THE IMMUNE RESPONSE IN CALVES VACCINATED WITH INFECTIOUS BOVINE RHINOTRACHEITIS OR BOVINE VIRAL DIARRHEA VIRUS VACCINES

by

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DEDICATION

To my parents for their

love and understanding

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ABBREVIATIONS

ATCC	American Type Culture Collection
BUN	Blood Urea Nitrogen
BVDV	Bovine Virus Diarrhea Virus
^{o}C	Degrees Celsius
Ca	Calcium
CBC	Complete Blood Count
cc	Cubic Centimeter
Ci	Curie
Cl	Chloride
CO_2	Carbon Dioxide
CPE	Cytopathic Effect
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
DPM	Disintegrations per Minute
EDTA	Ethylene Diamine Tetraacetic Acid
FCS	Fetal Calf Serum
g	Gravity
H_2O	Water
Hb	Hemoglobin
HS	Horse Serum
^{3}H-TdR	Tritiated Thymidine
IBRV	Infectious Bovine Rhinotracheitis Virus
IM	Intramuscular
IN	Intranasal

K	Potassium
LA Strain	Los Angeles Strain
MDBK	Madin Darby Bovine Kidney
MEM	Minimal Essential Media
mg	Milligram
ml	Milliliter
MLV	Modified Live Virus
mM	Millimole
Na	Sodium
NaCl	Sodium Chloride
NADL	National Animal Disease Laboratories
ng	Nanogram
P	Phosphorus
PBS	Phosphate Buffered Saline
PCV	Packed Cell Volume
PFU	Plaque Forming Unit
PHA	Phytohemagglutinin
PSK	Penicillin-Streptomycin-Kanamycin
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
SDH	Sorbitol Dehydrogenase
SGPT	Serum Glutamic Pyruvate Transaminase
SMA-12	Technicon 12 channel Sequential Multiple Analyzer
TCA	Trichloroacetic Acid
uCi	Microcurie
ug	Microgram

UV	Ultraviolet
WBC	White Blood Cells

SECTION I

INTRODUCTION

INTRODUCTION

Respiratory infections in cattle account for one of the most serious economic losses in the United States and many other parts of the world where the livestock industry has gained high level of economic importance. It is known that the agents responsible for causing respiratory disease syndrome are mainly viruses, bacteria and mycoplasma producing different clinical signs of the disease under varying environmental conditions (Omar, A. R., 1966; Rosenquist, B. D., 1974; Saunders, et al., 1964). The interaction of these infectious agents in a host with a complex immune system makes the clinical syndrome variable and a definite identification of the pathogens becomes difficult. A list of all infectious agents involved in the syndrome is likely to be incomplete as new viral agents are identified from time to time, but it is generally accepted that the main viral agents most frequently isolated are bovine virus diarrhea (BVD), infectious bovine rhinotracheitis (IBR) and parainfluenza-3 viruses. Circulating antibodies and/or hemagglutinating titers to all three viruses have been detected simultaneously in the same animal (Jensen and Mackay, 1971).

Commercial vaccines against both IBR and BVD, consisting of attenuated viruses grown in tissue cultures have been available, but are often thought to be responsible for post-vaccinal reactions (Bittle, 1968; Clark, 1968; McKercher, et al., 1968; Peacock, 1968; Rosner, 1968; Peter, et al., 1967; Kelling, et al., 1973; and Searl (1971). A strain of BVD virus grown in porcine cells has

been attenuated and used as a vaccine and is not associated with any adverse reaction in animals (Phillips, et al., 1975). Todd, et al. (1974) developed an IBRV vaccine for intranasal use that affords relatively early protection against the disease, however McKercher, et al. (1971) did not find any added advantages attributed to intranasal vaccination. Inactivated vaccines have been available for use against both IBRV (Matsuoka, et al., 1972; Kolar, et al., 1974; Haralambiev, 1976) and BVDV (Fernelius, et al., 1972; McClarkin, et al., 1975), but the duration and the type of immunity conferred by inactivated viral vaccines have been questioned.

Bovine respiratory disease problems are more frequently encountered in crowded situations such as in feedlots and during transportation where the animals are under conditions of stress. Physiological changes under stress affect the hormonal balance of the animal and alters the susceptibility of the host to viral infection (Kilbourne and Horsefall, 1951; Shwartzman, et al., 1952; Kass, et al., 1958; Kaufman, 1965; Smart, et al., 1966; Reinicke, 1965; Rytel, et al., 1966). Sheffy, et al. (1973) reported the reappearance of IBRV infection in animals during times of stress, and recovery of the virus has also been demonstrated following administration of corticosteroids (Davies, et al., 1974). Both in vitro and in vivo studies revealed that hormones can change the expression of viral genomes in cells resulting in increased virus synthesis (Joncas, et al., 1973; Hall, et al., 1977; Jones, et al., 1963). Corticosteroids are

known to be immunosuppressive, lympholytic and cause lesions on lymphoid tissues (Wallnerova, et al., 1971) and the administration of the hormone resulted in potentiation of BVD in calves (Shope, et al., 1977).

The objective of this work was to investigate the immune response in calves vaccinated with modified live and inactivated vaccines, and to study interaction of viruses and lymphocytes in the presence of corticosteroids.

SECTION II

REVIEW OF LITERATURE

LITERATURE REVIEW

History of the Viruses. Bovine virus diarrhea was first reported as a transmissible disease of cattle by Olafson et al. (1946) in New York; as an X disease of cattle in Canada by Childs in the same year, and as an epizootic enteritis in Sweden by Hedstrom et al. (1951). Ramsey and Chivers (1953) reported a mucosal disease in the Midwest. Wheat et al. (1954) isolated a virus diarrhea strain from cattle in California that was immunologically similar to the New York strain. Since then, the disease has been reported throughout the United States and many other countries.

Infectious bovine rhinotracheitis has been recognized as a disease of cattle since 1950 (Schroeder and Moys, 1954), and first described as a new respiratory tract disease of cattle in 1955 (Miller, N. J.). Soon it was also associated with infectious pustulo vulvo vaginitis (IPV) by Kendrick et al. (1958) and McKercher (1963). Since the IBR virus also causes IPV, it is often called IBR-IPV (Baker, et al., 1960; Studdert et al., 1964). Subsequently the disease has been recognized throughout the United States and has been reported in various parts of the world.

Epidemiology of the Diseases. Bovine virus diarrhea virus has been incriminated in a variety of cattle maladies such as respiratory illness, diarrhea, abortion and lesions of mucosa of the gastro-intestinal tract (Kahrs, 1971, Kahrs et al., 1970a,

Gillespie et al., 1967, Kahrs, 1968 and Kahrs et al., 1970b). The prominent signs in a respiratory tract involvement are varied as recorded in several descriptions of clinical BVD: cough of different types (Pritchard et al., 1954; Avellini et al., 1968; Schoop and Wachendorder, 1963; Olafson et al., 1946), increase in respiratory rate (Ballarini and Ferrari, 1969). Mills (1966) experimentally infected calves with the Dunkle strain of the BVD virus and made the following observations: ten days after inoculation, both in inoculated and in control animals, as a result of contact and manipulation, there was mild rhinitis. However, in calves killed after more than ten days post inoculation, the disparity between infected and control became marked. In calves killed 25 to 39 days after infection, the nasal mucosae were reddened and hemorrhagic. Virus was isolated from the lungs, bronchial lymph nodes, and trachea up to 56 days after infection by which time it had disappeared from all other tissues. According to Mills, transmission via the respiratory route was the probable mode of spread of this disease, but Pritchard (1963) and Howarth (1972) suggest that the BVD virus may also be excreted via urine. Apart from being recognized as a major etiological agent in the respiratory disease syndrome of cattle, recently BVDV has been shown to be responsible for abortion in cattle. Since the first report of BVD-MD in cattle, the virus has been considered an abortifaciant, and abortion has been described in pregnant cattle in disease outbreaks (Olafson et al., 1946). It is known that BVDV is a pathogen

that can cross the placental barrier of susceptible cattle and cause infection at any stage of gestation (Kahrs et al., 1973; Casaro et al., 1971; Lambert, G., 1966; Scott et al., 1973). The disease in calves which develops after an intrauterine infection depends on the stage of gestation when the fetus is infected. During the first trimester of pregnancy, if the fetus is infected, it may die or brain damage may occur resulting in underdevelopment or hypoplasia of cerebellum. Fetuses that develop an in utero infection during the second half of gestation may be weak or scouring (Lambert, 1966). Further, fetal BVD infection has been substantiated by viral isolation (Casaro, et al., 1971; Gillespie, et al., 1967; Kendrick, 1972, Scott, et al. (1973), and by detection of serum antibody in fetuses and colostrum-deprived newborn calves (Casaro, et al., 1971, Classick, et al., 1970, Kendrick, 1971, Kendrick, et al., 1972).

IBRV causes variable clinical symptoms in cattle. Primarily a respiratory problem, the disease begins with high fever, depression, anorexia and muco-purulent nasal discharge (Bruner, et al., 1973, Smith, et al., 1973). Congested mucous membranes, shallow ulcers, necrotic areas around the nostrils and muzzle, conjunctivitis and lacrimation are often seen in the respiratory form of the disease. Lesions also appear on the pharynx, larynx, trachea and bronchi (McKercher, 1959), and occasionally, fatal pneumonia ensues due to asphyxiation by swollen respiratory mucosae. Abinanti and Plumer (1961) noted that in a natural outbreak of the disease in feedlot cattle, conjunctivitis was the

only evidence of infection, there being no accompanying respiratory signs. British workers also described intense conjunctivitis with lacrimation as the predominant signs in a natural outbreak (Dawson, et al., 1962).

In addition to causing an upper respiratory disease in cattle, the virus has been implicated in vulvovaginitis (Gillespie, et al., 1957, encephalitis (Barenfus, et al., 1973; Johnston, et al., 1962), abortion (Chow, et al., 1964; McKercher, et al., 1964) and systemic infection in calves (Galloway, 1972). Abortion in cattle caused by IBR virus is the result of fetal death following the infection (Kendrick, 1973). The virus has been isolated from the placenta as early as the eighth day post inoculation, and abortion can occur as late as the third month post vaccination. There is also evidence to indicate that the localization of the virus during the long period from inoculation to abortion is in the placenta (Kendrick, et al., 1971).

Isolation and Classification of the Viruses.

Bovine Virus Diarrhea Virus. Underdahl, et al., in 1957 reported the isolation of two cytopathic agents but failed to identify the virus isolates as the causative agents of the disease. Gillespie, et al. in 1960 identified a cytopathic virus in Oregon, namely the C24V strain. The National Animal Disease Laboratory (NADL) strain was isolated by Gutekunst, et al. in 1962 from the spleen of a naturally-occurring case of BVD in a closed herd at NADL, Iowa, and was adapted to porcine kidney cell culture by Malmquist, et al. in 1965. In sub-clinical infection, the virus was isolated

from washed leucocytes even when serum antibody was present (Gutekunst, et al., 1964).

The virus is an RNA-helical-enveloped virus (Castrucci, 1968a, b). There is considerable variation on reports describing the size of the virion. Dutta, et al. (1964) described a size of 80 nm, while Hafez, et al. (1968) suggested a size between 22-90 nm. Ritchie, et al. (1969) suggested a size of 80-100 nm particles. Maess, et al. (1970) and Hornizek (1971) described a size of 54 nm with an inner core of 24 nm in diameter.

Horzinek, Maess and Laufs (1971) suggested that the virus should be classified as a togavirus (lipid containing RNA virus with cubic symmetry of the nucleocapsid), although BVDV differs from other togaviruses in their lower sedimentation rate and desntiy values, their nucleocapsid diameters and the unusual rosary envelope structure (Horzinek, et al., 1971).

Infectious Bovine Rhinotracheitis Virus. Madin, et al. (1956) first isolated the virus from nasal secretions of cattle and cultivated it on bovine embryonic kidney cells. Tousimis, et al. in 1958 studied the virus by electron microscopy and determined that the IBR particles produced in bovine kidney cell cultures had a diameter of 145-150 nm. Classified as a herpes virus (Armstrong, 1961) the IBRV is a double-stranded DNA (Deoxyribonucleic acid) virus that replicates in the cell nucleus. The morphology of the virion is also similar to other mature herpes viral particles (Bruner, et al., 1973). It has an icosahedral

capsid with an envelope which appears to contain both viral and host components as the virus capsid develops in the nucleus.

Vaccination. The development of vaccines for control of BVD-MD syndrome was based on the attenuation of the BVD virus by serial passages in rabbits (Baker, et al., 1958) or in bovine kidney cell cultures (Coggins, et al., 1961). Commercial vaccines consisting of cell cultures attenuated BVD strain C24V (Bittle, 1968; Coggins, et al., 1961) or NADL strain adapted to swine kidney cell line (PK-15) (Gutekunst, 1968) are usually multivalent, containing more than one live viral or bacterial component (Baker, et al., 1958; Bittle, et al., 1968; and Tamoglia, 1968). Unfortunately, ever since the live attenuated vaccines were marketed in 1964, it was observed that they were often accompanied by post-vaccinal reactions in animals (Clark, 1968; McKercher, 1968; Peacock, 1968; Peter, et al., 1967; and Rosner, 1968) or were contaminated with other viral agents (McKercher, 1968; Peacock, 1968; Tamoglia, 1968). Evaluation of the efficacy of BVD vaccines is difficult because experimentally produced disease is mild whether challenged via the respiratory route (Mills et al., 1968; Tyler, et al., 1968), or through the intravenous route (Malmquist, 1968; Tyler, et al., 1968). A soluble antigen vaccine was investigated by Fernelius, et al., 1971 and vaccinated calves developed high titer of neutralizing antibodies against BVD virus. Fernelius, et al. (1972) studied the evaluation of beta-propiolactone inactivated and chloroform-treated virus vaccines against bovine viral diarrhea-mucosal disease,

and reported that it was effective in high antibody production in animals. In both these methods, two injections were recommended to stimulate antibody response sufficient to protect cattle. However, McClarkin, et al. (1975) demonstrated that if a killed vaccine was concentrated ten-fold only one injection was sufficient to elicit the immune response which protected the fetus in pregnant cattle experimentally exposed to BVDV. Phillips, et al. (1975) attenuated BVD in continuous porcine cell line (JS-PK 278) with good immunization results and without post-vaccinal reactions.

