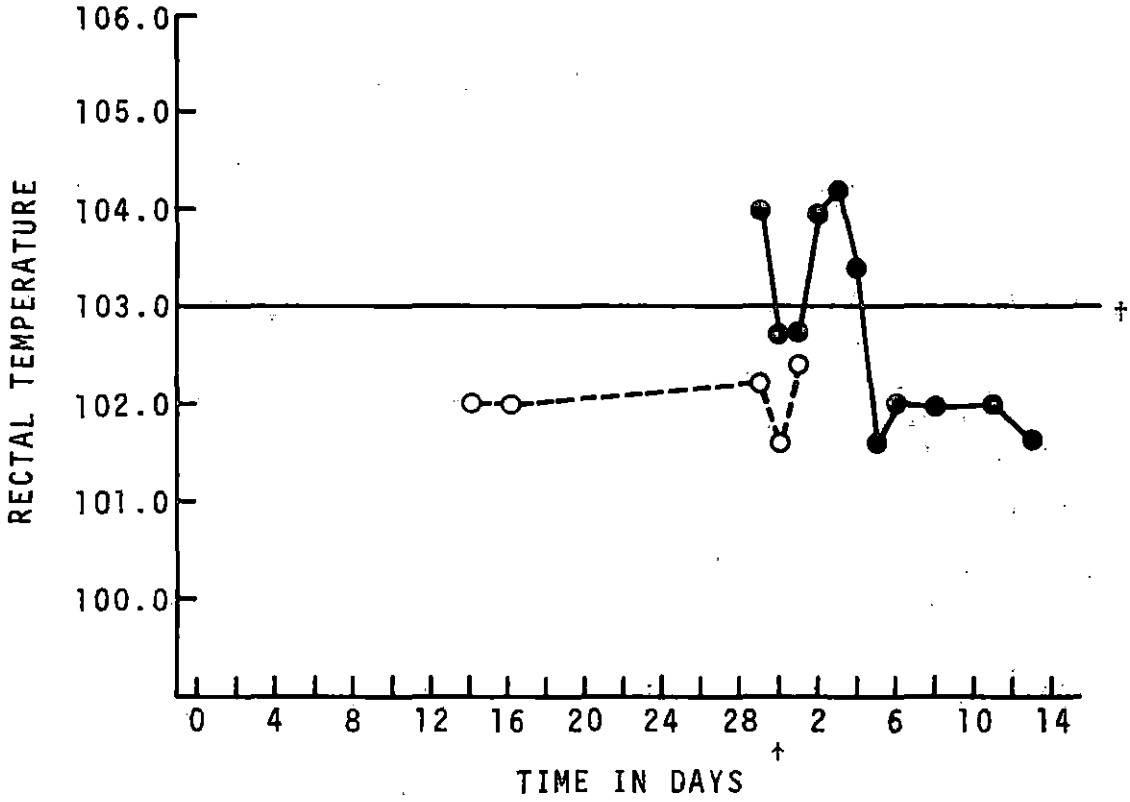
[a]Received DIVEMA 48 hours before challenge.



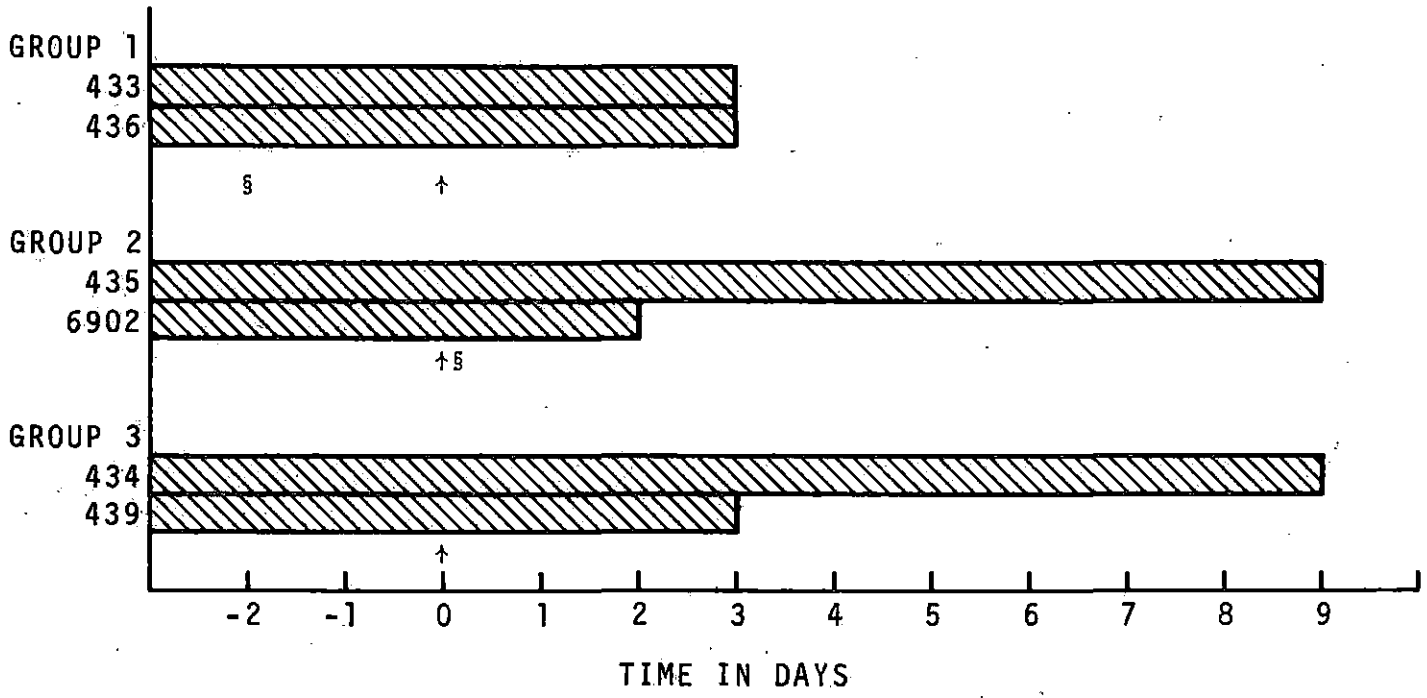
---- Calf 444

____ Calf No. Number

†Challenged.

