To the Graduate Council:

I am submitting herewith a dissertation written by James S. Guy entitled "Distribution of Specific Antibody Activity Among Serum Immunoglobulin Isotypes Following Bovine Herpesvirus I Infection of Cattle." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

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The Graduate School

DISTRIBUTION OF SPECIFIC ANTIBODY ACTIVITY AMONG SERUM IMMUNOGLOBULIN ISOTYPES FOLLOWING BOVINE HERPESVIRUS I INFECTION OF CATTLE

A Dissertation

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ABSTRACT

The kinetics of antibody formation in Holstein-Friesan heifers following primary and secondary intranasal inoculation of bovine herpesvirus I (BHVI), reactivation of latent BHVI (RLI), and BHVI-induced abortion was determined. Sera were fractionated by gel filtration and ion exchange chromatography. The antibody activity within serum immunoglobulin isotypes was assessed by a virus plaque reduction assay (PRA).

The primary immune response to BHVI infection was characterized by the appearance of IgM and IgG antibodies in serum 7 days post inoculation (pi). Maximal IgG antibody activity occurred at 35 day pi in nonpregnant heifers and 14 days pi in pregnant heifers. Thereafter antibody activity in this class slowly declined. Maximal IgM antibody activity occurred at 14 days pi in both groups of heifers and declined rapidly thereafter. The IgG antibody activity during primary immune responses was localized to the IgG1 subclass primarily.

Secondary responses were characterized by anamnestic IgG antibody responses. Secondary IgM antibody formation was elicited by abortion induced by the intraamniotic inoculation of BHVI and RLI, but not by reexposure by the intranasal route. Antibody activity was present within both IgG subclasses during secondary immune responses. However, the increase in antibody activity during this period was in the IgG2 subclass primarily.

Abortion occurred in one heifer 28 days after intranasal BHVI inoculation. Abortion in this animal failed to stimulate a secondary antibody response.

The nature of BHVI antigenic exposure in the bovine was determined by assessing the relative distribution of anti-BHVI antibody activity among the serum immunoglobulin isotypes, IgM, IgG1, and IgG2. The formation of IgM antibody, with the exception of secondary intranasal exposure, indicated recent BHVI antigenic exposure. Primary immune responses were differentiated from secondary immune responses by the presence of IgG1 antibody and the absence of IgG2 antibody.

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I. INTRODUCTION

Bovine abortion represents a major constraint to livestock production. It results in tremendous economic losses and the wastage of a food resource. In addition to the loss of the fetus, a period of uterine disease resulting in infertility, often follows abortion. This results in additional economic loss due to the maintenance within the herd of an unproductive member.

Despite the economic importance of this disease, attempts at determining the causes of abortion are frequently unproductive. The etiologic diagnosis of bovine abortion cases by diagnostic laboratories is notoriously poor. Based on published reports from these laboratories, a definitive diagnosis is obtained in only a small percentage of submitted cases. The failure to obtain a diagnosis in most cases of abortion is due to a number of factors including the absence of fetal tissues, autolysis of fetal tissues, the disappearance of the etiologic agent by the time of abortion, the absence of paired serum samples, and difficulty in the interpretation of serological data. Fetal tissues often are not submitted to the diagnostic laboratory, therefore precluding diagnosis based on histopathology, virus isolation, and antigen detection. Similarly, fetal tissues when submitted, often are autolytic and therefore unsuitable for diagnostic analysis. The autolysis of aborted fetal tissues is due to the frequent delay between fetal death and fetal expulsion. As a result of this delay, viral isolation attempts may be futile presumably because many viruses causing abortion are labile at

body temperature, and sensitive to the enzymatic activity present in fetal tissues undergoing autolysis.

The absence of fetal tissues, or the unsuitability of such tissues, for histopathology, virus isolation, and antigen detection methods, places a heavy burden on serological diagnosis. Serology could be an important means of diagnosing bovine abortion since serum is often the only clinical material available for diagnostic analysis. However, the diagnosis of abortion based on serology is hindered by the frequent absence of paired serum samples, and the frequent difficulty in the interpretation of serological data when paired serum samples are tested.

Conventional serological diagnosis requires demonstration of a fourfold increase in specific antibody titer, or a seroconversion from negative to positive antibody titer over a two- to three-week period. The initial sample is usually taken during the acute phase of the disease. Since many of the viruses causing abortion are ubiquitous in most bovine populations, the mere presence of antibody to these viruses, in a single serum sample, has no diagnostic meaning.

Cows frequently are infected several weeks to months prior to abortion and therefore have relatively high levels of specific antibody at the time of abortion. Some evidence suggests that secondary antigenic stimulation occurs during abortion to the virus responsible for the abortion. It has been suggested also that a rise in specific antibody, may not occur due to the presence of high levels of specific antibody. This may explain why there is difficulty often in the interpretation of serological data even when paired serum samples are tested. Antibody

formation in the bovine occurring as a consequence of secondary antigenic exposure during abortion has not been extensively studied. Perhaps the difficulties related to the interpretation of serological data reflects a poor understanding of antibody formation at this time, or the use of insensitive serological techniques to measure such a response.

The difficulty of obtaining paired serum samples is also a problem in the serologic diagnosis of human viral infections. Serological techniques, for diagnosis of human viral infections, have been devised to circumvent this problem which include the detection of specific anti-viral IgM antibody, and the detection of specific antibody directed against certain virus-specific antigens. Since the IgM antibody response is transient, detection of specific IgM antibody represents a means of determining a recent viral infection based on a single serum sample. In the acute viral infections of man such as rubella, hepatitis A, and influenza, the detection of specific IgM antibody has been shown to be useful in differentiating primary and secondary infection. The secondary response to these viruses, following reinfection or immunization with attenuated strains of these viruses, does not result in secondary IgM antibody formation.