Infectious Bovine Rhinotracheitis Virus Vaccine. The first IBR vaccine developed was a modified live vaccine by Swartz, et al. in 1957 and was intended for intramuscular inoculation. This vaccine, which is still in use, has some disadvantages. It is contraindicated for use in pregnant animals and if used on calves less than six months old a second vaccination is essential because of the possibility of interference by colostrally acquired maternal antibodies. Apart from abortions the use of live vaccines for intramuscular use has also resulted in other problems. In 1971 there was evidence suggesting that IBR-vaccinated calves shed vaccine virus which then infected pregnant dams (Kelling, et al. 1973; Searl, R. D., 1971). Despite the controversy the intramuscular live virus vaccine is still widely used and the recommendations for revaccinating cattle after the first vaccination is varied among practicing veterinarians.

In an attempt to stimulate both local and parenteral defense mechanisms a modified live virus intranasal vaccine was introduced in 1969 by Todd, et al. Unlike vaccines for intramuscular use that can cause abortion, it has been claimed that the intranasal vaccines are usually safe for pregnant cattle (Kahrs, et al., 1973; Todd, et al., 1976; Todd, et al., 1972; Todd, et al., 1971). McKercher, et al. (1971) reported no distinct advantage of intranasal vaccination over parenteral injection. In studies related to the development of the vaccine, interferon production was detected in vaccinated animals and they were protected when challenged with virulent IBRV 40-72 hr. post-vaccination (Todd, et al., 1971). It is also claimed that intranasal vaccination of attenuated IBR vaccine virus into calves with maternal antibodies elicited active immunity (Todd, 1973). However, intranasal vaccination is laborious as head restraint is necessary and occasionally vaccinated cattle develop fever, excess nasal discharge and intranasal plaques from vaccination (Kahrs, et al., 1973).

Production of inactivated IBRV vaccine is one approach to eliminate the problems observed in animals given live modified vaccines. Inactivated IBR vaccine has been available intermittently in combination with a bacterin containing two Pasteurella species and inactivated parainfluenza-3 vaccine, but the immunogenic response to it has been poor (Matsuoka, et al., 1972, Schipper, et al., 1975). However, inactivated vaccines prepared by addition of an adjuvant have produced adequate antibody titres

in animals. Kolar, et al., 1972, using formalin-killed polyvalent vaccine produced high antibody levels in cattle.

Juhasz, et al. (1974) using formalin-killed IBRV vaccine which was adjuvated with aluminum-hydroxide gel adsorbent produced high titers of serum antibodies that protected calves to a large quantity of virus given simultaneously by intramuscular, intratracheal routes by spraying on the nasal mucosa and by scarification into vaginal mucosa. The symptoms were mild and of short duration.

Haralambiev (1976) used inactivated IBR vaccine prepared by ethanol and saponin treatment (Haralambiev, et al., 1967) into the nasal mucosa and demonstrated production of antibodies and interferon in the nasal discharge. Vaccinated animals resisted challenge with virulent virus. Animals given subcutaneous injection of the same preparation developed clinical illness following the challenge, thus supporting the efficacy of submucosal vaccination.

Blastogenesis Studies. In recent years, protection of the animals to infection with microorganisms have been focused not only on the humoral aspect of the immune system but also on the production of cell-mediated immunity (CMI). One of the methods of measuring CMI is to study blastogenesis of lymphocytes by mitogenic substances, i.e. PHA which is a non-specific mitogen as described for the first time by Nowell (1960). The stimulation of lymphocytes by PHA results in their transformation, termed as blastogenesis and is characterized by a rise in the synthesis of

deoxyribonucleic acid (DNA) reaching a maximum after 72 hrs. incubation. Blomgren, et al. (1971) and Janossy, et al. (1971) have shown that PHA stimulates selectively thymus-dependent lymphocytes (T lymphocytes).

In earlier studies lymphocyte response to mitogens was evaluated microscopically on the basis of cell morphology. Recently Sorensen, et al. (1969) have developed a method for measuring the incorporation of ^{3}H-thymidine into stimulated cells. At present a number of methods for quantitative assessment of lymphocyte response to PHA are available (Phillips, et al., 1970; Fernald, et al., 1971; Fauguet, 1976; Lazary, et al., 1974).

BVDV and Cell-Mediated Immunity. Necrotic foci can be observed in the lymphatic tissues like lymph nodes, spleen, and thymus of cattle fatally infected with BVD virus. Gross lesions also include destruction of Peyer's patches in the small intestines and loss of differentiation between cortex and medulla in lymph nodes. There is severe leukopenia and anemia which may persist for weeks (Blood, et al. (1963). The responsiveness of lymphocytes to PHA was depressed by addition of viable bovine virus diarrhea virus into cell cultures. This phenomenon was not observed when heat-killed or UV-irradiated virus was used (Muscoplat, et al., 1973). Abnormalities in surface immunoglobulin of circulating lymphocytes from animals with chronic bovine virus diarrhea have been studied (Muscoplat, et al., 1973) and there are indications that the infected animal has a possible metabolic defect resulting in inability of lymphocytes to secrete

immunoglobulins. Calves immunosuppressed with corticosteroid did not respond to BVDV for antibody production and were consequently not protected against challenge with the virus. However, calves with passive BVD antibodies, when given corticosteroid were protected (Shope, et al., 1976). These results may point out to a major role of humoral antibodies in protection of calves from BVDV. Popisil, et al. (1976) experimentally infected calves with BVDV and showed a decline in the phytohemagglutin responsiveness of lymphocytes from the infected calves, which again tends to support the evidence that the BVDV is an immunosuppressant.

IBRV and CMI. Rouse, et al. (1974) studied blastogenesis of peripheral blood lymphocytes of cattle immunized or infected with IBRV. They measured the uptake of ^{3}H-TdR in lymphocytes after stimulation with UV-inactivated IBR virus. The specificity of the blastogenic response was demonstrated as cells from animals immunized with IBR failed to be stimulated with two other non-specific herpes virus antigens (herpes simplex and equine rhinopneumonitis viruses). IBR specific antibodies also inhibited the uptake of ^{3}H-thymidine into cell cultures. The specificity of CMI response to IBR was further demonstrated by preventing the production of CPE by IBR virus in cell cultures in the presence of stimulated lymphocytes (Babuik, et al., 1974).

Corticosteroids. Corticosteroids are endogenous hormones secreted by the adrenal cortex, which have multiple physiological functions,

such as control of carbohydrate metabolism, acting as anti-inflammatory agents and regulating the number of circulating lymphocytes. At concentrations higher than normal physiological levels, corticosteroids change the profile of circulating white blood cells. There is a significant decrease in the absolute number of lymphocytes, monocytes, and eosinophils, with an increase in the number of circulating neutrophils (Fauci, et al., 1974; Yu, et al., 1974). Yu, et al. (1974) also reported that the number of T lymphocytes decreased to a greater extent as compared to B lymphocytes, thus having a direct bearing on the immune system. Nowell (1961) described the inhibition of mitotic activity of normal human lymphocytes cultures by prednisolone 21-phosphate and established a direct relationship between the concentration of steroid and percent decrease in mitotic activity. Roath and Cuppari (1965) suppressed the in vitro lymphocyte transformation by addition of dexamethasone, hydrocortisone, cortisone, and prednisolone. Aldosterone, hydroprogesterone and estradiol had no effects. It had also been observed by Coburg, et al. (1970) that skin test reactivity is decreased to a variety of antigens applied two hours after a high dose of corticosteroids. In addition to the suppression of T-cell function, Peter (1971) reported that corticosteroids diminish the effects of T-cell cytotoxicity by interfering with the effect of lymphotoxin on target cells.

Corticosteroids also affect the humoral response but to a lesser degree. High levels of corticosteroids over a period of

time significantly suppress the production of immunoglobulin-G (IgG) in the bone marrow (Butler, 1975). High doses of corticosteroids depressed the production of antibodies whereas physiologic levels stimulated the immunoglobulin production (Smith, 1972; Ambrose, 1970).

Corticosteroids and Binding Sites. Munck and Brinck-Johnson (1968) observed that rat thymocytes had well-defined steroid-binding sites and explained the kinetics of the binding process by the following mechanisms: the first, non-specific binding is independent of the concentration of the steroid and is peculiar to each hormone. The second, which is the specific binding, is concentration-dependent and directly related to the glucocorticoid activity. The specifically bound hormone elutes very slowly at 37° C when exposed to 0.9% saline, but there is no elution at 4° C. It was indicated that the specific binding is directly related to the activity of the steroid in the cell (Munck, 1968).

Corticosteroids and Bovine Respiratory Diseases. During conditions of stress in cattle, like overcrowding in feedlots or transport, the concentration of corticosteroids in blood may be elevated up to ten-fold or more (Ray, et al., 1972; Volkes, et al., 1973). IBRV which often produces subclinical infection in cattle (Snowdon, 1965) reappeared in time of stress. By administration of corticosteroid, Sheffy, et al. were able to recover IBRV from secretions and tissues of respiratory tract of cattle. Isolation and recrudescence of IBRV following

hormonal administration were subsequently shown by other workers (Sheffy, et al., 1972; Sheffy, et al., 1973; Davies, et al., 1973; Davies, et al., 1974). In vitro studies done by Hall, et al. (1977) revealed a ten to twelve fold increase in IBRV progeny in cells treated with corticosterone acetate. Shope, et al., (1976) potentiated the severity of BVDV infection in calves by administration of dexamethasone.

SECTION III

(Part I)

STUDIES ON BOVINE RESPIRATORY DISEASES

1. Immunological Responses of Calves to IBR and BVD Vaccines

Abstract

Twenty-four calves vaccinated with a commercial polyvalent modified live vaccine against infectious bovine rhinotracheitis virus (IBRV), bovine virus diarrhea virus (BVDV), and parainfluenza-3 virus, were studied for humoral and cellular immunity. Maximum serum antibody titers of 1/80 and 1/160 were detected against IBRV and BVDV, respectively. However, no cell mediated immune response was detected by lymphocyte blastogenesis studies.

Formalized inactivated vaccines with Freund's incomplete adjuvant prepared against IBRV and BVDV were inoculated into two calves. Serum antibody response was observed one week after inoculation reaching maximum titers of 1/640 and 1/1280 between six to nine weeks against IBRV and BVDV, respectively. In addition, cell mediated immune response was also evident in the calf inoculated with IBRV inactivated adjuvated vaccine as indicated by incorporation of ^{3}H-thymidine into lymphocytes stimulated by specific virus antigen.

Introduction. The bovine respiratory disease complex is one of the most serious disease problems of the cattle industry in the United States (McKercher, 1968). The etiology of the disease is complex, but it is now generally accepted that a number of viral agents, bacteria and mycoplasmas may be involved (Omar, 1966; Rosenquist, et al., 1970; Rosenquist, et al., 1974; Sanders, et al., 1964). Among the known pathogens, bovine virus diarrhea virus (BVDV) and infectious bovine rhinotracheitis virus (IBRV) have received considerable attention due to their wide distribution and the clinical disease they cause in cattle of all ages.

Vaccines against IBR and BVD have been available for the past decade but have been the subject of controversy because of their post-vaccinal syndromes (Clark, 1968; Peacock, 1968; McKercher, 1968; Peter, et al., 1971). Kelling, et al., (1973) and Searl (1971) reported the shedding of vaccine virus from calves vaccinated intramuscularly with IBRV vaccine. An IBRV vaccine developed for intranasal use (Todd, et al., 1974) has been claimed to afford early protection, however McKercher, et al., (1971) did not find any added advantages attributed to internasal vaccination. Inactivated vaccines have been available for use against both IBRV (Matsuoka, et al., 1972; Kolar, et al., 1974; Haralambiev, 1976) and BVDV (Fernelius, et al., 1972; McClarkin, et al., 1975) but the duration and type of immunity conferred by inactivated viral vaccines have not been illucidated.

The present study was undertaken to investigate the immune response in animals inoculated with live or inactivated virus vaccines.

Materials and Methods.

Animals. Twenty four calves, one to one and a half years old at the dairy farm, Kansas State University, were immunized against infectious bovine rhinotracheitis (IBR), bovine virus diarrhea (BVD) and parainfluenza-3 viruses at three months of age using Nasalgen. Between eight to twelve months of age they were revaccinated by intramuscular inoculation of modified live IBR and BVD viruses. In addition, six calves (four to five months old) from farms in the vicinity of Manhattan were selected on the basis of their susceptibility to IBRV and BVDV or both and were grouped as follows: Two calves (D3 and 293): free of serum antibodies to both IBRV and BVDV were used as control. Two calves (#9083 and #9100): free of serum antibodies to BVDV were inoculated with BVDV vaccine. Two calves (#1859 and #1861): free of serum antibodies to IBRV were incoulated with IBRV vaccine.

Pre-Inoculation Procedures. Prior to inoculation, body temperatures were established and complete blood counts were determined. Serum profiles of the animals were studied in the Sequential Multiple Analyzer (SMA-12), 12/60 micro (Technicon Instruments Corporation, Tarrytown, NY). Both blood and serum analysis were performed at the Clinical Pathology Laboratory, Department of Infectious Diseases, Kansas State University.

Cell Culture. Madin Darby bovine kidney (MDBK) cells were grown and maintained in Minimal Essential Medium (MEM), Eagles' containing 0.1% sodium bicarbonate, 10% horse serum (H.S., Kansas

City Biologicals), and an antibiotic mixture of penicillin 100 units/ml, streptomycin 1 mg/ml and kanamycin 5 ng/ml (PSK). Cells were cultured at 37° C in glass prescription bottles or in tissue culture dishes.

Viruses. The bovine virus diarrhea virus (BVDV), National Animal Disease Laboratory (NADL) strain and infectious bovine rhinotracheitis virus (IBRV), Los Angeles (LA) strain were obtained from ATCC and used in these studies. Both of these viruses produced cytopathic effects (CPE) on MDBK cells.

Virus Growth. Monolayer cultures of MDBK cells were inoculated with BVDV or IBRV at a multiplicity of about five Plaque Forming Units (PFU) and maintained in Medium 199. Cultures were harvested when 90% cells showed cytopathic effect (CPE) and stored at -80° C. Following two cycles of freezing and thawing, the suspension was centrifuged at 2,000 g for 30 minutes (mins) at 4° C, supernatant collected and spun at 100,000 g for five hours in an ultracentrifuge. The pellet was resuspended in five ml of MEM and stored in aliquots of 0.5 ml each.