Graph 7. Temperatures of calves in Group G[a].

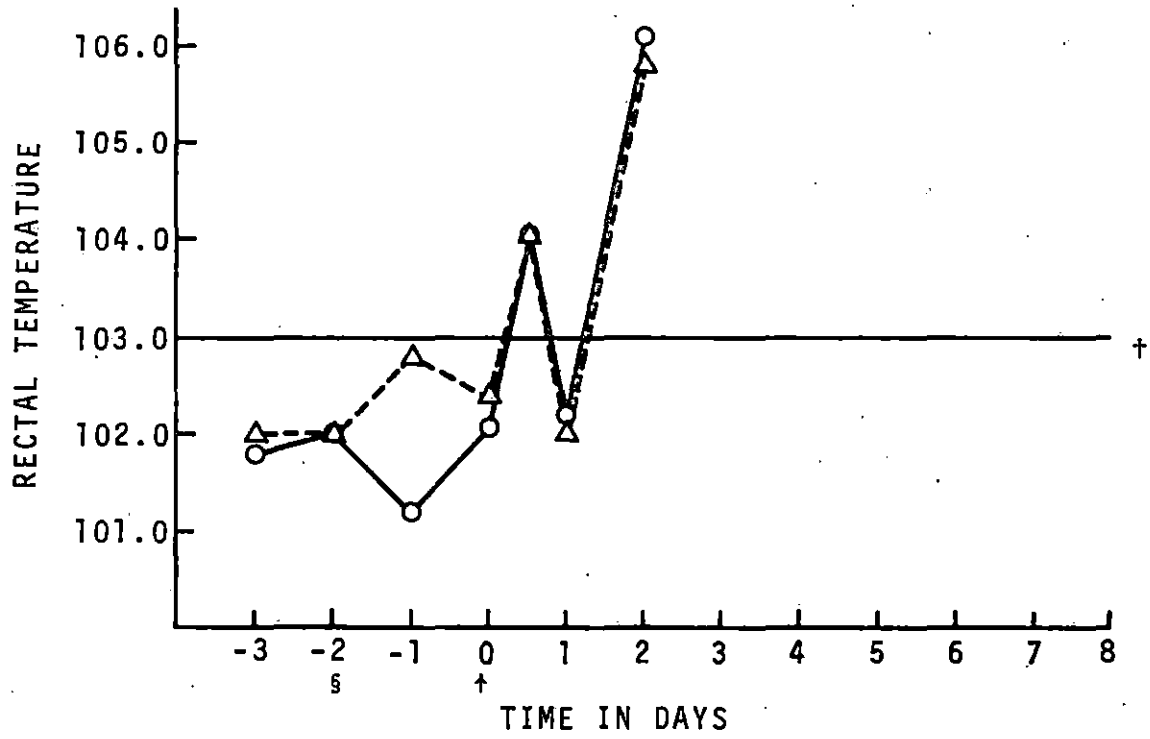
[a]Control group - did not receive vaccine or DIVEMA.



§DIVEMA administered.

†Challenged.

Graph 8. Mortality of calves in Experiment II.



---- Calf 433

—— Calf 436

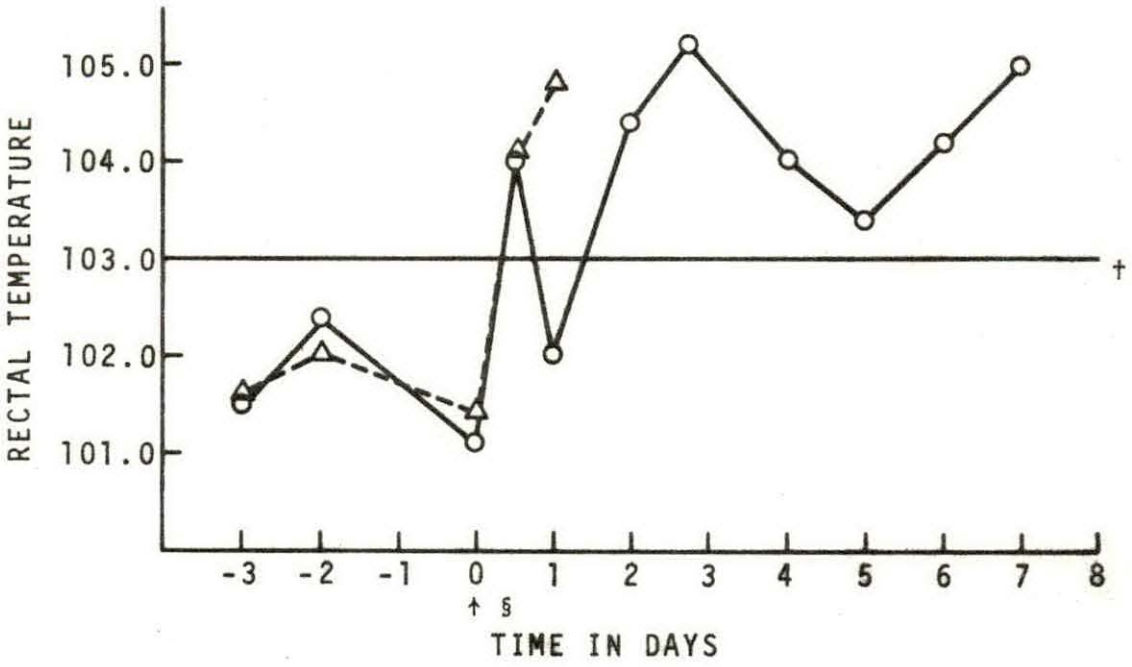
†Temperatures above this line considered above normal range.