Virus Assay. All viruses were assayed by the method described by Dulbecco (1952) for animal viruses with some modifications. 0.2 ml of each serial log dilutions of virus was inoculated in duplicate MDBK monolayers in 60 x 15 mm tissue culture dishes. Following one hour adsorption at 37° C, cultures were overlaid with eight ml of a mixture of 2% purified agar and 2 X MEM with

5% H.S., and further incubated. Monolayers were stained with the media mixture containing 0.01% neutral red after appropriate incubation of inoculated cultures (three days for IBRV and four days for BVDV). Plaques were counted the following day and recorded as PFU/ml.

Detection of Serum Antibodies by Plaque Reduction Method. Two fold dilutions of heat inactivated serum were mixed with an equal volume of BVDV or IBRV to contain 100 PFU/0.2 ml of the mixture. After one hour incubation at 25° C, 0.2 ml of the virus-serum mixture was inoculated in duplicate plates of MDBK cell monolayers and adsorbed for one hour at 37° C. Plaque assay was done as described previously and the serum titers on the basis of 50% reduction in PFU's were recorded.

Inactivation of Viruses by Formaldehyde. BVDV, 1.5×10^{8} to 3×10^{8} PFU/ml and IBRV, 1×10^{9} to 3×10^{9} PFU/ml were each treated with a final dilution of 1:1000 of formaldehyde. The mixture was incubated at room temperature for six hours, then overnight at 4° C and dialyzed in a 1/4 inch dialyzing tubing for 24 hours at 4° C with three changes of Phosphate Buffered Saline (PBS). The formalized virus was tested for complete inactivation by plaque assay.

Preparation of UV Irradiated Viruses. BVD and IBR viruses were inactivated by placing one ml of 1×10^{7} PFU/ml in a 60 x 15 mm dish and exposing for five minutes to a source of U.V. light (U.V. Germicidal Sterilamp, G30T8, 30 watt, Westinghouse Electric

Co., Bloomfield, NJ) at a distance of 10 cms. The virus was tested for total inactivation by plaque assay following U.V. treatment.

Inoculation of Calves with Formalized Vaccines. Calf #1859 and calf #9083 were inoculated intranasally (IN) with 1.5×10^8 PFU and 1×10^9 PFU of formalized IBRV and BVDV, respectively. Control calf #D3 was sham inoculated with one ml of formalized cell lysate (from MDBK cells prepared in Medium 199).

Three weeks post IN inoculation, one ml of formalin inactivated IBRV (3×10^9 PFU/ml) and three ml of formalin inactivated BVDV (3×10^8 PFU/ml) were homogenized with equal volumes of Freund's incomplete adjuvant (Grand Island Biological) and administered intramuscularly (IM) in the gluteal muscles to the respective animals. Control calf received a homogeneous mixture of cell lysate and Freund's incomplete adjuvant.

Inoculation of Modified Live Vaccines. Calf #1861 was inoculated IN with modified live IBRV (Nasalgen I, Monovalent, Serial 511), and calf #9100 inoculated IM with BVD monovalent vaccine. Both vaccines were kindly provided by Dr. Donald Todd, Jensen-Salsbery Laboratory, Kansas City, MO). Uninoculated calf #293 served as control.

Post-Inoculation Procedures. Body temperatures and hematologic norms were followed as previously described and calves were observed regularly for any abnormal signs. Nasal swabs and serum samples were collected at weekly intervals.

Testing of Nasal Secretions. Wooden applicators with sterile cotton tips were introduced in both nostrils and kept in five ml of cold PBS. Suspensions of nasal swabs (0.2 ml) were inoculated into duplicate cultures of MDBK cells and passaged three times in MDBK cells to observe CPE, if any, due to the presence of virus. Specificity of virus isolates was determined by plaque reduction test.

For detection of specific virus neutralizing factors, nasal secretion samples in five ml cold PBS were concentrated 5X in Minicon concentrators B15 (Amicon Corporation, Lexington, MA) and 0.2 ml was incubated with specific virus for one hour at 25° C and plaque assayed. For detecting non-specific virus neutralizing factors, monolayers of MDBK cells were treated overnight with 0.2 ml of 5 X concentrated sample and challenged the following day with 50-100 PFU of appropriate virus and plaqued. (IBRV was used as non-specific virus for BVD vaccinated calves and vice versa).

Isolation of Lymphocytes by Ficoll Isopaque Gradient. The isolation procedure included centrifugation of heparinized blood to obtain buffy coat cells, which were carefully aspirated with a pasteur pipette. The cells were suspended in three ml of PBS, gently overlaid on four ml of Ficoll Isopaque (Winthrop Laboratories, NY) (density 1.077 gm/ml) and centrifuged at 400 g for 20 minutes. Lymphocytes were collected by aspirating the layer of cells at the interface between the Ficoll and PBS.

Isolation of Bovine Lymphocytes by Lymphoprep. Cells from the buffy coat were recovered as described and resuspended in three ml of PBS and gently laid on lymphoprep (Nyegaard and Co., Oslo, Norway), which is a mixture of 9.6% sodium metrizoate and 5.6% Ficoll.

Lymphocytes were recovered from the interface after centrifugation as described earlier.

Isolation of Bovine Lymphocytes by Distilled Water Technique. Lymphocytes were isolated by the method described by Rossi, et al. (1977) with some modifications. Peripheral blood was collected by venipuncture in heparinized containers (five units of heparin/ml of blood). Five ml of blood was treated with 10 ml of distilled water (not deionized) for 15-20 seconds to lyse erythrocytes, followed by a treatment with 10 ml of 1.7% sodium chloride solution to restore isotonicity. The mixture was centrifuged for 10 minutes at 200 g. The procedure was repeated to completely eliminate RBC's. The recovery of viable mixed lymphocytes as determined by 0.04% trypan blue exclusion method was more than 99% by this procedure. Cells were washed and suspended in 10 ml of RPMI Medium 1640 (Roswell Park Memorial Institute Medium 1640) with PSK. In the present study, this technique was adopted for isolation of lymphocytes.

Separation of Bovine Mononuclear Cells. For separation of mononuclear cells, essentially the method of Julius, et al. (1973) was adopted. Nylon fiber from Fenwall Leukopaks (Fenwall,

Deerfield, IL) was washed in 0.2N hydrochloric acid for two hours and allowed to dry. 0.2 to 0.3 grams of this nylon fibre was packed in a six ml capacity plastic syringe up to the four or five ml mark and sterilized. The column was flushed with RPMI containing 10% calf serum (CS) free of IBR and BVD antibodies and placed in a CO_2 incubator one hour at 37^{o} C.

Mixed lymphocytes 5 x 10^7 cells suspended in one ml of warm RPMI were gently overlaid on the column and incubated for 45 minutes. The mononuclear cells were eluted from the column with dropwise addition of a total volume of 20 ml warm RPMI medium and collected in a tube.

Isolation of Macrophages. Mixed lymphocytes were suspended in RPMI with 10% C.S. at a cell concentration of about 5 x 10^6 cells/ml. About 1.5 to two ml of this suspension was dispersed in 60 x 15 mm tissue-culture plastic dishes and incubated for 30 minutes to allow macrophages to settle and adhere to the dish. The medium was then discarded and replaced by two ml of RPMI with 10% C.S. and reincubated for 30 minutes. This procedure was repeated two to three times to eliminate any nonadherent cells. Macrophages were obtained by scraping the layer of adherent cells with a sterile rubber policeman.

Preparation of Lymphocyte Cultures for Blastogenesis Studies. Duplicate or triplicate cultures, each containing 1 x 10^6 cells were suspended in two ml of RPMI with 10% C.S. and Phytohemagglutinin (PHA, Lectin type 'V', Sigma Chemical, St. Louis, MO)

2.5 ug/ml or 0.2 ml of 1 x 10^6 PFU/ml of U.V. treated virus was added to the cultures. Following 48 hours incubation, 0.1 ml media containing 1 uCi ^{3}H-thymidine (^{3}H-TdR, specific activity 5 Ci/mmole) was added. After a further incubation of 24 hours, the cultures were centrifuged, washed three times with cold Phosphate Buffered Saline (PBS) and precipitated with 5% trichloroacetic acid (TCA). They were left on ice bath for 10 to 15 minutes and the acid insoluble precipitates were collected on glass fiber filters (GF/C, 2.4 cms/ Whatman, England) in a sampling manifold (Millipore Corporation, Model 3025, Bedford, MA). Tubes were washed twice with 5% TCA and contents transferred on filters, which were placed in scintillation vials containing 10 ml of scintillation fluid. Samples were counted for 10 minutes in a scintillation spectrometer (Packard Tri-Carb, Model 3002, Downers Grove, IL).

Results.

Clinical Observations. Following intranasal or intramuscular inoculation of inactivated vaccines, none of the calves showed signs of illness. Appetite was normal and body temperatures remained in the range of 101 to 102° F. There were no changes observed in white blood cell counts after vaccination.

The calf vaccinated intranasally with Nasalgen 1 had a slight increase in body temperature (103.8° F) on the fourth day post-vaccination which returned to normal the following day. The calf that received modified live BVD vaccine IM did not show

any signs of illness. In both calves, no changes in white blood cell counts were observed after vaccination.

Virus Excretion. Attempts to isolate virus from nasal swabs after the administration of inactivated vaccines were negative. However, IBR virus was isolated from calf 1861 vaccinated with Nasalgen 1 on day three and subsequently on day four (Table 1). Specificity of the virus was determined with IBR and BVD antisera in plaque reduction test.

Assay of Virus Neutralizing Factors in Nasal Swabs. Plaque reduction assays did not reveal the presence of either specific or non-specific virus inactivating factors in the nasal swab suspensions of calves following vaccination with either inactivated or modified live vaccines.

Humoral Response. All the 24 calves from Kansas State University Dairy Farm had antibodies to IBRV and BVDV. Serum samples from 10 calves were titrated and had IBR and BVD antibody titers of 1/80 and 1/80 to 1/160, respectively. The intranasal vaccination with inactivated IBRV and BVDV in experimental calves free of neutralizing antibodies did not produce any humoral response. Serum samples collected one week after IM inoculation of these calves with inactivated-adjuvated vaccines produced serum antibody titers of 1/160 for IBRV (Figure 1), and 1/80 for BVDV (Figure 2), respectively. The IBR titers gradually increased, reaching a maximum of 1/640 by the fifth week, declined to 1/320 and remained constant thereafter (Figure 1). The BVD antibody

titer reached a peak of 1/1280 on the ninth week and did not change during the rest of the study (Figure 2).

In contrast, maximum serum antibody titers in calves inoculated with modified live IBR and BVD virus vaccines were 1/80 and 1/640, respectively (Figures 1 and 2).

Lymphocyte Studies. Table 2 demonstrates the percent recovery of mixed and purified lymphocytes by the methods described. A maximum recovery of 98% and 30% was observed for mixed and purified lymphocytes, respectively. Figure 3 demonstrates that the viability of lymphocytes was 100% upon initial isolation. The viability of mixed lymphocytes with PHA was 25-35% higher at 24 and 48 hours than cells without PHA. The number of viable cells declined to 60% by 72 hours.

Lymphocyte Stimulation by PHA. Figure 4 demonstrates the effect of different concentration of PHA on the stimulation of lymphocytes. Maximum stimulation occurred with 2.5 ug/ml of PHA as indicated by the incorporation of ^{3}H-TdR in cells. Higher levels of PHA resulted in decreased uptake of the label. Figure 5 illustrates that the time course of maximum DNA synthesis in lymphocytes was at 72 hours after addition of 2.5 ug/ml of PHA. The maximum incorporation of ^{3}H-TdR in PHA stimulated lymphocytes when exposed to the label was found to be between 12 and 24 hours as shown in Figure 6.

Lymphocyte Stimulation by Specific Viral Antigen. The results of stimulation of lymphocytes from calves by specific viral antigen

are shown in Tables 3 to 7. Lymphocytes from 24 dairy calves inoculated with live modified vaccine did not respond to stimulation by either IBR or BVD virus antigen (Table 3).

Mixed lymphocytes obtained from calf vaccinated with inactivated IBR virus and adjuvant were stimulated by IBR virus antigen as indicated by increase in stimulation index (Table 4). The stimulation index increased from 1.76 to 7.4 by the third week reaching a maximum of 20.69 by the 11th week. The mean stimulation index for the period of study was 11.53.

Lymphocytes of calves inoculated with inactivated BVD virus and adjuvant and IBR or BVD modified live vaccines did not respond to stimulation by specific virus antigens (Tables 5-7). Their stimulation indices did not change appreciably throughout the course of studies (mean stimulation indices ranged from 0.7 to 1.4).

Effect of Adherent Cells on Lymphocyte Stimulation. Nylon wool purified lymphocytes from calf vaccinated with inactivated IBR and adjuvant were not stimulated by IBR antigen. However, when they were mixed with a population of adherent cells (macrophages), stimulation was evidenced by blastogenesis and incorporation of ^{3}H-TdR (Figure 7).

Discussion. The absence of clinical signs in calves vaccinated with inactivated IBRV and BVDV is in accordance with the findings of other workers (Fernelius, et al., 1971; Fernelius, et al., 1972; McClarkin, et al., 1975; Kolar, et al., 1972; Juhasz, et al.,

1974; A. Schipper, et al., 1975). The rise in temperature recorded in calf given live modified IBR vaccine and subsequent recovery of the virus from its nasal passages indicated that the IBR virus replicated at the sites of entry. Recovery of IBR virus from nasal swabs of calves following vaccination with Nasalgen has also been reported by Hyland and Easterday (1975). The replication of virus observed in our studies may have conferred early protection to the animal by means of interferon synthesis before the production of humoral immunity (Todd, et al., 1971). Contrary to the findings of Peter, et al., 1967; Bittle, 1968; the virus shedding was not observed in calf vaccinated with modified live BVDV vaccine, and the vaccination was not accompanied by any untoward clinical reactions.

Calves inoculated with inactivated adjuvated vaccines demonstrated higher serum antibody titers as compared to calves immunized with modified live virus vaccines (Figures 1 and 2). It is also evident from Figure 1 that vaccination with inactivated-adjuvated IBR virus brought about an earlier serum antibody response as opposed to Nasalgen 1. Fernelius, et al. (1971, 1972) reported high antibody response with soluble and inactivated BVD virus vaccines after two or more vaccinations; and McClarkin, et al. (1975), using killed BVD virus, elicited adequate humoral response (1/256-1/512) by inoculation of a 10-fold concentrated BVDV antigen. In our study, a single inoculation of inactivated BVD virus with adjuvant produced a titer of 1/1280 serum antibodies (Figure 2). There have been many investigations as regards

to the use of inactivated IBR vaccines. Schipper (1975) reported poor and delayed serological response following administration of inactivated IBRV vaccine; Haralambiev (1976) produced secretory antibodies and interferon in nasal mucosa, but low serum antibody titers with saponin-ethanol inactivated IBRV. Kolar, et al. (1972), demonstrated high antibody titers by two inoculations of formalized inactivated IBRV (Colorado strain) with adjuvant, while Juhasz used inactivated IBRV adjuvated with aluminum hydroxide gel adsorbent in calves and showed high degree of serum antibodies. In our study, a single inoculation of inactivated-adjuvated IBRV elicited a rapid humoral response which reached a high antibody titer of 1:640 by the fifth week. The mode of action of oily adjuvants is well documented and described by many workers. McKinney, et al. (1960) demonstrated that oily adjuvants caused slow release of virus over long period of time and Hobson (1973) showed that the amount of virus needed to produce a maximum humoral response was much less when administered with an adjuvant. Influenza virus initiated a high antibody response when given with adjuvant 65 (Woodhour, et al., 1969). Higher serum antibodies response of inactivated-adjuvated vaccine in our studies may have been due to a similar mode of action as described by these investigators.

It was surprising to find that one of the control calves, previously free of BVD serum antibodies, showed titers of 1/80 after inoculation of cell lysate and adjuvant. Tests in rabbits using the lysate showed no viral contamination whatsoever

(Table 8). It is possible that the presence of adjuvant in the lysate might have changed the expression of previously undetectable serum antibody, or affected the ratio of various antibody classes resulting in appearance of humoral antibodies (White, 1967).

The maximum stimulation of lymphocytes was obtained when a concentration of 2.5 ug/ml of PHA Type V was used. Muscoplat, et al. (1974) reported a maximum stimulation of bovine lymphocytes with a concentration of 5 ug/ml of PHA (Type M), while other workers have obtained best stimulation at concentrations lower or higher than 2.5 ug/ml using different types of PHA preparations (Popisil, et al., 1976; Gerber, et al., 1974). The optimum culture period of 72 hours and the time of maximum uptake of ^{3}H-TdR when PHA stimulated cells are exposed to the label are similar to the findings of other investigations (Muscoplat, et al., 1974; Rouse, et al., 1974).

Cultures of mixed lymphocytes from calf vaccinated with inactivated-adjuvated vaccine were stimulated in vitro with specific IBR virus antigen suggesting the presence of cellular immunity (Table 4). Rouse, et al. (1974) have investigated the blastogenic response of IBR-primed lymphocytes with specific virus antigen. In their studies, lymphocytes were obtained from calves that were initially injected with a virulent strain of IBRV (Colorado strain), and Freund's complete adjuvant, followed by monthly intramuscular administration of live IBR virus, or calves infected intranasally with virulent virus. However, the mycobacterium antigen present in the adjuvant used

by them might have influenced the stimulation seen. In our studies specific stimulation was obtained by inoculation of a calf with a single dose of inactivated IBRV with Freund's incomplete adjuvant, and the stimulation index remained at high levels till the completion of this study (Table 4).

Rouse, et al., 1974 observed that purified lymphocytes from nylon wool column were specifically stimulated by IBRV antigen. In the present study purified lymphocytes from vaccinated calf failed to respond to IBRV antigen in contrast to the findings of Rouse, et al. (1974). However, when adherent cells (macrophages) were added, blastogenic effect was restored. This finding is in accordance with that of Alder and Rabinowitz (1973), indicating that macrophages are important in eliciting the cell mediated immune response.

These prototype studies have demonstrated that serum antibodies to both IBRV and BVDV may be elicited by a single inoculation of inactivated viruses with Freund's incomplete adjuvant and the antibody titers were relatively greater as compared to humoral response observed with modified live vaccines. In addition, cell mediated immune response was also evident in calf vaccinated with inactivated IBR virus with adjuvant. It is suggested that further studies on the role of inactivated vaccines in producing humoral and cell mediated immunity should be done using biologically degradable adjuvant and a larger sample size of animals.

Figure 1. Serum antibody titers of calves vaccinated with IBRV vaccines. Two fold dilutions of heat inactivated serum were mixed with an equal volume of IBR virus to contain 500 PFU/0.2 ml of the mixture. After one hour incubation at 25° C, 0.2 ml of the serum virus mixture was inoculated on duplicate monolayers of MDBK cells and adsorbed for one hour at 37° C. Monolayers were overlaid with a mixture of 2 X MEM and 2% purified agar containing 5% horse serum. After three days of incubation, monolayers were stained with media-agar mixture containing 0.01% neutral red, and serum antibody titers were determined on the basis of 50% reduction in the number of PFU as compared to virus control. Symbols: —○— Modified Live Virus Vaccine; —●— Formalized Virus Vaccine with Adjuvant.

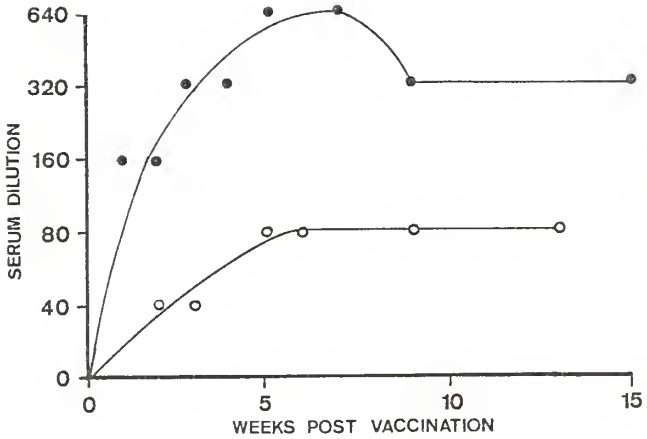

640
320
160
80
40
0
SERUM DILUTION
0
5
10
15
WEEKS POST VACCINATION

Figure 2. Serum antibody titers of calves vaccinated with BVDV vaccines. Two fold dilutions of heat inactivated serum were mixed with an equal volume of BVD virus to contain 100 PFU/0.2 ml of the mixture. After one hour incubation at 25° C, 0.2 ml of the serum virus mixture was inoculated on duplicate monolayers of MDBK cells and adsorbed for one hour at 37° C. Monolayers were overlaid with a mixture of 2 X MEM and 2% purified agar containing 5% horse serum. After four days of incubation, monolayers were stained with media-agar mixture containing 0.01% neutral red, and serum antibody titers were determined on the basis of 50% reduction in the number of PFU as compared to virus control. Symbols: —○— Modified Live Virus Vaccine; —●— Formalized Virus Vaccine with Adjuvant.

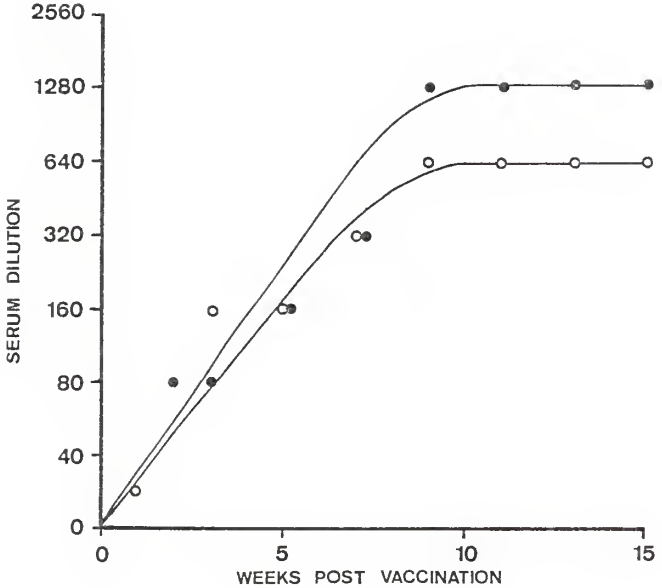

2560
1280
640
320
160
80
40
0
SERUM DILUTION
0
5
10
15
WEEKS POST VACCINATION

Figure 3. Viability of lymphocytes in culture. Mixed or purified lymphocytes, 1 x 10^6 cells were suspended in 1.9 ml R.P.M.I. with 10% calf serum. Media (0.1 ml) containing or lacking PHA was added to cultures that were incubated at 37° C. At 0, 24, 48, 72 and 96 hours, samples from cultures were mixed with 0.04% trypan blue, and examined under the microscope. Cells which excluded the dye were regarded as viable and counted on a hemocytometer. Symbols: —⊙—Mixed Lymphocytes with PHA; ---□--Mixed Lymphocytes without PHA; —●—Purified Lymphocytes with PHA; —▲— Purified Lymphocytes without PHA.

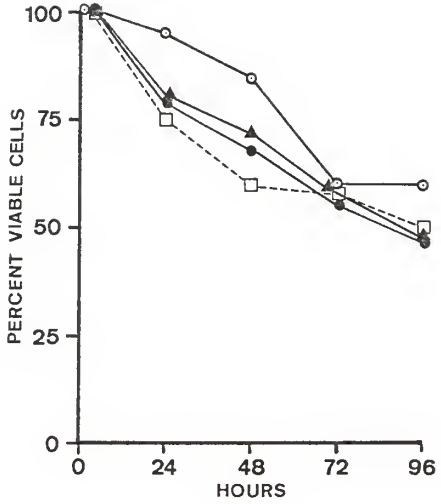

100
75
50
25
0
PERCENT VIABLE CELLS
0
24
48
72
96
HOURS

Figure 4. Stimulation of purified lymphocytes by PHA. Phytohemagglutin in concentrates of 1, 2, 2.5, 5, 7.5 and 10 ug/ml was added at 0 hour to lymphocytes cultures containing 1 x 10^6 cells in two ml of RPMI with 10% calf serum. At 48 hours, 1 uCi of ^{3}H-TdR was added and cultures terminated at 72 hours. Cells were harvested and processed for counting in scintillation spectrometer.

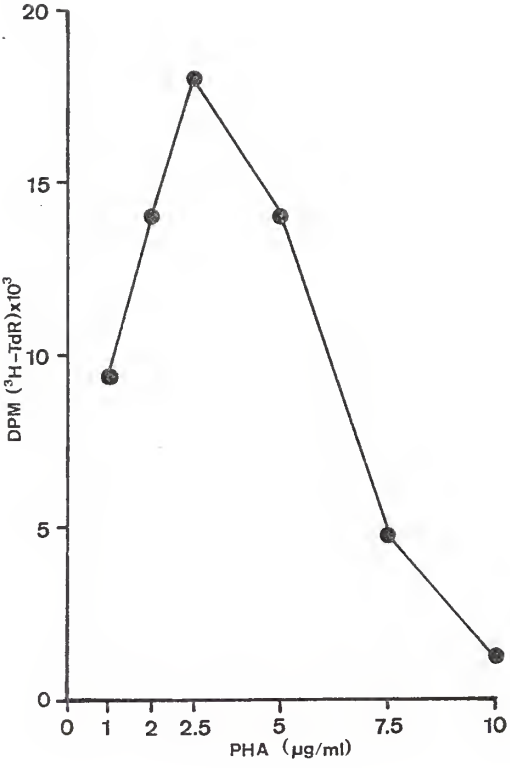

20
15
10
5
0
DPM (^{3}H-TdR)x10^3
0
1
2
2.5
5
7.5
10
PHA (µg/ml)

Figure 5. Time course of DNA synthesis in PHA stimulated lymphocytes. Lymphocyte cultures containing 2.5 ug/ml of PHA were incubated for different lengths of time up to 96 hours. Samples were labelled with ^{3}H-TdR 24 hours prior to harvest. At termination of culture, cells were processed for counting and specific activity on the basis of cell numbers (DPM/10^{5} cells) was determined. Symbols: —●— Mixed Lymphocytes; --□-- Purified Lymphocytes.

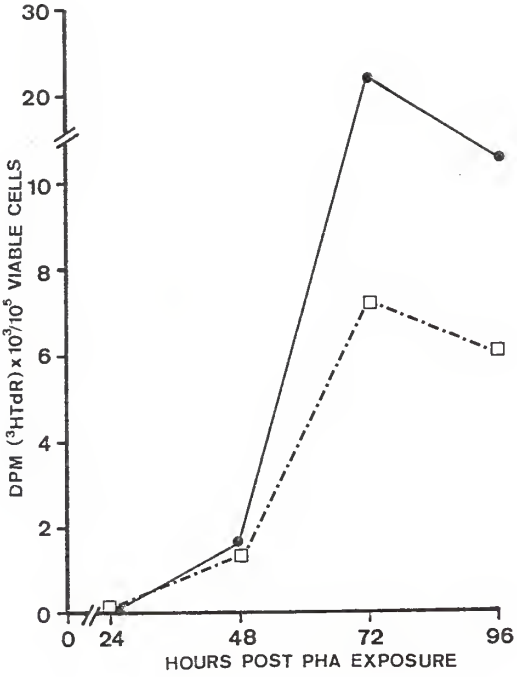

30
20
10
8
6
4
2
0
0
24
48
72
96
DPM (^{3}HTdR) x 10^3/10^5 VIABLE CELLS
HOURS POST PHA EXPOSURE

Figure 6. Incorporation of ^{3}H-thymidine in PHA stimulated lymphocyte. Lymphocyte cultures containing 2.5 ug PHA/ml were incubated. Beginning at 48 hours, ^{3}H-TdR was added to different sets of cultures at four hour intervals. All cultures were harvested at 72 hours, processed and DPM's determined.