§DIVEMA administered.

†Challenged.

Graph 9. Temperatures of calves in Group 1[a].

[a]Received DIVEMA 48 hours before challenge.



____ Calf 435

---- Calf 6902

†Temperatures above this line above normal range.

§DIVEMA administered.

†Challenged.

Graph 10. Temperatures of calves in Group 2[a].

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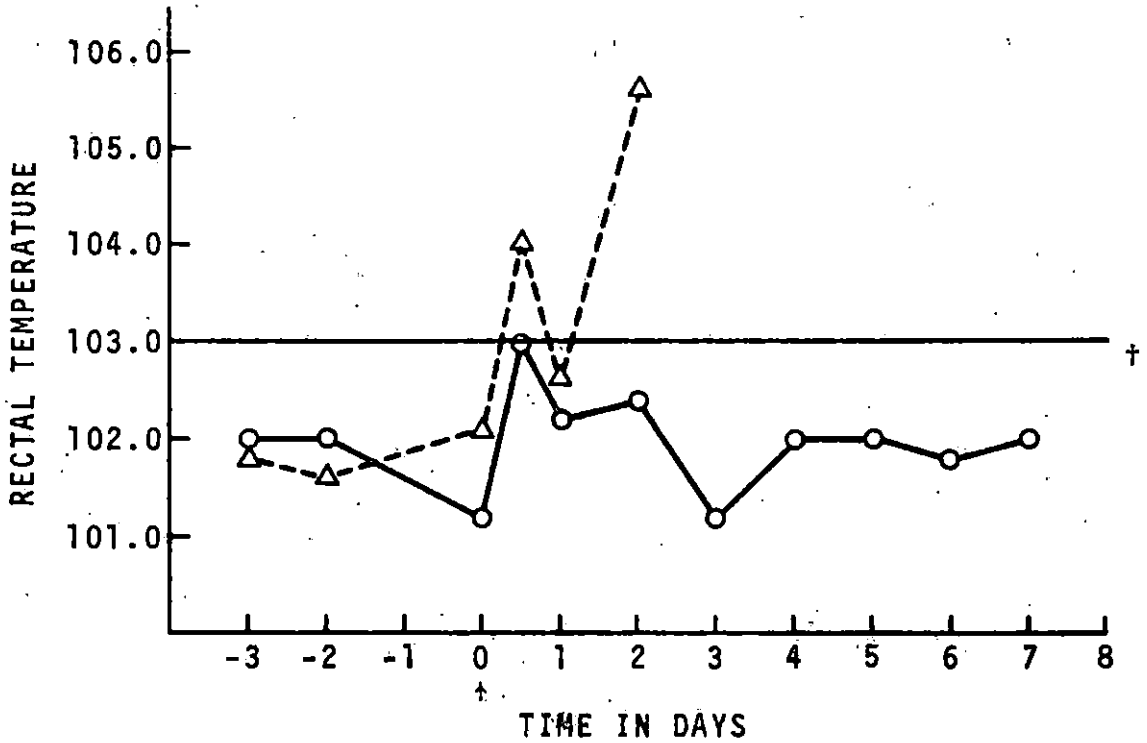
[a]DIVEMA administered 8 hours after challenge.



—— Calf 434

---- Calf 439

†Temperatures above this line above normal range.

†Challenged.

Graph 11. Temperatures of calves in Group 3[a].

[a]Control group - received no DIVEMA.

Table 1. WBC and differentials of Group A calves[a]

	Calf 433				Calf 436			
Day	WBC	Bands[b]	Segs[c]	Lymphs[d]	WBC	Bands	Segs	Lymphs
0	8,350	0	14	86	7,500	-	21	71
1	8,150	5	21	72	6,450	3	21	65
2	9,700	10	18	66	6,500	2	14	81
3	6,700	5	23	71	6,850	3	20	71
4	6,250	3	28	69	4,800	3	21	74
5	6,550	1	26	73	6,750	14	14	68
11	7,950	5	22	68	7,050	0	27	69
18	10,500	13	16	71	6,200	16	12	67
25	8,300	21	5	72	7,500	5	5	88
29	7,500	7	16	78	6,300	1	15	84
30[e]	7,900	5	18	77	7,100	5	19	76
1	6,800	6	21	72	6,300	8	16	76
2	blood clotted				5,500	5	28	65
3	8,900	15	23	61	8,240	19	33	46
4	9,880	11	14	75	11,060	14	41	44
5	8,000	12	17	71	6,400	9	21	69
6	9,110	7	23	67	7,300	9	10	79
8	7,900	7	17	75	5,100	2	11	81
11	8,000	-	-	-	5,300	-	-	-
13	5,500	-	-	-	5,300	-	-	-

[a]Received one dose of vaccine.

[b]Immature neutrophils.

[c]Mature neutrophils.

[d]Lymphocytes.

[e]Day challenged.

Table 2. WBC and differentials of Group B calves[a]

Day	Calf 434				Calf 439			
	WBC	Bands[b]	Segs[c]	Lymphs[d]	WBC	Bands	Segs	Lymphs
0	9,400	1	21	86	8,100	2	35	62
1	9,150	4	28	67	8,200	11	30	59
2	9,800	3	6	81	5,100	10	21	68
3	8,700	3	20	69	6,050	3	30	65
4	8,600	2	17	76	5,900	2	14	84
5	7,750	1	13	81	5,950	8	10	82
11	7,000	3	13	8	6,650	2	16	74
18	12,800	1	3	93	6,700	12	5	79
25	9,300	10	6	83	5,500	12	18	69
29	10,500	11	13	75	5,700	4	10	81
30[e]	10,500	2	14	82	6,100	2	20	75
1	10,500	7	23	65	5,700	9	25	63
2	8,500	2	22	65	5,700	3	31	64
3	8,200	9	21	61	7,300	14	34	52
4	9,540	13	13	72	7,530	12	24	62
5	8,000	7	18	73	5,400	7	12	80
6	8,540	7	14	78	5,350	12	18	69
8	7,300	1	10	87	4,300	3	7	89
11	7,500	-	-	-	4,200	-	-	-
13	8,400	-	-	-	4,300	-	-	-

[a]Received one dose vaccine and one dose DIVEMA.

[b]Immature neutrophils.

[c]Mature neutrophils.

[d]Lymphocytes.

[e]Day challenged.

Table 3. WBC and differentials of Group C calves[a]

Day	Calf 435				Calf 6902			
	WBC	Bands[b]	Segs[c]	Lymphs[d]	WBC	Bands	Segs	Lymphs
0	11,700	5	37	57	9,000	3	27	68
1	10,500	13	21	65	7,450	1	35	60
2	10,050	13	26	61	7,500	1	23	73
3	-	-	-	-	8,800	5	35	51
4	9,900	10	33	59	8,700	4	39	51
5	10,150	8	25	67	8,600	16	17	65
11	11,450	3	32	60	10,500	0	14	82
14	10,200	31	18	51	9,000	4	18	74
15	11,600	28	12	57	11,500	4	36	54
16	7,800	8	27	63	9,900	3	38	53
17	6,800	9	29	60	7,300	9	31	59
18	6,600	9	9	75	7,800	6	17	74
25	9,800	11	12	73	7,700	5	4	87
29	10,400	8	22	70	11,800	1	18	73
30[e]	11,000	6	16	77	13,200	5	19	69
1	8,900	4	35	69	11,300	8	38	52
2	7,900	6	42	51	9,900	19	35	46
3	8,460	8	12	76	16,600	13	44	42
4	9,260	9	16	74	17,700	16	17	67
5	8,400	12	11	76	7,100	9	18	69
6	8,900	7	11	81	9,090	7	15	70
8	4,300	13	-	85	5,600	2	14	81
11	7,300	-	-	-	7,200	-	-	-
13	7,000	-	-	-	7,100	-	-	-

[a]Received two doses of vaccine.

[b]Immature neutrophils.

[c]Mature neutrophils.

[d]Lymphocytes.

[e]Day challenged.

Table 4. WBC and differentials of Group D calves[a]

Day	Calf 416				Calf 426			
	WBC	Bands[b]	Segs[c]	Lymphs[d]	WBC	Bands	Segs	Lymphs
0	10,500	2	19	71	10,150	1	15	84
1	11,700	9	21	67	8,200	7	27	64
2	8,800	7	36	55	8,200	6	16	78
3	7,250	-	15	82	7,800	-	21	74
4	6,700	-	7	91	7,800	-	21	75
5	6,850	4	6	89	6,550	10	23	65
11	7,500	-	11	87	9,000	1	11	88
14	7,600	1	12	83	-	-	-	-
15	9,000	5	8	87	11,700	23	20	56
16	7,400	-	8	89	7,600	3	20	71
17	6,400	-	15	85	6,000	1	21	76
18	7,500	4	6	90	8,700	1	9	90
25	5,500	6	11	83	6,900	5	17	76
29	11,200	2	6	91	8,800	2	17	78
30[e]	12,700	-	-	100	16,700	6	12	81
1	7,800	1	5	91	8,000	7	21	70
2	8,100	1	8	87	7,300	6	41	53
3	6,290	11	5	82	11,100	11	38	50
4	8,900	11	17	70	11,900	23	20	56
5	7,300	3	12	84	8,100	8	19	67
6	8,350	2	7	90	7,700	5	22	73
8	6,100	2	4	92	6,300	1	3	95
11	6,900	-	-	-	8,300	-	-	-
13	7,300	-	-	-	8,100	-	-	-

[a]Received two doses of vaccine and two doses DIVEMA.

[b]Immature neutrophils.

[c]Mature neutrophils.

[d]Lymphocytes.

[e]Day challenged.

Table 5. WBC and differentials of Group E calves[a]

Day	Calf 428				Calf 412				Calf 438			
	WBC	Bands[b]	Segs[c]	Lymphs[d]	WBC	Bands	Segs	Lymphs	WBC	Bands	Segs	Lymphs
0	8,450	2	19	78	8,900	-	4	94	7,450	-	13	84
1	7,100	7	19	74	10,400	12	26	62	6,550	4	29	61
2	7,800	5	18	76	7,750	25	26	49	-	-	-	-
3	8,000	-	28	70	10,700	-	15	82	-	-	-	-
4	6,600	2	20	73	8,300	-	13	86	-	-	-	-
5	6,150	6	16	72	8,600	16	17	65	-	-	-	-
11	5,650	-	-	-	4,900	-	2	97	-	-	-	-
14	6,600	1	11	88	-	-	-	-	-	-	-	-
15	9,250	24	22	54	-	-	-	-	11,000	22	35	43
16	5,500	1	13	86	-	-	-	-	6,000	5	28	64
17	5,400	1	10	88	-	-	-	-	5,600	1	15	84

[a]Calves 428 and 413 received one dose DIVEMA on day 1. Calf 438 received one dose DIVEMA on day 15. Calf 428 received one dose DIVEMA on day 14 and one dose vaccine on day 14.

[b]Immature neutrophils.

[c]Mature neutrophils.

[d]Lymphocytes.