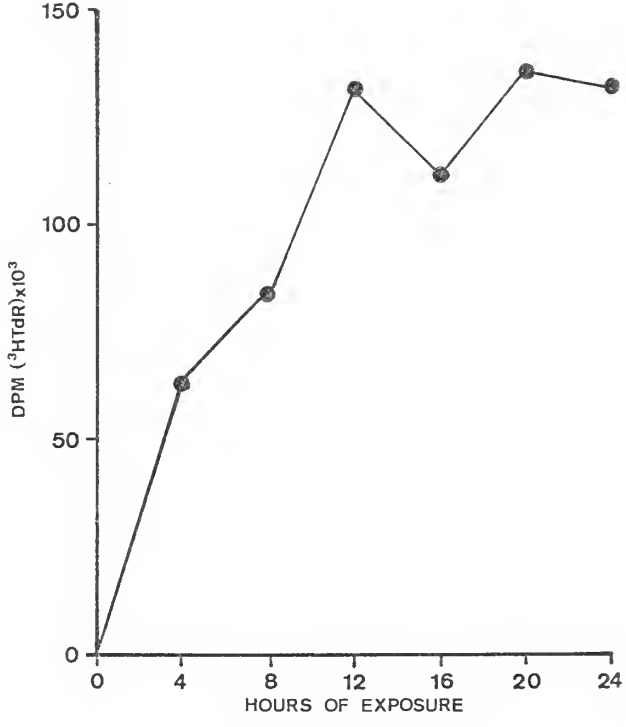

150
100
50
0
DPM (^{3}HTdR)x10^3
0
4
8
12
16
20
24
HOURS OF EXPOSURE

Figure 7. Stimulation of lymphocytes (mixed and purified) by specific virus antigen. Mixed and purified lymphocytes, 1 x 10^6 cells, obtained from calf vaccinated with formalized-adjuvated vaccine were stimulated in vitro by IBRV (U.V. irradiated). A set of purified lymphocytes cultures, to which macrophages from the same animal were added, was treated in a similar way, and the stimulation indices of the different cultures were determined after the samples were processed and DPM calculated. Symbols: ☐ Mixed Lymphocytes; ▨ Mixed Lymphocytes + IBRV Antigen; ⁘ Mixed Lymphocytes + BVDV Antigen; ▥ Purified Lymphocytes; ▩ Purified Lymphocytes + IBRV Antigen; ▤ Purified Lymphocytes + Macrophages + IBRV Antigen.

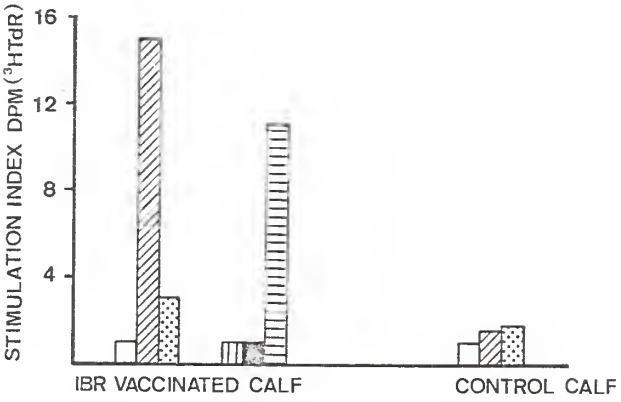

STIMULATION INDEX DPM (^{3}HTdR)
16
12
8
4
IBR VACCINATED CALF
CONTROL CALF

TABLE 1

ISOLATION OF VIRUS FROM NASAL SWABS OF VACCINATED CALVES

Days Post Vaccination	P.F.U./ml			
	Inactivated Virus Vaccine		Modified Live Virus Vaccine	
	BVD	IBR	BVD	IBR
3	0	0	0	1×10^4
4	0	0	0	1×10^5
5	0	0	0	0

TABLE 2

RECOVERY OF BOVINE LYMPHOCYTES BY DIFFERENT METHODS

Method	Per Cent Recovery
Distilled Water:	98
Ficoll Isopaque:	25
Lymphoprep:	20
Nylon Wool Column:*	30

*Recovery of purified lymphocytes

TABLE 3

STIMULATION INDICES OF MIXED LYMPHOCYTE CULTURES OF IMMUNIZED CALVES FROM K.S.U. DAIRY FARM

Calves No.	Serum Antibody Titer to IBRV	Serum Antibody Titer to BVDV	S.I.*	(IBR)	S.I.*	(BVD)
461	--	--	1.01		1.07	
462	1:80	1:80	1.34		1.08	
463	1:80	1:160	0.94	(0.36)	1.01	(0.33)
464	--	--	2.69		0.93	
466	--	--	2.40		1.08	
467	1:80	1:160	3.56		1.26	
468	1:80	1:160	2.40		0.90	
469	1:80	1:160	2.82	(1.50)	1.75	(3.11)
470	1:80	1:160	2.39		1.12	
471	1:80	1:160	2.02		0.83	
472	--	--	0.86		2.38	
473	1:80	1:160	0.69		2.40	
474	--	--	1.49	(2.14)	2.60	(0.50)
475	--	--	1.70		1.00	
476	--	--	4.60		1.05	
477	--	--	2.31		1.90	
478	--	--	1.39	(1.43)	1.14	(2.14)
480	--	--	0.70		0.69	
481	--	--	1.20		1.40	
482	--	--	1.53		1.58	
483	--	--	1.27		1.33	
484	1:80	1:160	2.00	(1.17)	2.13	(1.17)
486	1:80	1:160	1.26		1.19	
487	--	--	1.32		0.74	
24 Dairy Calves			Mean Stimulation Index (±S.D.) With IBRV 1.82 (±0.92 S.D.)		With BVDV 1.36 (±0.53 SD)	

Stimulation Index = $\frac{\text{DPM (cells + antigen)}}{\text{DPM (cells only)}}$

*S.I. Stimulation Index.
Numbers in parentheses represent stimulation indices of control calf.

TABLE 4

STIMULATION INDICES OF MIXED LYMPHOCYTE CULTURES FROM VACCINATED CALVES

	Calf Immunized With Formalized IBRV Vaccine		Control Calf	
	S.I. With IBRV Antigen	S.I. With BVDV Antigen	S.I. With IBRV Antigen	S.I. With BVDV Antigen
Pre-Vaccination	1.76	--	1.52	--
Weeks Post-Vacc.				
3	7.41	--	1.23	--
4	6.23	1.43	0.98	1.05
5	6.16	0.75	0.53	0.88
6	8.51	1.93	0.36	1.07
8	14.87	2.66	1.41	1.62
11	20.69	3.55	1.07	2.88
13	12.90	4.7	3.15	4.77
14	11.95	--	0.74	0.54
16	9.0	1.08	0.33	0.20
17	9.19	1.75	2.14	0.88
18	17.04	2.05	1.43	2.82
19	14.50	1.88	1.17	1.60
Mean Stimulation Index ($\pm$S.D.)	11.53 ($\pm$4.38)	2.18 ($\pm$1.12)	1.21 ($\pm$0.76)	1.66 ($\pm$1.27)

TABLE 5

STIMULATION INDICES OF MIXED LYMPHOCYTE CULTURES FROM VACCINATED CALVES

	Calf Immunized With Formalized BVD Vaccine		Control Calf	
	S.I. With BVDV Antigen	S.I. With IBRV Antigen	S.I. With BVDV Antigen	S.I. With IBRV Antigen
Pre-Vaccination	2.8	--	.2	--
Weeks Post-Vacc.				
3	0.34	--	.25	--
4	0.39	0.22	.86	0.2
5	0.24	0.38	.23	0.4
6	0.36	1.20	2.05	0.63
8	1.61	0.27	0.9	1.20
10	0.20	1.04	0.36	0.69
11	0.90	1.75	0.33	0.36
13	2.22	1.25	3.11	1.50
14	0.5	1.67	0.50	0.80
16	0.86	1.20	2.14	0.88
17	0.87	1.29	1.17	1.60
Mean Stimulation Index ($\pm$S.D.)	0.77 ($\pm$0.60)	1.02 ($\pm$0.52)	1.08 ($\pm$0.9)	0.82 ($\pm$0.45)

TABLE 6

STIMULATION INDICES OF MIXED LYMPHOCYTE CULTURES FROM VACCINATED CALVES

	Calf Vaccinated With Modified Live BVDV Vaccine		Control Calf	
	S.I. With BVDV Antigen	S.I. With IBRV Antigen	S.I. With BVDV Antigen	S.I. With IBRV Antigen
Pre-Vaccination	1.29	1.44	--	--
Weeks Post-Vacc.				
2	0.36	0.69	0.28	0.41
3	1.21	1.09	0.33	0.36
5	1.58	1.31	2.04	0.60
6	1.53	1.17	3.11	1.57
7	1.35	1.34	0.50	0.80
8	1.03	1.50	0.33	0.20
10	1.40	2.43	2.14	0.88
12	0.90	1.60	1.17	1.60
Mean Stimulation Index ($\pm$S.D.)	1.17 ($\pm$0.37)	1.39 ($\pm$0.47)	1.23 ($\pm$1.0)	0.80 ($\pm$.49)

TABLE 7

STIMULATION INDICES OF MIXED LYMPHOCYTE CULTURES FROM VACCINATED CALVES

	Calf Immunized With Modified Live IBRV Vaccine		Control Calf	
	S.I. With IBRV Antigen	S.I. With BVDV Antigen	S.I. With IBRV Antigen	S.I. With BVDV Antigen
Pre-Vaccination	1.4	0.90	--	--
Weeks Post-Vacc.				
2	0.9	1.43	1.35	1.2
3	1.3	1.27	0.36	0.33
5	0.8	1.51	0.60	2.04
6	2.45	1.41	8.25	6.81
8	1.05	0.87	0.5	0.8
10	3.5	4.4	0.88	0.2
12	0.23	1.3	1.60	1.6
Mean Stimulation INdex (±S.D.)*	1.4 (±1.04 S.D.)	1.74 (±1.1 S.D.)	1.93 (±2.6 S.D.)	1.85 (±2.10 S.D.)

$$\text{Stimulation Index} = \frac{\text{DPM (cells + antigen)}}{\text{DPM (cells only)}}$$

*Standard Deviation

TABLE 8

DETECTION OF SERUM NEUTRALIZING ANTIBODIES

	Percent Virus Neutralized				
	Weeks Post Inoculation				
Treatment	1	2	3	4	6
Rabbit inoculated with inactivated BVD + adjuvant	0	70	32	46	53
Rabbit inoculated with lysate + adjuvant	0	0	0	0	0
Rabbit inoculated with lysate	0	0	0	0	0
Uninoculated rabbit	0	0	0	0	0

SECTION IV

(Part II)

STUDY ON BOVINE RESPIRATORY DISEASES

II. Effect of Steroids on Lymphocytes of Immune and Non-immune Calves, *In Vitro*

Abstract

The interaction of bovine lymphocytes with steroid hormones demonstrated specific receptors for cortisol. Increasing concentrations of cortisol (0-5,000 ng/ml) failed to influence the degree of adsorption of infectious bovine rhinotracheitis virus (IBRV) or bovine virus diarrhea virus (BVDV) to bovine lymphocytes indicating a lack of competition between the steroid and the viruses for cellular receptors.

Cortisol inhibited the incorporation of ^{3}H-thymidine (^{3}H-TdR) in PHA stimulated lymphocytes. Lymphocytes from the calf vaccinated with inactivated-adjuvated IBRV were specifically stimulated by virus antigen and the presence of cortisol in culture inhibited blastogenesis as indicated by lack of incorporation of ^{3}H-TdR into cells.

Introduction. Bovine respiratory disease problems are frequently encountered in crowded animal situations such as in feedlots, during transportation or under adverse weather conditions. Under such circumstances, the animals are under stress which may affect the physiological hormonal balance, often leading to an increase in the level of serum or plasma cortisol (Weiss, 1977; Christison, G. J., et al., 1972, 1970). Susceptibility of the host to viral diseases have been shown to be altered by the increase in steroid concentrations (Kilbourne and Horsefall, 1951; Shwartzman, et al., 1952; Kass, et al., 1958; Reinicke, 1965; and Rytel, et al., 1966). Sheffy, et al. (1973) reported the reappearance of IBRV infection in animals during times of stress and the recovery of the virus was demonstrated following administration of corticosteroids (Davies, et al., 1974). Shope, et al. (1977) observed that administration of cortisol resulted in potentiation of BVDV infection in calves. Both in vitro and in vivo studies revealed that hormones can change the expression of viral genomes in cells resulting in increased virus synthesis (Joncas, et al., 1977; Jones, et al., 1963). Hall, et al. (1977) demonstrated a ten-fold increase in IBRV synthesis in cells treated with corticosterone. In addition, steroid hormones are known to be immunosuppressive, lympholytic and cause lesions on lymphoid tissues (Wallnerova, et al., 1971).

The objective of this study was to investigate the interaction of IBR or BVD viruses with bovine lymphocytes in the presence of cortisol.

Materials and Methods.

Animals. Six calves, five to six months old, two each immunized against BVDV or IBRV, and two control calves were used in these studies. The animals were tested for the production of antibodies to the viruses by 50% plaque reduction method. Serum antibody titers ranged from 1/80 to 1/1280. The immune status of the animals has been described in detail in Part 1.

Cells. Madin Darby Bovine Kidney (MDBK) cells were propagated in Eagles' Minimal Essential Medium (MEM) containing 10% horse serum as previously described.

Viruses. The bovine virus diarrhea virus, National Animal Disease Laboratory (NADL) strain, and infectious bovine rhinotracheitis virus, Los Angeles (LA) strain were obtained from ATCC. Both viruses produced cytopathic effects in MDBK cells.

Preparation of Radioactively Labelled Viruses. Monolayers of MDBK cells grown in tissue culture dishes (100 x 20 mm, Falcon) were infected with IBRV or BVDV at a multiplicity of three to five Plaque Forming Units (PFU) per cell and maintained in medium 199, containing 0.25 uCi/ml of either ^{3}H-thymidine (^{3}H-TdR, specific activity 90 Ci/mmole, New England Nuclear, Boston, MA) for IBRV inoculated cultures or ^{3}H-Uridine (specific activity 23.5 Ci/mmole) for BVD virus infected cells. Cultures were harvested as previously described, frozen and thawed twice and centrifuged at 1,500 g to remove the cellular debris. The supernatant was ultracentrifuged for five hours at 100,000 g.

The virus pellet was suspended in five ml of MEM and treated with DNase and RNase at final concentration of 10 ug/ml in IBR and BVD virus preparations, respectively. The mixture was incubated for 30 minutes at 25° C and ultracentrifuged for 1 1/2 hours at 200,000 g. The pellet was washed in cold MEM to remove the enzymes, resuspended and dialyzed (1/4 inch dialyzing tubing, size one, Fisher Scientific, Pittsburgh, PA) against MEM in an ice bath for three hours and stored at -80° C.

Virus Assay. Viruses were assayed by the method described by Dulbecco (1952) for animal viruses with some modifications. (Details of this procedure were described in Part 1.)

Isolation of Bovine Lymphocytes. Bovine lymphocytes were isolated by the method described by Rossi, et al. (1977) with some modifications. Peripheral blood was collected in heparinized containers and treated with distilled water and 1.7% sodium chloride solution. For separation of mononuclear cells, the method of Julius, et al. (1973) was adopted.