Table 5. (continued)

	Calf 428				Calf 412				Calf 438			
Day	WBC	Bands	Segs	Lymphs	WBC	Bands	Segs	Lymphs	WBC	Bands	Segs	Lymphs
18	9,700	-	12	85	8,700	28	14	57	10,400	16	12	67
25	6,300	4	3	93	6,200	25	13	62	6,100	4	1	93
29	8,320	1	3	95	9,720	7	25	66	6,600	5	8	85
30	7,500	13	13	74	9,000	9	10	80	6,900	9	12	78
1	5,600	1	10	87	8,300	3	22	71	6,200	1	19	77
2	5,300	2	9	86	6,700	4	12	83	5,800	7	25	65
3	7,220	7	21	72	7,000	9	24	65	8,180	14	23	62
4	7,900	7	14	77	10,400	28	13	60	10,100	16	10	74
5	7,700	10	18	69	6,500	1	19	77	7,400	13	13	74
6	9,910	7	12	81	7,620	2	7	90	8,390	8	7	85
8	5,500	2	7	88	4,500	6	3	89	5,000	2	8	87
11	7,100	-	-	-	6,100	-	-	-	6,100	-	-	-
13	6,200	-	-	-	5,600	-	-	-	6,000	-	-	-

Table 6. WBC and differentials of Group F calves[a]

	Calf 445				Calf 447			
Day	WBC	Bands[b]	Segs[c]	Lymphs[d]	WBC	Bands	Segs	Lymphs
28	7,550	-	-	-	4,950	-	-	-
29	6,420	7	21	69	5,810	5	20	74
30[e]	6,800	8	20	70	5,700	7	38	54
1	6,700	16	26	55	3,600	12	14	74
2	6,900	15	26	57	12,200	1	13	85
3	6,260	13	26	61	4,840	9	10	81
4	9,400	30	18	52	5,800	13	28	58
5	9,000	30	16	52	4,900	8	5	83
6	7,920	7	32	59	5,900	9	14	77
8	3,400	5	30	65	3,400	3	1	94
11	4,900	-	-	-	3,200	-	-	-
13	5,000	-	-	-	3,200	-	-	-

[a]Received DIVEMA 48 hours before challenge.

[b]Immature neutrophils.

[c]Mature neutrophils.

[d]Lymphocytes.

[e]Day challenged.

Table 7. WBC and differentials of Group G calves[a]

	No number				Calf 444			
Day	WBC	Bands[b]	Segs[c]	Lymphs[d]	WBC	Bands	Segs	Lymphs
29	13,400	7	18	70	6,420	12	26	59
30[e]	14,400	13	33	54	6,900	21	26	53
1	12,900	10	35	53	5,600	23	53	24
2	3,100	9	16	71	Died			
3	11,900	7	27	65				
4	14,000	12	9	79				
5	10,300	2	22	71				
6	10,100	3	17	73				
8	6,900	6	7	87				
11	7,500	-	-	-				
13	7,800	-	-	-				

[a]Received no DIVEMA or vaccine.

[b]Immature neutrophils.

[c]Mature neutrophils.

[d]Lymphocytes.

[e]Day challenged.

Table 8. Results of the micro-titer serum neutralization tests

Group	A		B		C		D		E			F		G	
Day	433	436	434	439	435	6902	416	426	428	413	438	445	447	No number	444
0	0	0	0	0	0	0	0	0	0	0	0	-[a]	-	-	-
1	0	0	0	0	0	0	0	0	0	0	0	-	-	-	-
2	0	0	0	0	0	0	0	0	0	0	0	-	-	-	-
3	0	0	0	0	0	0	0	0	0	0	0	-	-	-	-
4	0	0	0	0	0	0	0	0	0	0	0	-	-	-	-
5	0	0	0	0	0	0	0	0	0	0	0	-	-	-	-
11	0	0	0	0	0	7	0	0	0	0	0	-	-	-	-
14	0	0	0	0	5	4	0	0	0	0	0	-	-	-	-
15	0	0	0	0	0	0	0	0	0	0	0	-	-	-	-
16	0	0	0	0	7	0	0	0	0	0	0	-	-	-	-
17	0	0	0	0	0	0	0	0	0	0	4[b]	-	-	-	-
18	0	0	0	0	0	0	0	0	0	0	0	-	-	-	-
25	0	0	0	0	0	0	0	0	0	0	0	0[c]	0[c]	0	0
28	-	-	-	-	-	-	-	-	-	-	-	0[c]	0[c]	0	0

[a]Not done.

[b]Titers that were negative on plaque reduction.

[c]Titers that were positive on plaque reduction.

Table 8. (continued)

Group	A		B		C		D		E			F		G	
Day	433	436	434	439	435	6902	416	426	428	413	438	445	447	No number	444
29	0	0	0	0	0	0	0	0	0	0	0	6	17	0	0
30[d]	0	0	0	0	0	0	0	0	0	0	0	5	0[c]	0	2[b]
1[e]	0	0	0	0	5	0	6	2[b]	0	0	0	0[c]	10	0	died
2	0	0	0	0	0	0	0	0	0	0	0	0[c]	0[c]	0	
3	0	0	0	6	0	0	16	0	0	5[b]	0	64	8	0	
4	0	0	0	0	0	3	0	0	0	0	0	32	8	0	
5	0	0	0	0	0	3	0	0	0	0	0	38	8	5	
6	0	0	0	0	0	3	0	0	0	0	0	45	7	0	
8	10	5	6	20	29	37	47	8	6	3	7	53	10	6	
11	128	76	54	219	128	>512	514	54	32	10	17	58	10	8	
13	151	130	30	153	219	>512	350	306	76	158	28	27	7	10	

[d]Challenged.

[e]Post challenged.

Table 9. Results of the plaque reduction serum neutralization tests

Group	A		B		C		D		E			F		G	
Day	433	436	434	439	435	6902	416	426	428	413	438	445	447	No number	444
0	0	0	0	0	0	0	0	0	-[a]	-	0	-	-	-	-
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	0	-	-	-	-	0	0	-	-	-	-	-	-	-
3	-	-	-	-	0	-	0	0	-	-	-	-	-	-	-
4	-	0	-	-	-	-	0	0	-	-	-	-	-	-	-
5	-	0	-	-	0	-	0	-	-	-	-	-	-	-	-
11	0	0	0	0	0	7	0	0	-	-	-	-	-	-	-
14	-	-	-	-	4	4	0	-	-	-	-	-	-	-	-
15	-	-	-	-	0	-	0	0	-	-	0	-	-	-	-
16	-	-	-	-	2	-	0	0	-	-	-	-	-	-	-
17	-	-	-	-	-	-	0	0	-	-	0	-	-	-	-
18	-	0	-	-	-	-	0	0	-	-	0	-	-	-	-
25	-	0	-	-	-	-	-	0	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-	-	32	8	-	-
29	0	0	0	0	-	-	0	0	-	-	-	26	8	0	0
30	-	0	-	-	-	-	0	0	-	-	-	30	11	0	0

[a]Not done.