Blastogenesis Studies with PHA and Specific Virus Antigen. Triplicate cultures, each containing 1×10^6 cells were suspended in two ml of RPMI medium with 10% calf serum and Phytohemagglutinin (PHA) 2.5 ug/ml or 0.2 ml of 1×10^6 PFU/ml of U.V. inactivated virus was added to the cultures. Following 48 hours incubation, 1 uCi ^{3}H-thymidine in 0.1 ml media was incorporated. Cultures were further incubated for 24 hours, centrifuged, washed three times with cold phosphate buffered saline (PBS) and precipitated

with 5% trichloroacetic acid (TCA). The acid insoluble precipitates were collected on glass fiber filters (GF/C, 2.4 cms) in a sampling manifold. Tubes were washed twice with 5% TCA, contents transferred on glass fiber filters that were placed in scintillation vials and counted.

Adsorption of Steroids to Bovine Lymphocytes. Mononuclear cells or mixed lymphocytes (5×10^6 cells in two ml of RPMI) were added to sets of triplicate tubes each containing a mixture of 0.2 uCi of a radioactive hormone and a concentration of the corresponding non-radioactive hormone. Cultures were washed twice with cold PBS, cells were transferred on glass fiber filters in a sampling manifold and subsequently placed in scintillation vials for counting.

Adsorption of Radioactive Viruses on Lymphocytes in the Presence of Cortisol. Different concentrations of cortisol (0-500 ng) were added to a suspension of mixed or purified lymphocytes (1×10^6 cells) in triplicate cultures. After four hour incubation all cultures received a constant amount of ^{3}H-IBR (1.4×10^6 PFU/ml, 3,000 counts/mins/ml or ^{3}H-BVD (3×10^7 PFU/ml, 1,700 counts/mins/ml. Cultures were further incubated for two hours and were washed twice with cold PBS. The cell pellet was resuspended in two ml of PBS and 0.2 ml was saved from each sample for plaque assay. The remaining cell suspension was transferred on glass fiber filters and processed as described previously for counting in scintillation spectrometer.

Results.

Adsorption of Cortisol Binding to Lymphocytes. Figure 1 illustrates that the adsorption of ^{3}H-cortisol to lymphocytes increased linearly as a function of cell number. Decreased incorporation of ^{3}H-cortisol in the presence of non-radioactive cortisol (500 ng) occurred with more than 50% reduction in cultures containing 10 million cells.

Specificity of Cortisol Binding to Bovine Lymphocytes. Binding of radioactive cortisol to receptor sites on bovine lymphocytes declined with increasing concentration of non-radioactive hormone. Reductions of 25-50% in the adsorption of the labelled hormone was observed with the addition of 5-500 ng of the competing hormone suggesting specificity of binding. There was no difference in the degree of adsorption of the hormone to cells of control and vaccinated animals.

Adsorption of Radioactive Viruses to Lymphocytes in Presence of Cortisol. Tables 1 and 2 illustrate the adsorption of ^{3}H-IBR or ^{3}H-BVD viruses to lymphocytes of control and vaccinated calves in the presence of varying concentrations of cortisol. In these cultures, no appreciable differences were observed in DPM's and PFU's indicating lack of competition between the viruses and the hormone for the same cellular receptor sites. Purified or mixed lymphocytes in these experiments yielded identical results (Tables 3 and 4).

Inhibitory Effects of Cortisol on Lymphocyte Stimulation. Lymphocytes from both control and vaccinated calves when stimulated for blastogenesis studies in the presence of cortisol demonstrated low incorporation (10-15%) of ^{3}H-TdR (Tables 5-7). Maximum suppression (90%) of label uptake was observed at the lowest concentrations (5 ng) used, with no further suppression at higher levels. Stimulation by specific virus antigen in cultures from immunized animal demonstrated some degree of inhibition of blastogenic response in the presence of the hormone.

Discussion. Munck, et al. (1968) demonstrated saturation of the specific binding sites of thymus cells with increase in the concentration of the hormone. In our studies, binding of ^{3}H-cortisol decreased as the concentration of the competing hormone was increased (Figure 2). It seems that a saturation point was reached at a concentration of 500 ng/ml of the hormone and there was no further decline in label incorporation at the highest concentration (5,000 ng/ml). In the experiment cortisol bound non-specifically would elute from cells since all cultures were incubated at 37^{o} C for four hours after addition of the hormone (Munck, 1968). Thus, the binding of cortisol to bovine lymphocytes was most likely due to the presence of specific receptors (Figure 2). Eurenius, et al. (1968) reported that the binding of cortisol to rat and rabbit thymocytes was related to mean cell volume. Figure 1 demonstrates that incorporation of ^{3}H-cortisol increased in cultures with higher number of cells.

Since bovine lymphocytes have specific receptors for cortisol, an increase in the number of lymphocytes would lead to a higher number of receptor sites, thus resulting in increased incorporation of the labelled hormone in cultures as observed in our experiments (Figure 1).

Tables 1 and 2 indicate that cortisol, IBRV and BVDV have different receptors on the bovine lymphocyte. The presence of different concentrations of the hormone did not influence the adsorption of a constant amount of radioactively labelled virus as measured by the recovery of DPM's and PFU from cells to both of control and vaccinated calves. Tables 3 and 4 illustrate no difference in adsorption of viruses on mixed and purified lymphocytes, suggesting that the adsorption is mostly an interaction between virus and lymphocyte and is independent of the presence of other cells in a mixed population.

Nowell (1961) reported that cortisol in concentrations of 10 ng/ml inhibited mitogenesis by PHA in cultures of human peripheral blood lymphocytes. Muscoplat, et al. (1975) showed that corticosteroid given in vivo altered the response of peripheral blood lymphocytes of calves, as indicated by a decrease in their response to PHA, in vitro. In our studies, we presented data indicating that the addition of cortisol, as low as 5 ng per ml to lymphocyte cultures of calves resulted in a sharp decline in their responsiveness to both PHA and specific virus antigen in vitro (Tables 5-7). It is probable that the nature of inhibitory action produced by cortisol is of a general type of

suppression of the lymphocyte rather than an activity restricted to surface membranes since cortisol and the IBRV antigen have been shown to have different receptor sites. The mode of suppression by the hormone has been explained earlier by Feldman, et al. (1972); the steroid-receptor complex is capable of entering the nucleus and combining with the chromatin which influences the DNA transcription leading to specific alterations in the cell functions.

The recovery of viruses and recrudescence of diseases reported by earlier workers (Sheffy, et al. 1973; Davies, et al. 1973; Cribbs, et al. 1975) following administration of corticosteroids or during times of stress when hormonal levels are high, may be the result of a general suppression of the T-cell function as observed in our studies, coupled with inhibition of antibody production as described by Butler (1975).

In this study, we have demonstrated that bovine lymphocytes have specific receptors for cortisol and the adsorption of IBR and BVD viruses to bovine lymphocytes was not influenced by the presence of the hormone. The inhibitory action of the hormone has also been investigated and the data presented, suggest that the inhibition may be the result of changes at cellular level brought about by association of the hormone in the cell DNA, as postulated by Feldman, et al. (1972).

Figure 1. Adsorption of ^{3}H-cortisol to bovine lymphocytes. Mixed lymphocytes (0.5 to 10 million cells in two ml RPMI) were added to sets of triplicate tubes containing or lacking 500 ng non-radioactive cortisol. A constant amount of ^{3}H-cortisol (0.2 uCi) was added to all cultures and incubated for four hours. Cultures were washed twice with cold PBS and processed for counting in scintillation spectrometer and DPM's determined. Symbols: —●— ^{3}H-cortisol, —○— ^{3}H-cortisol + 500 ng non-radioactive cortisol; —✱— cells only.

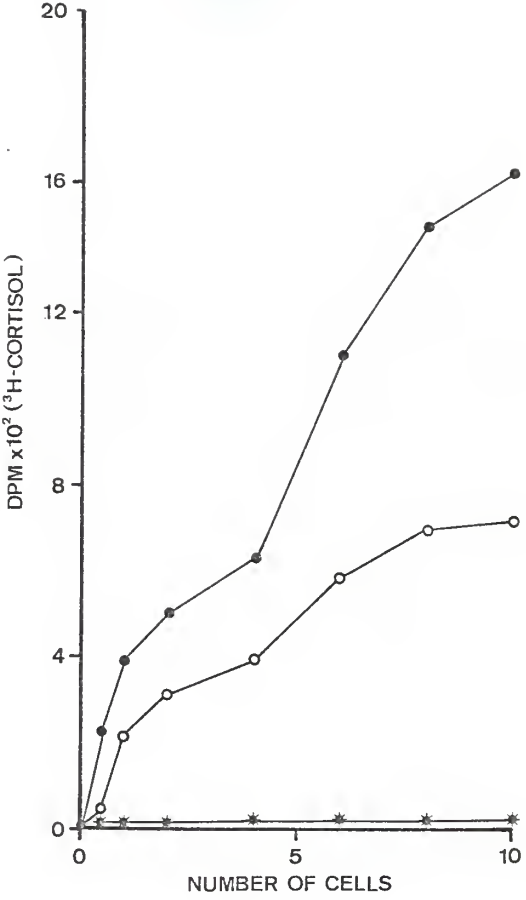

20
16
12
8
4
0
DPM x10² (³H-CORTISOL)
0
5
10
NUMBER OF CELLS

Figure 2. Specificity of adsorption of cortisol to bovine lymphocytes. Different concentrations of cortisol (0-5,000 ng/ml) were added to a suspension of 5×10^6 lymphocytes cultures containing a constant amount (0.2 uCi) of ^{3}H-cortisol. Cultures were incubated for four hours before they were washed twice with cold PBS and processed for counting and DPM's determined. Symbols: —●— Control Calves; —□— IBRV Vaccinated Calves; —△— BVDV Vaccinated Calves.

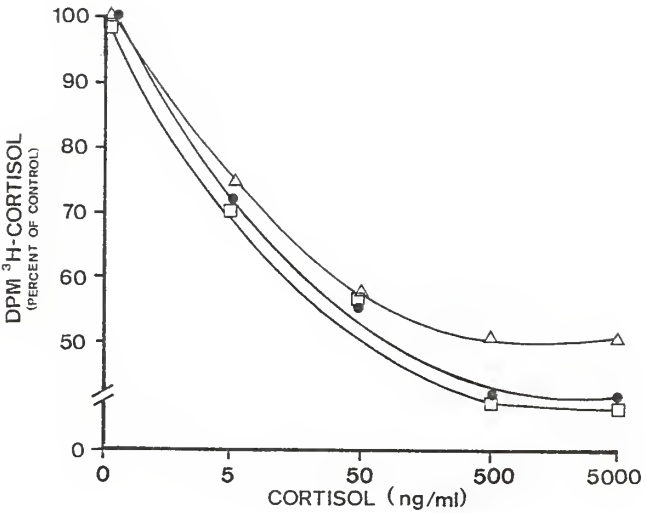

DPM ^{3}H-CORTISOL
(PERCENT OF CONTROL)
100
90
80
70
60
50
0
0
5
50
500
5000
CORTISOL (ng/ml)

TABLE 1

ADSORPTION OF ^{3}H-BVDV TO MIXED LYMPHOCYTES IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF CORTISOL

Treatment	Mean of Two BVDV Vaccinated Calves		Mean of Two Control Calves	
	DPM	PFU	DPM	PFU
^{3}H-BVDV Only	788	1.15×10^{3}	814	1.1×10^{3}
^{3}H-BVDV + 5 ng Cortisol	815	1.2×10^{3}	826	1.12×10^{3}
^{3}H-BVDV + 50 ng Cortisol	776	1.15×10^{3}	791	1.16×10^{3}
^{3}H-BVDV + 500 ng Cortisol	796	1.21×10^{3}	769	1.13×10^{3}

TABLE 2

ADSORPTION OF ^{3}H-IBRV TO MIXED LYMPHOCYTES IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF CORTISOL

Treatment	IBR Vaccinated Calf		Control Calf	
	DPM	PFU	DPM	PFU
^{3}H-IBRV Only	1421	3.6×10^{3}	1507	3.6×10^{3}
^{3}H-IBRV + 5 ng Cortisol	1468	3.4×10^{3}	1448	3.6×10^{3}
^{3}H-IBRV + 50 ng Cortisol	1419	3.6×10^{3}	1374	3.4×10^{3}
^{3}H-IBRV + 500 ng Cortisol	1392	3.3×10^{3}	1435	3.7×10^{3}

TABLE 3

ADSORPTION OF ^{3}H-BVDV TO BOVINE LYMPHOCYTES (MIXED AND PURIFIED) IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF CORTISOL

Treatment	BVDV Vaccinated Calf				Control Calf			
	Mixed Lymphocytes		Purified Lymphocytes		Mixed Lymphocytes		Purified Lymphocytes	
	DPM	PFU	DPM	PFU	DPM	PFU	DPM	PFU
^{3}H-BVDV Only	960	1.12×10^{3}	836	1.15×10^{3}	801	1.11×10^{3}	730	1.15×10^{3}
^{3}H-BVDV + 5 ng cor.	930	1.01×10^{3}	846	1.2×10^{3}	894	1.12×10^{3}	738	1.11×10^{3}
^{3}H-BVDV + 50 ng cor.	850	1×10^{3}	842	1.1×10^{3}	816	1.1×10^{3}	807	1.15×10^{3}
^{3}H-BVDV + 500 ng cor.	952	1.12×10^{3}	882	1.1×10^{3}	862	1.1×10^{3}	742	1.12×10^{3}

TABLE 4

ADSORPTION OF ^{3}H-IBRV TO BOVINE LYMPHOCYTES (MIXED AND PURIFIED) IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF CORTISOL

Treatment	IBRV Vaccinated Calf				Control Calf			
	Mixed Lymphocytes		Purified Lymphocytes		Mixed Lymphocytes		Purified Lymphocytes	
	DPM	PFU	DPM	PFU	DPM	PFU	DPM	PFU
^{3}H-IBRV Only	1573	3.6×10^{3}	1447	3.4×10^{3}	1547	3.6×10^{3}	1426	3.6×10^{3}
^{3}H-IBRV + 5 ng cor.	1593	3.4×10^{3}	1510	3.3×10^{3}	1610	3.6×10^{3}	1438	3.4×10^{3}
^{3}H-IBRV + 50 ng cor.	1593	3.2×10^{3}	1452	3.4×10^{3}	1524	3.4×10^{3}	1415	3.6×10^{3}
^{3}H-IBRV + 500 ng cor.	1436	3.5×10^{3}	1406	3.4×10^{3}	1520	3.7×10^{3}	1325	3.3×10^{3}

TABLE 5

EFFECT OF CORTISOL ON PHA STIMULATED LYMPHOCYTES

D.P.M. x 10^3 (^{3}H-TdR)

Treatment	IBRV Vaccinated Calf	Control Calf
Cells Only	6.4	5.3
Cells + PHA	808.9 (100%)	119.6 (100%)
Cells + PHA + Cortisol 5 ng	115.7 (14.3%)	5.4 (4.6%)
Cells + PHA + Cortisol 50 ng	111.2 (13.7%)	4.0 (3.4%)
Cells + PHA + Cortisol 500 ng	85.3 (10.5%)	2.7 (2.26%)

The number in parenthesis represents percent incorporation of ^{3}H-TdR.