Table 9. (continued)

Group	A		B		C		D		E			F		G	
Day	433	436	434	439	435	6902	416	426	428	413	438	445	447	No number	444
1[b]	-	0	-	-	-	-	0	0	-	-	-	21	12	-	0
2	-	0	-	8	-	0	0	0	-	0	0	>16	>16	0	-
3	-	0	-	-	-	-	-	0	-	-	-	>16	>16	-	-
4	-	0	-	-	-	-	-	0	-	-	-	>16	>16	-	-
5	-	0	-	-	-	-	-	0	-	-	-	>16	>16	-	-
6	-	0	-	-	-	-	-	0	-	-	-	>16	>16	-	-
8	-	-	-	6	-	16	-	30	-	-	-	>16	-	-	-
11	128	-	>128	-	-	-	-	332	-	-	-	18	-	-	-
13	>128	>128	>128	>128	-	>128	-	384	-	-	-	16	64	-	-

[b]Post challenged.

Table 10. Results of the interferon assay

Group	A		B		C		D		E			F		G	
Day	433	436	434	439	435	6902	416	426	428	413	438	445	447	No number	444
0	0[a]	0[a]	0[b]	0[b]	0[a]	0[a]	15[b]	2[b]	12[c]	8[c]	22	NS[d]	NS	NS	NS
1	0	0	2	0	0	0	15	2	3	6	NS	NS	NS	NS	NS
2	0	0	2	0	0	0	15	2	2	6	NS	NS	NS	NS	NS
3	0	0	0	8	0	0	10	0	0	3	NS	NS	NS	NS	NS
4	0	0	0	2	0	0	20	0	8	3	NS	NS	NS	NS	NS
5	0	0	0	0	0	0	11	0	4	3	NS	NS	NS	NS	NS
11	0	0	0	0	0	0	8	3	-[e]	8	NS	NS	NS	NS	NS
14	NS	NS	NS	NS	0[a]	0[a]	4[b]	2[b]	-[b]	NS	NS	NS	NS	NS	NS
15	NS	NS	NS	NS	0	0	2	2	-	NS	NS[c]	NS	NS	NS	NS
16	NS	NS	NS	NS	2	0	2	0	5	NS	0	NS	NS	NS	NS
17	NS	NS	NS	NS	2	4	40	0	0	NS	0	NS	NS	NS	NS

[a]Administered vaccine.

[b]Administered DIVEMA + vaccine.

[c]Administered DIVEMA.

[d]No sample.

[e]Not done.

Table 10. (continued)

Group	A		B		C		D		E			F		G	
Day	433	436	434	439	435	6902	416	426	428	413	438	445	447	No number	444
18	0	2	0	0	4	8	11	0	3	8	35	NS	NS	NS	NS
25	0	2	0	0	11	0	2	2	0	9	35	NS	NS	NS	NS
28	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	2[c]	2[c]	0	0
29	0	0	0	0	8	3	0	2	0	8	20	0	0	0	0
30[f]	0	0	0	2	7	2	0	0	0	6	3	4	0	2	0
1[g]	0	0	0	4	7	2	0	2	0	6	2	10	4	16	Died
2	4	2	2	NS	8	11	7	4	6	14	7	68	85	14	
3	5	3	4	8	57	28	20	22	34	57	6	21	30	11	
4	11	6	8	14	38	28	24	16	40	57	34	21	26	2	
5	8	3	24	14	64	48	32	19	28	34	11	20	16	0	
6	6	3	16	11	32	34	30	10	16	21	6	0	9	0	
8	0	0	4	8	4	4	2	0	2	8	2	2	2	0	
13	0	0	4	3	2	2	2	NS	0	4	2	2	2	0	

[f]Challenged.

[g]Post challenged.

Table 11. Characterization of bovine serum interferon

Procedure or criterion	Titers		Result
	Before treatment	After treatment	
Dialysis	33	22	Not dialyzable
Ultracentrifugation (100,000g for 1 hrs.)	43	40	Not sedimented
Acid treatment for 1 hr. at pH 2	43	24	Activity retained
Shorten contact with cells (4 hours)	54	16	Less activity
Heat inactivation 1 hour 56 C	33	2	Loss of activity
Direct neutralization with VSV (1 hour) 50 $TCID_{50}$		0	No reduction in virus plaque forming ability

Table 12. WBC and differentials of Group 1 calves

Day	WBC	Differential		
		Neutrophils		
		Bands	Segmented	Lymphocytes
		Calf 433		
-2	6,600	1	14	78
-1	10,500	21	35	43
0[a]	6,100	5	16	67
0[b]	11,100	23	39	35
1	7,700	18	35	45
2	9,000	20	37	40
3	Died			
		Calf 436		
-2	7,000	1	13	79
-1	12,400	13	26	57
0[a]	6,500	0	16	81
0[b]	12,300	14	37	40
1	12,200	10	33	49
2	13,700	12	35	45
3	Died			

[a] 8:30 AM.

[b] 4:30 PM.

Table 13. WBC and differentials of Group 2 calves

Day	WBC	Differential		
		Neutrophils		
		Bands	Segmented	Lymphocytes
		Calf 435		
-2	9,600	0	10	81
-1	ND[a]			
0[b]	11,900	8	15	68
0[c]	21,000	14	38	45
1	15,300	19	23	55
2	10,900	18	22	54
3	ND			
4	15,300	23	28	45
9	Killed			
		Calf 6902		
-2	13,600	2	13	78
-1	ND			
0[b]	7,800	3	22	68
0[c]	26,100	20	56	20
1	24,600	10	33	49
2	Died			

[a]Not done.

[b]8:30 AM.

[c]4:30 PM.

Table 14. WBC and differentials of Group 3 calves

Day	WBC	Differential		
		Neutrophils		
		Bands	Segmented	Lymphocytes
		Calf 434		
-2	12,900	1	11	80
-1	ND[a]			
0[b]	13,900	4	8	79
0[c]	19,100	14	16	62
1	17,200	8	33	49
2	18,100	10	31	51
3	ND			
4	15,500	9	25	57
9	Killed			
		Calf 439		
-2	8,600	1	15	81
-1	ND			
0[b]	11,600	7	13	79
0[c]	13,300	7	59	33
1	12,500	14	46	36
2	12,200	11	43	43
3	Died			

[a]Not done.

[b]8:30 AM.

[c]4:30 PM.

DISCUSSION

The experiments were a preliminary investigation to examine the effectiveness of DIVEMA against both bacterial and viral agents and its enhancement of the immune response to a killed vaccine. Experiments in mice had shown that the multi-faceted mechanisms of action of DIVEMA could possibly enable this substance to be useful in combating the microorganisms involved in the BRDC.

There were two major aspects to Experiment I. The first was to determine whether DIVEMA could enhance the immune response to a killed virus vaccine and the second to see if DIVEMA alone could protect calves from IBR infection.

A host's defense system against viruses is complicated. The type and degree of resistance depends upon the age, species, genetic makeup, state of health and immune status of the host as well as the virulence. size of the inoculum and portal of entry of the virus. Immune responses can be artificially divided into two groups, cell mediated responses and humoral responses; these two groups, however, are neither physiologically nor functionally mutually exclusive of each other. With many virus infections, including herpes infection (24), cellular immunity is of particular importance.

Cell mediated immune reactions may be responsible for most of the host's defense against IBR; however, humoral antibody levels are frequently measured to evaluate the host's immune status. I measured humoral antibody against IBR in this research as an indication of resistance to the agent.

Table 8 will show that the killed IBR vaccine, even though it contained $10^{9.5}TCID_{50}$'s before beta-propiolactone inactivation, did not provide protection for any of the calves either when given alone or when given with DIVEMA. Calves in Group C showed transient titers of neutralizing antibody and yet they were not protected. This could be because of a lack of antigenicity of the inactivated vaccine or the levels of specific viral antigens were too low to properly sensitize immuno-competent cells.

Since Braun et al. (3) reported DIVEMA enhanced specific responses of antibody formation, it was hoped that DIVEMA would present the killed antigen to the host's immune system in a manner that would mock the viral presentation of the successful modified-live vaccines. The vaccine was given IM and the DIVEMA IV. This was not a successful combination in activating the host's immune system as none of the calves in groups A-D were protected against the IN IBR challenge. Further studies should be made using different combinations of routes of administration of vaccine and DIVEMA. It would be advantageous to have a small laboratory animal as the host so that a variety of tests may be performed.

At the time these experiments were conducted the effects of DIVEMA had not been measured in the calf. In human studies researchers reported toxic effects as indicated by elevated WBC counts and fever (29). Pigs receiving DIVEMA suffered adverse reactions, including abdominal pain and fevers. One pig died from a severe peritonitis that resulted from an IP injection of DIVEMA (23).

Calves from three herds were purchased and subsequently penned together. The animals developed fevers, diarrhea, and coughing during this initial holding period. Two calves died before the experiment was started; however, the other calves responded to antibiotic therapy. Attempts to isolate virus were negative. The cause of death was not determined. Because the calves had been ill, animals from the fourth herd were kept separate from the others until day 12 of the experiment. Calves in Groups F and G were the youngest animals in the experiment and were not purchased until 10 days before challenge. These calves came from the same herd as four others that had been purchased at the beginning of the experiment. Two days before challenge the calves were moved to a location several miles away where the live virus could be administered without danger of infecting other cattle at VMRI. Because the calves may have been stressed by the moves, change of environment, and change of diet, bacteria that would not ordinarily be expressed might be expressed. This could result in inapparent infections causing elevated WBCs, hence making interpretation of the data difficult. WBC counts and temperatures were used to evaluate the effects of DIVEMA in the calf host as well as the response of the calves to the vaccine.

Some of the calves did have elevated WBCs and temperatures, as shown in Graphs 1-7 and Tables 1-7 that might be attributed to the DIVEMA. Calf 416 was the only calf that had an adverse reaction. He had a very high temperature which lasted for 5 days. In addition the calf was lethargic and off feed. He had an interferon titer the day of vaccination which could suggest that there was a subclinical infection. One can not draw

any conclusions from one calf but it would be wise to check for toxic effects of DIVEMA in further studies. Except for calf 416 temperatures and WBCs were no higher in the calves receiving vaccine and DIVEMA and only DIVEMA than in the calves receiving only vaccine.

The second aspect of Experiment I was to ascertain if DIVEMA would be effective in preventing viral infections in calves. DIVEMA had been effective in preventing a number of viral infections in mice (6,7,25,26,28, 30,33,37). The mechanism of resistance has been suggested to be the production of interferon. However, DIVEMA has been reported to protect against viral diseases for as long as 55 days (45), while interferon is short-lived.

If we accept the fact that the vaccine used in Groups A-D was not effective then we can look at these calves to evaluate the effectiveness of DIVEMA along with the calves in Groups E and F. DIVEMA given to calves in one or two doses 30 days or 14 days before challenge did not prevent infection by IBR virus. Any long term effectiveness that was hoped would prevail did not occur at the dosage given.