TABLE 6

EFFECT OF CORTISOL ON PHA STIMULATED LYMPHOCYTES

	D.P.M. x 10^3 (^{3}H-TdR)	
Treatment	BVDV Vaccinated Calf	Control Calf
Cells Only	0.7	3.7
Cells + PHA	62.7 (100%)	18.5 (100%)
Cells + PHA + Cortisol 5 ng	7.5 (11.9%)	2.0 (10.8%)
Cells + PHA + Cortisol 50 ng	7.3 (11.6%)	2.7 (14.5%)
Cells + PHA + Cortisol 500 ng	7.2 (11.4%)	1.6 (8.65%)

The number in the parenthesis represents percent incorporation of ^{3}H-TdR.

TABLE 7

EFFECT OF CORTISOL ON LYMPHOCYTES FROM IBR VACCINATED CALF STIMULATED BY SPECIFIC ANTIGEN

	DPM X 10^2 (^{3}H-TdR)
Cells only	36.7
Cells + IBR	209.6 (100%)
Cells + IBR + Cortisol 5 ng	80.8 (38.6%)
Cells + IBR + Cortisol 50 ng	79.6 (37.9%)
Cells + IBR + Cortisol 500 ng	73.6 (35.11%)

The number in parenthesis represents the percent incorporation of ^{3}H-TdR.

SECTION V

APPENDIX

APPENDIX

Inactivation of Viruses by Formalin. BVDV, 1.5×10^8 to 3×10^8 PFU/ml and IBRV, 1×10^9 to 3×10^9 PFU/ml were treated with formalin at final concentrations of 1:1000, 1:2000, 1:4000, 1:8000, 1:16000 and 1:32000 (40% formaldehyde solution, Fisher Scientific, Fairlawn, NJ). The mixture was incubated for six hours at room temperature, then overnight at 4° C and dialyzed in a 1/4 inch dialyzing tubing for 24 hours at 4° C with three changes of PBS. The virus was plaqued to determine the percent activity remaining (Figure 1).

Inactivation of Viruses by U.V. Irradiation. One ml of BVDV or IBRV, 1×10^7 PFU/ml was placed in a 60 x 15 mm dish and exposed for various lengths of time to a source of U.V. light (U.V. Germicidal Sterilamp, G30T8, 30 watt, Westinghouse Electric Co., NJ) at a distance of 10 cms. Percent activity of the virus was determined by plaque assay (Figure 2).

Growth of Viruses in Lymphocytes. BVD or IBR virus, 1×10^6 PFU/ml was added to sets of mixed lymphocytes cultures (1×10^6 cells) in two ml of RPMI, with 10% calf serum, tested free of anti-IBRV and anti-BVDV, and incubated at 37° C. At 0, 24, 48, 72 and 96 hours, cultures were washed three times with cold PBS and resuspended in two ml media. 0.2 ml from each sample was inoculated on MDBK cells and plaque assayed (Table 1).

Adsorption of ^{3}H-Cortisol to Bovine Lymphocytes. Table 2 illustrates the adsorption of radioactive cortisol to bovine lymphocytes. Samples were processed for counting as described previously at 0, 0.5, 4, 8, 24 and 48 hours of incubation.

Adsorption of ^{3}H-Hormones to Bovine Lymphocytes. A suspension of 5 x 10^{6} mixed lymphocytes were added to triplicate tubes containing 0.2 uCi of ^{3}H-hormone and 0 to 500 ng of the corresponding non-radioactive hormone. Cultures were placed in CO_2 incubator for four hours. At the end of incubation, cells were washed twice with cold PBS and transferred on glass fiber filters in a sampling manifold and subsequently placed in scintillation vials for counting (Table 3).

Hematological and Serum Profiles of Calves. Tables 4 to 9 show the hematological and serum profiles of control and vaccinated calves before and after inoculation of the vaccines.

Figure 1. Formalin inactivation of IBRV and BVDV. IBR and BVD viruses were treated with formalin at final concentrations of 1:1000, 1:2000, 1:4000, 1:8000, 1:16000 and 1:32000. The virus was plaque assayed to determine the percent activity remaining. Symbols: —●— IBRV; —○— BVDV.

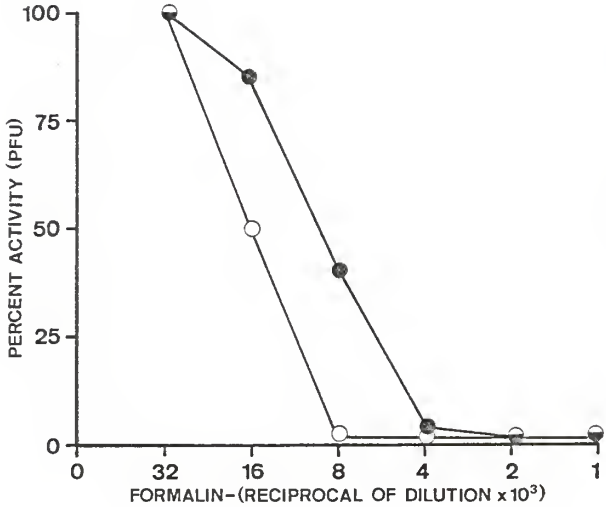
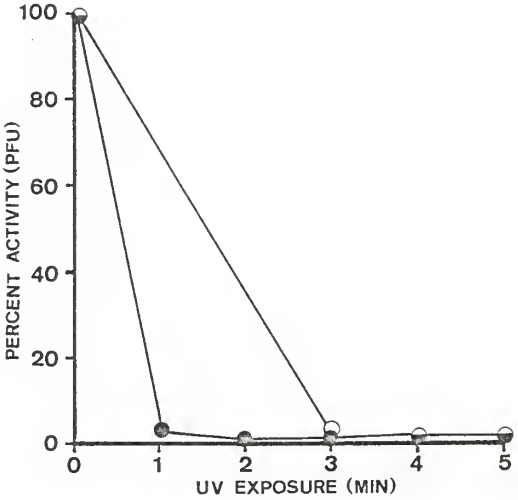

100
75
50
25
0
PERCENT ACTIVITY (PFU)
0
32
16
8
4
2
1
FORMALIN-(RECIPROCAL OF DILUTION x 10³)

Figure 2. Inactivation of IBRV and BVDV by UV irradiation. One ml of virus (1×10^7 PFU) was U.V. irradiated for 0-5 minutes. The virus was collected and plaque assayed to determine the percent activity remaining. Symbols: —●— IBRV; —○— BVDV.


100
80
60
40
20
0
PERCENT ACTIVITY (PFU)
0
1
2
3
4
5
UV EXPOSURE (MIN)

SECTION VI

DETAILED METHODOLOGY AND REAGENTS

TABLE 1

GROWTH OF IBR AND BVD VIRUSES IN BOVINE LYMPHOCYTES

P.F.U. Recovered per 0.2 ml

Length of Culture (hours)	IBRV		BVDV	
	Original Sample	10^{-1} Dilution	Original Sample	10^{-1} Dilution
0	Lysis	33	Lysis	Lysis
24	95	8	Lysis	75
48	51	6	29	3
72	19	0	13	1
96	0	0	0	0

TABLE 2

ADSORPTION OF ^{3}H-CORTISOL TO BOVINE LYMPHOCYTES

Hours of Incubation	DPM
0	86
0.5	352
4	370
8	349
24	330
48	284

TABLE 3

MEAN DPM OF LYMPHOCYTES OF SIX CALVES FROM TWO EXPERIMENTS

ng/ml of Non-Radioactive Hormone*	Corticosterone	Estradiol	Progesterone	Testosterone
0 ng	1010	4228	1193	672
5 ng	1031	4765	1031	633
50 ng	858	4405	1064	614
500 ng	912	4494	1160	509

*All samples had constant amount of ^{3}H-hormone.

TABLE 4

CALF INOCULATED WITH INACTIVATED-ADJUVATED IBRV VACCINE
on 12-29-77

HEMATOLOGICAL AND SERUM PROFILES

	Pre-Inoculation Date		Mean	Post-Inoculation Date				Mean
	12-12-77	12-28-77		1-4-78	1-17-78	1-31-78	2-14-78	
PCV Vol %	37	40	38.5	35	36	36.5	38	36.3
Hemoglobin	11.7	12.8	12.5	11.3	11.3	11.7	12.4	11.6
WBC/Cu mm	9800	11900	10850	9000	11000	10700	10800	10375
Bands/Cu mm	294	119	206		111			
Neutrophil/Cu mm	3136	2610	2873	2160	3330	2247	2268	2501
Lymphocyte/Cu mm	5390	7021	6205	6300	5883	7811	7884	6969
Monocyte/Cu mm	882	696	789	360	888	214	570	383
Eosinophil/Cu mm	98	1071	584	180	888	428	342	459
Basophil/Cu mm								
SDH I.U.	7.8	23	15.4	5.5	7.8	7.2	8.6	7.28
Cl_2 meq/L	100.5	101.5	101	100	101	100	98	99.7
Na meq/L	142	140	141	140	143	138	137	139
K meq/L	4.6	4.8	4.7	3.9	4.3	4.4	4.5	4.2
CO_2 meq/L	24	20	22	26	27	29	24	26.5
BUN mg%	16	15	15.5	15	13	17	19	16
ALK Ptase mu/ml	180	131	155	210	174	145	132	122.7
SGPT mu/ml	30	0	15	10	0	7	2	4.7
T.P. gm%	6.5	7	6.75	7.4	7.1	7.3	6.8	7.1
Alb gm%	4.1	4.2	4.15	4.5	4.2	4.2	4.2	4.2
Ca mg%	10	9.8	9.9	11	11.1	10.4	9.5	10.5
Phos mg%	7.5	9.3	8.4	7.9	8.5	7.8	6.9	7.7
Gluc mg%	75	88	81.5	85	103	90	85	90.7
Creat mg%	1.2	0.9	1.05	0.9	1.3	1	1	1.05

TABLE 5

CALF INOCULATED WITH MODIFIED LIVE IBRV VACCINE
on 2-22-78

HEMATOLOGICAL AND SERUM PROFILES

	Pre-Inoculation Date		Mean	Post-Inoculation Date			Mean
	1-31-78	2-14-78		3-7-78	3-14-78	3-28-78	
PCV Vol %	33	33	33	37.5	36	33	35.5
Hemoglobin	11.5	11	11.2	12.4	11.7	11.1	
WBC/Cu mm	10200	10000	10100	10800	10300	8300	9800
Bands/Cu mm					412		
Neutrophil/Cu mm	2210	2173	1191	2268	2575	1825	2222
Lymphocyte/Cu mm	7310	7540	7425	7884	6592	5307	6594
Monocyte/Cu mm	340	354	347	432	515	1095	680
Eosinophil/Cu mm	196	212	204	216	206	23	148
Basophil/Cu mm							
SDH I.U.	8	8.7	8.3	9.6	6.5	8.4	8.1
Cl_2 meq/L	100	97	98.5	99	97	105	100.3
Na meq/L	136	135	135.5	140	135	135	136.6
K meq/L	5.4	5.2	5.3	4.4	4.5	4.8	4.5
CO_2 meq/L	29	28	28.5	26	32	30	29.3
BUN mg%	14	16	15	15	12	11	14
ALK Ptase mu/ml	150	154	152	127	100	102	109.3
SGPT mu/ml	15	15	15	0	1	0	8
T.P. gm%	6.8	6.8	6.8	6.3	6.5	6.4	6.4
Alb gm%	4.0	3.8	3.9	4.2	3.8	4.1	4.03
Ca mg%	10.1	10.2	10.1	9.2	10	9	9.4
Phos mg%	6.9	7.1	7	7.8	6.7	6.5	7
Gluc mg%	60	55	57.5	85	75	80	80
Creat mg%	0.9	0.8	0.85	0.8	0.8	0.9	0.83

TABLE 6

CALF INOCULATED WITH MODIFIED LIVE BVDV VACCINE
on 2-22-78

HEMATOLOGICAL AND SERUM PROFILES

	Pre-Inoculation Date		Mean	Post-Inoculation Date			Mean
	1-31-78	2-10-78		3-7-78	3-14-78	3-28-78	
PCV Vol %	42	33	37.5	36	36	36	36
Hemoglobin	13.6	12	12.8	12.4	11.9	12	12.1
WBC/Cu mm	7300	6000	6650	8500	8200	6900	7866
Bands/Cu mm							
Neutrophil/Cu mm	2190	1880	2035	3315	2132	1725	2390
Lymphocyte/Cu mm	4380	4100	4240	4590	5330	4416	4778
Monocyte/Cu mm	438	405	421	595	574	759	616
Eosinophil/Cu mm	292	197	244	--	164	--	--
Basophil/Cu mm							
SDH I.U.	8.6	11	9.8	10	6.4	6.7	8.8
Cl_2 meq/L	102	101	101.5	101	100	102	101
Na meq/L	140	135	137.5	145	138	135	139.3
K meq/L	4.2	5	4.6	4.6	4.2	4.2	4.3
CO_2 meq/L	26	25	25.5	25	31	30	28.6
BUN mg%	13	13	13	16	15	14	15
ALK Ptase mu/ml	153	150	151.5	152	140	135	142
SGPT mu/ml	4	45	24.5	3	2	4	3
T.P. gm%	6.6	7.3	6.9	6.8	6.5	4.0	5.7
Alb gm%	4.3	3.8	4.05	4.5	4	4.1	4.2
Ca mg%	9.8	10.3	10.05	9.5	10.2	13	10.9
Phos mg%	8.8	6.3	7.5	5.1	5.2	5.1	5.13
Gluc mg%	95	55	75	85	65	70	73.3
Creat mg%	1	0.9	0.95	0.9	0.9	0.9	0.9

TABLE 7

CONTROL CALF INOCULATED WITH LYSATE AND ADJUVANT
on 12-29-77

HEMATOLOGICAL AND SERUM PROFILES

	Pre-Inoculation Date		Mean	Post-Inoculation Date				Mean
	12-12-77	12-28-77		1-4-78	1-17-78	1-31-78	2-14-78	
PCV Vol %	40	41	40.5	38	39	37.5	37	37.8
Hemoglobin	13.2	12.8	13	12.4	12.4	12.2	12.2	12.3
WBC/Cu mm	9400	13200	10800	14000	14800	14300	15400	14625
Bands/Cu mm								
Neutrophil/Cu mm	1840	2904	2372	2940	2664	3861	4620	3521
Lymphocyte/Cu mm	6888	7920	7404	9240	10508	8294	9394	9359
Monocyte/Cu mm	672	1320	996	1400	888	1573	1386	1311
Eosinophil/Cu mm	--	1056	--	420	740	572	--	577
Basophil/Cu mm								
SDH I.U.	7.9	10.2	9.05	10.2	8.6	8.3	8.1	8.8
Cl_2 meq/L	99	101.5	100.25	101.5	101.5	99.5	99	100.38
Na meq/L	146	142	144	142	145	138	145	142.5
K meq/L	4.4	4.5	4.4	4.5	4.6	3.8	4.6	4.38
CO_2 meq/L	28	26	27	26	28	32	25	27.7
BUN mg%	15	12	13.5	12	15	15	16	14.5
ALK Ptase mu/ml	340	270	305	270	289	280	288	281
SGPT mu/ml	30	0	--	0	0	4	3	--
T.P. gm%	6.3	6.7	6.5	6.7	6.5	6.3	6.6	6.5
Alb gm%	4	4.2	4.1	4.2	4.2	4.1	4.5	4.2
Ca mg%	10	10	10	10	11.1	10.2	9.5	10.2
Phos mg%	8.3	10.8	9.5	10.8	8.5	7	5.1	7.85
Gluc mg%	90	85	87.5	85	103	102	85	93.7
Creat mg%	1	0.8	0.9	0.8	1.3	1	1	1.03

TABLE 8

CALF INOCULATED WITH INACTIVATED-ADJUVATED BVDV VACCINE
on 12-29-77

HEMATOLOGICAL AND SERUM PROFILES

	Pre-Inoculation Date		Mean	Post-Inoculation Date				Mean
	12-12-77	12-28-77		1-4-78	1-17-78	1-31-78	2-14-78	
PVC Vol %	42	38	40	35	36	37	37.5	36.3
Hemoglobin	13.6	12.8	13.2	11.5	12	11.9	12.8	12.05
WBC/Cu mm	7300	8700	8000	6900	10800	9300	9700	9175
Bands/Cu mm	219	87	153			93		
Neutrophil/Cu mm	876	2610	1743	966	2808	2883	3395	2513
Lymphocyte/Cu mm	5110	5220	5165	4140	5508	4836	5238	4930
Monocyte/Cu mm	730	696	713	690	1404	651	679	856
Eosinophil/Cu mm	365	87	226	1104	972	744	388	802
Basophil/Cu mm					108	93		
SDH I.U.	7.6	8.3	7.9	7.2	7.7	8.6	8.4	7.9
Cl_2 meq/L	97	101.5	99.2	101	101	102	99	100.7
Na meq/L	145	141	143	139	141	139	138	139.2
K meq/L	4.6	4.5	4.55	3.9	4.4	4.3	4.0	4.1
CO_2 meq/L	26.5	26	26.2	26	28	30	23	26.7
BUN mg%	15	12	13.5	15	15	13	15	14.5
ALK Ptase mu/ml	160	140	150	290	289	153	144	219
SGPT mu/ml	30	8	19	10	0	15	0	--
T.P. gm%	6.5	6.6	6.55	6.5	6.4	6.7	6.5	6.5
Alb gm%	4.2	4.3	4.25	4.6	4.2	4.3	4.4	4.3
Ca mg%	9.2	9.9	9.5	11	10.8	10.2	9.9	10.4
Phos mg%	9.7	8.8	9.2	6.7	9	7.5	6.1	7.3
Gluc mg%	65	76	70.5	95	86	90	88	89.7
Creat mg%	1.1	0.9	1	1.1	1.1	1.0	0.9	1.03

TABLE 9

UNINOCULATED CALF

	Dates				Mean
	1-31-78	2-14-78	3-7-78	3-28-78	
PCV Vol %	35	37.5	36	32	35.1
Hemoglobin	11.9	12.4	11.7	10.7	11.6
WBC/Cu mm	14000	15500	16000	16500	15500
Bands/Cu mm					
Neutrophil/Cu mm	2520	2790	3200	2145	2663
Lymphocyte/Cu mm	9940	11470	11360	12045	11203
Monocyte/Cu mm	560	930	800	1485	943
Eosinophil/Cu mm	980	310	640	825	688
Basophil/Cu mm					
SDH I.U.	7.9	9.8	7.4	6	7.7
Cl_2 meq/L	97.5	99	99	102	99.3
Na meq/L	141	149	136	139	141
K meq/L	4.2	4.5	4.4	4.3	4.3
CO_2 meq/L	30	25	31	32	29.5
BUN mg%	12	15	12	13	13
ALK Ptase mu/ml	140	156	125	114	133
SGPT mu/ml	5	0	10	0	--
T.P. gm%	7.5	7.3	7.4	7.2	7.4
Alb gm%	4.0	4.5	4.5	4.2	4.3
Ca mg%	9.9	9.4	9.9	9.3	9.6
Phos mg%	7.8	5.8	6.9	7.9	7.1
Gluc mg%	80	84	80	59	75
Creat mg%	0.9	1.0	1	1.1	1

SECTION VI

DETAILED METHODOLOGY AND REAGENTS

Reagents and Addresses

Absolute Alcohol (analytical grade)	
Agar	BBL, Cockeysville, MD
Dimethyl Sulfoxide	Mallinckrodt Chemical Co., St. Louis, MO
Dioxane	Scientific Products, IL
DNase (pancreatic)	Sigma Chemical Co., St. Louis, MO
EDTA	Mallinckrodt Chemical Co., St. Louis, MO
Fetal Calf Serum	Kansas City Biological Co., Kansas City, KS
Fungizone	E. R. Squibb and Sons, NY
Hormones (non-radioactive)	Calbiochem, Los Angeles, CA
Hormones (tritiated)	New England Nuclear, Boston, MA
Horse Serum	Kansas City Biological Co., Kansas City, KS
Kanamycin (Kantrex)	Bristol Laboratories, Syracuse, NY
Ketamine Hydrochloride	Bristol Laboratories, Syracuse, NY
Medium 199	Grand Island Biological Co., Grand Island, NY
Minimal Essential Media	Grand Island Biological Co., Grand Island, NY
Napthalene (recrystalized)	Mallinckrodt Chemical Co., St. Louis, MO
Neutral Red	Fisher Scientific Co., Fairlawn, NJ
Omnifluor	New England Nuclear, Boston MA
Penicillin G	E. R. Squibb and Sons, NY
Phytohemagglutinin	Sigma Chemical Co., St. Louis, MO
RPMI-1640	Grand Island Biological Co., Grand Island, NY

RNase	Sigma Chemical Co., St. Louis, MO
Streptomycin Sulfate	Eli Lily, Indianapolis, IN
Toluene	Mallinckrodt Chemical Co., St. Louis, MO
Trichloroacetic Acid	Mallinckrodt Chemical Co., St. Louis, MO
Tritiated Thymidine	New England Nuclear, Boston, MA
Trypsin	Difco, Detroit, MI

Reagents

Agar: 2% purified agar dissolved in deionized water and autoclaved for use. A mixture of 50% agar - 50% (2X) MEM is used for plaquing.

Antibiotics:

Penicillin (1,000,000 units)

Streptomycin (1 g)

Kanamycin (50 mg) is dissolved in 100 ml of sterile deionized water. One ml of PSK stock solution is added to give a final concentration of: 100 units penicillin, 1 mg streptomycin and 5 ng kanamycin/ml media.

Dimethyl Sulfoxide $(CH_3)_2SO_4$:

For freezing cells, this is made to an appropriate concentration (10%) and autoclaved 10 minutes at 15 minutes.

DNase: 2 mg/ml, stock. A final concentration of 10 ug/ml is used.

RNase: 2 mg/ml, stock. A final concentration of 10 ug/ml is used.

Fungizone: 50 mg (one vial) plus 20 ml sterile deionized water. Add 0.2 ml/liter of media.

Hormones:

^{3}H-cortisol - specific activity (S.P.) 44.3 Ci/mmol

^{3}H-corticosterone (S.P.) 47.5 Ci/mmol

^{3}H-extradiol (S.P.) 44 Ci/mmol

^{3}H-progesterone (S.P.) 55.7 Ci/mmol

^{3}H-testosterone (S.P.) 25 Ci/mmol

All tritiated hormones diluted in R.P.M.I. to a concentration of 1 uCi/ml. In adsorption studies, 0.2 ml of this preparation is used.

Non-radioactive hormones, 25 mg, dissolved in 5 ml of acetone-alcohol (1:1) mixture to give stock of 5 mg/ml. Diluted in R.P.M.I. to 5, 50, 500, 5000 ng/ml and used.

Neutral Red: A solution (0.015%) for plaquing is prepared from powder with deionized water and dissolved in a 37° C water bath. Whatman #2 filter paper is used for filtration. Sterilize by autoclaving and store at 4° C, protected from light. 1.5 ml stock/100 ml agar and media.

Phytohemagglutinin: 50 ug/ml in RPMI 1640 stock. A final concentration of 2.5 ug/ml is used.

Phosphate-Buffered Saline (PBS):

Solution 1

$Na_2HPO_4 \cdot 7H_2O$	134 g/l deionized H_2O

Solution 2

$NaH_2PO_4 \cdot H_2O$	69.1 g/l deionized H_2O

Buffer Stock

$Na_2HPO_4 \cdot 7H_2O$	386 ml solution 1
$NaH_2PO_4 \cdot H_2O$	114 ml solution 2
Deionized Water	500 ml
	pH 7.15

PBS:

80 ml buffer stock

17 g NaCl

1920 ml deionized water

pH 7.2

Autoclave at 15 lbs. for 15 minutes

Scintillation Fluid:

360 ml	Toluene
360 ml	Dioxane
80 g	Napthalene
216 ml	Absolute alcohol
4 g	Omnifluor

Thymidine, tritiated:

Specific activity 43 Ci/mmol

Stock of 10 uCi/ml made in RPMI. For blastogenesis studies, 0.1 ml of this dilution is used.

Trypsin:

1 g/400 ml trypsin in PBS made and frozen for use.

Working Solution:

20 ml	0.25% trypsin
30 ml	PBS
0.5 ml	10% EDTA

SECTION VII

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THE IMMUNE RESPONSE IN CALVES VACCINATED WITH INFECTIOUS BOVINE RHINOTRACHEITIS OR BOVINE VIRAL DIARRHEA VIRUS VACCINES

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Abstract

Humoral and cell mediated immune response was studied in twenty-four dairy calves inoculated with a commercial modified live polyvalent vaccine containing both infectious bovine rhinotracheitis virus (IBRV) and bovine virus diarrhea virus (BVDV). Plaque reduction and lymphocyte blastogenesis assays were used to measure serum antibodies and cellular immunity, respectively. Serum antibody titers of 1/80 and 1/160 were observed in these animals against IBRV and BVDV, respectively. However, cell mediated immune response was not detected as demonstrated by lack of incorporation of ^{3}H-thymidine (^{3}H-TdR) in lymphocyte cultures of these calves in the presence of specific virus antigens.

Post vaccinal reactions due to modified live viruses have been reported by many investigators. Cell mediated immune response to inactivated vaccines against IBR and BVD viruses have not been illucidated. A prototype study in calves vaccinated with inactivated-adjuvated vaccines was undertaken. The vaccines were prepared by inactivating IBR or BVD virus with formalin (1:1000) and homogenizing with Freund's incomplete adjuvant. Each vaccine was inoculated into a calf. Two other calves were vaccinated each with a modified live IBR virus intranasally or modified live BVDV intramuscularly. Humoral response was elicited earlier in calves given inactivated virus vaccines as compared to those vaccinated with

modified live virus (MLV) vaccines. Serum antibody titers of 1/160 and 1/80 for IBRV and BVDV, respectively, were observed one week post vaccination, reaching maximum titers of 1/640 and 1/1280 by six to nine weeks. In addition, cell mediated immune response was detected in the calf vaccinated with formalized adjuvated IBRV, as demonstrated by lymphocyte blastogenesis studies. The mean stimulation index, as measured by the ratio of radioactive thymidine counts of cells stimulated by antigen to that of cells without antigen, was 11.5 during the course of study. All other calves did not show any appreciable rise in stimulation indices, as indicated by lack of response and failure of incorporation of ^{3}H-TdR when their lymphocytes were stimulated with specific virus antigens.

The role of steroid hormones in the immune response to bovine respiratory disease viruses has not been thoroughly investigated. Corticosteroid levels in calves have been shown to increase during times of stress in feedlots or during transportation. The interaction of IBR or BVD virus with lymphocytes in the presence of cortisol was studied. Radioactively labelled IBRV and BVDV were prepared by growing them in Madin Darby bovine kidney cells supplemented with ^{3}H-thymidine or ^{3}H-uridine, respectively. The viruses were treated with DNase or RNase, ultracentrifuged at 200,000 g and resuspended in culture medium.

The specificity of receptors for cortisol on bovine lymphocytes from immune and control calves was demonstrated by decreased uptake of ^{3}H-cortisol in the presence of the competing

non-radioactive hormone. Increasing concentrations of cortisol failed to influence the degree of adsorption of labelled IBRV or BVDV on lymphocytes from control and vaccinated calves as indicated by recovery of similar number of radioactive counts and plaque forming units. This indicated the presence of different receptors on bovine lymphocytes for cortisol and the viruses.

Physiological concentrations of cortisol in cultures of lymphocytes from the calf vaccinated with inactivated IBRV inhibited the stimulation by specific virus antigen suggesting depression of cell mediated immune response in the presence of the hormone.

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