Calves in Group F were given DIVEMA 48 hours before challenge. These were the only calves in this experiment that did not show any signs of a clinical infection. Unfortunately there is a problem of evaluating this data. Pre-purchase serums from the calves had been screened by the microtiter serum neutralization test and found to be negative for IBR antibodies. In light of this the test results from the daily bleedings as shown in Table 8 are puzzling. Calf 445 had no detectable antibody to IBR 2 days pre-challenge but had measurable titers the day before chal-

lenge and on the day of challenge. Calf 447 had no detectable titer 2 days before challenge but had measurable titers 1 day before challenge and on PC day 1. A plaque reduction test was conducted as it was thought this test would be more sensitive. The results of this showed all of the serums for these calves that were negative were now positive for IBR antibodies.

There are two ways in which the calves could have antibodies to IBR. They could have had a recent infection but this is doubtful in that the antibody pattern does not show a rise in titer that would reflect a recent infection. There is also a lack of any amnestic response after challenge that would be suggestive of a prior active infection. The calves came from the same herd that supplied me with six other calves which did not have antibodies against IBR and it seems unlikely that these two calves would be the only ones to get an IBR infection.

The second possibility is that they had maternal antibodies and this appears to be the most likely explanation. Their antibody level remained low but steady during the post-challenge period. These calves were the youngest of the herd and were approximately 6-8 weeks old. If cell mediated immunity is the key to recovery from a herpes infection as discussed earlier, then the maternal antibodies in the serum may not protect the calf from IBR virus. It is a possibility then that the administration of DIVEMA 48 hours pre-challenge was instrumental in prevention of the viral infection. This possibility can be resolved by further studies.

Serum levels of interferon are frequently measured as an indication of anti-viral resistance an animal may have, even though interferon production in the nasal cavity may be responsible for the host's defense.

The summary of the results of the interferon assays as shown in Table 10 show four items of interest. First there are low levels of interferon which appear 2 days after DIVEMA administration and reach peak levels at either 4 or 5 days. This was seen in approximately 50% of the calves that received DIVEMA.

Second, there were five calves that had low interferon titers the day of challenge and yet they were not protected from infection. These low levels could have been induced as a result of the move to the new location.

Third, the IBR challenge virus produced higher titers than that induced by DIVEMA. This is consistent with reports from other workers (26) that viral infections produce higher levels of interferon than do synthetic interferon inducers. The interferon appeared at PC day 2 and reached peak levels at PC days 3, 4 and 5. Interferon was probably ultimately responsible for the calves' recovery.

Fourth, the calves in Group F, which had been given DIVEMA 48 hours before challenge, had the highest titers of any of the groups. The peak levels of interferon were reached on PC day 2, which was the fourth day after administration of DIVEMA. It appears that the combination of stimulation from the challenge and DIVEMA produced high titers. While the possibility of antibodies providing the defense mechanism exists, as mentioned earlier, it is likely that interferon contributed to the protection. The serum levels of interferon were at their highest at the time the virus was probably multiplying rapidly in the host.

If interferon induction is the mechanism of defense, further testing should be done to establish time intervals of effectiveness. Also one

could try intranasal administration of the DIVEMA. This route of induction would possibly eliminate some of the toxic side effects.

If DIVEMA is to be effective against the micro-organisms of the BRDC it would necessarily have to be effective against many bacteria as well as many virus. DIVEMA has been reported to have antibacterial properties (27,35,41).

In the second experiment DIVEMA's antibacterial properties were tested against a H. somnus infection. A review of Graph 8 shows that the DIVEMA treated calves did not survive the bacterial infection and further there was no sparing effects in the treated calves. Necropsies on the calves also revealed that there were no differences between the treated calves and the controls.

An interesting response to the Hemophilus infection, which has not been reported for experimentally infected calves, was the sharp rise is temperature and WBCs hours after infections. Temperatures and WBCs then subsided and were found to be normal 24 hours after infection. This reaction is not consistent with an endotoxin response in that the WBC count rose rather than having an immediate drop. The most logical explanation is a rapidly disseminating bacterial infection unless DIVEMA alters the reaction normally observed with an endotoxin.

Calf 6902's temperature and WBC did not return to normal but continued to rise and the animal died 24 hours before the calves in Group 1 and one control calf (as shown in Graph 8). The possibility exists that there was an adverse reaction from the DIVEMA that hastened its death. Munson and Regelson (31) reported that DIVEMA produces a sensitization to

endotoxin. Their experiments show that the amount of endotoxin required to produce lethal effects is 300 times less in the DIVEMA treated mice than in nontreated animals. Calf 435 was also treated with DIVEMA 8 hours after infection. It was almost dead when it was killed on the 9th day after infection. One could surmise that DIVEMA may have prolonged its life since it lived longer than the others except for one control. Control calf 434 which was the only one to recover from the infection and calf 435 came from the same herd and were the oldest calves and the only two Jerseys in this study. Age and genetic factors could have made them more resistant to the infection and perhaps calf 435 would have recovered had it not received DIVEMA.

The possibilities discussed in the preceding paragraph can at best be only conjectures. The small number of calves used in this study do not allow one to draw conclusions except that the calves treated with DIVEMA as outlined in Materials and Methods did not confer resistance to H. somnus infection.

It is doubtful that DIVEMA can be used in the treatment or in prevention of cattle suffering from respiratory diseases. It had been hoped the diverse mechanisms of action of DIVEMA could protect the calves from the many micro-organisms that makeup the BRDC. Unfortunately this diversity also works against the usefulness of DIVEMA. Whether a host treated with DIVEMA had decreased or increased resistance to infection depends on a number of factors such as route of administration, time interval between treatment and infection and pathogenesis of the infection. DIVEMA did not enhance the immune response to the killed IBR vaccine and

did not confer any resistance to H. somnus infection; however, there is some evidence to suggest that DIVEMA when administered 2 days before challenge protected the calves against IBR infection.

In conclusion DIVEMA did not provide any protection against bacterial challenge of calves in the H. somnus study. Additional work must be completed to determine the role of this compound in the protection of cattle against specific viral infections.

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