The detection of specific antibody activity among the other immunoglobulin isotypes, and the utilization of this information in the serologic diagnosis of viral infections, has not been investigated. However, several reports suggest that the distribution of specific antibody activity among the different IgG subclasses may be useful in differentiating primary and secondary antibody responses.

A number of studies have cast doubt on the applicability of specific IgM antibody detection in the serological diagnosis of bovine viral infections. These studies have indicated that the formation of specific IgM antibody occurs following secondary antigenic exposure. However, these studies were based on antigenic exposure to soluble proteins emulsified in adjuvant, and to viruses inoculated by unnatural routes. Therefore one must question whether these studies represent antibody formation as it occurs following natural infection.

Bovine herpesvirus I (BHVI), a herpesvirus infection of cattle, is the cause of several different clinical syndromes including respiratory tract infection, conjunctivitis, encephalitis, genital infection, and abortion. This virus is believed to be the most important cause of bovine abortion in the United States since several reports indicate that BHVI is the most frequently diagnosed cause of bovine abortion.

Bovine herpesvirus I, like other herpesviruses, tends to remain in a latent state within the host following primary infection. The reactivation of latency may occur periodically following the establishment of latent infection, and clinical signs of infection are seen again. The effect of periodic reactivation of latent infection on the humoral immune response has not been examined in the bovine, but has received considerable attention in the human. Specific IgM antibody formation occurs following the reactivation of certain human herpesvirus infections. This suggests that the demonstration of specific IgM antibody response cannot differentiate primary and secondary herpesvirus infections.

The importance of BHVI reactivation in the pathogenesis of BHVI-induced disease has not been examined. The inability to

differentiate primary infection from the reactivation of latent infection has precluded an examination of this question. This differentiation cannot be based on virus isolation or antigen detection techniques, and therefore is dependent upon serological methods. However, serological methods cannot be applied because of inadequate knowledge of the kinetics of antibody isotype formation in the bovine.

The object of this study was to investigate immunoglobulin isotype formation in the bovine, and determine its application in the serological diagnosis of bovine viral infections. The kinetics of immunoglobulin isotype formation following primary BHVI infection, secondary (intranasal) BHVI infection, reactivation of latent BHVI infection, and BHVI-induced abortion was determined. The nature of viral antigenic exposure was differentiated by comparing patterns of immunoglobulin isotype formation. The feasibility of single serum sample diagnosis based on the distribution of specific antibody activity among the immunoglobulin isotypes was determined.

II. SURVEY OF THE LITERATURE

Bovine Abortion

Bovine abortion results in tremendous economic loss and the considerable wastage of a nutritional resource (1). Along with the loss of the fetus, a period of uterine disease in the affected cow often follows abortion (2). This results in additional economic loss due to the maintenance within the herd of an unproductive member (2).

The importance of infectious agents in the pathogenesis of bovine abortion is well established (3). Infectious agents represent 80-90% of the diagnosed cases of bovine abortion (3). There is evidence also to suggest that a significant proportion of undiagnosed cases are due to infectious agents, because histopathologic lesions suggestive of infection are present in a large percentage of these cases (4).

A number of different infectious agents have been shown to be capable of eliciting abortion in the bovine. Among these are Brucella abortus, Leptospira species, Campylobacter species, Listeria monocytogenes, Corynebacterium species, Eschericia coli, Staphylococcus aureus, Streptococcus species, bovine herpesvirus I (BHVI), bovine virus diarrhea virus, bluetongue virus, rift valley fever virus, parainfluenza virus III, malignant catarrhal fever virus, foot and mouth disease virus, Chlamydia psittaci, and various mycotic agents (1). Of these agents, BHVI is believed to be the most important cause of infectious abortion in the United States since BHVI is the most frequently diagnosed cause of bovine abortion (3, 4, 5).

The diagnosis of bovine abortion by veterinary diagnostic laboratories is notoriously poor (3). Reports from these laboratories indicate a definitive diagnosis is obtained in only 20-25% of submitted cases (3, 4, 5). Establishing a diagnosis is often difficult for a number of reasons (1) fetal tissue or placenta are not available, (2) autolysis of fetal tissue, (3) etiologic agent is not detectable at the time of abortion, (4) paired serum samples not available, (5) suitable serological tests not available for certain infectious agents, and (6) difficulty in the interpretation of serological data.

Often the fetus and placenta are not submitted to the diagnostic laboratory and often when such tissues are submitted, extreme autolysis precludes histopathologic examination (6). Autolysis of fetal tissue is due to the frequent delay between fetal death and fetal expulsion in infectious abortion (7). This delay also precludes the isolation of certain agents since the conditions in utero such as body temperature (37°C) and autolytic enzymatic activity, are conditions which are unfavorable for a number of the agents associated with bovine abortion (8).

The importance of serology in the diagnosis of bovine abortion is evident (6). The absence of fetal tissue or the condition of such tissue often prevents diagnosis based on histopathology, the detection of specific antigen or recovery of the agent involved (6). Serum is often the only clinical material, or the only suitable clinical material, with which a laboratory has to make a diagnosis (6). However, serological diagnosis is hampered often by the difficulty in obtaining paired serum samples and the difficulty in interpreting serological data generated from paired serum samples.

Bovine Herpesvirus I

Infections bovine rhinotracheitis (IBR) is a herpesvirus infection of cattle caused by bovine herpesvirus I (BHVI). The respiratory form of IBR was recognized first in 1954 by Miller (9) who described it as a new respiratory tract infection of feedlot cattle. It was recognized soon thereafter in dairy cattle (10), and later associated with several clinical manifestations other than respiratory tract disease (11). The virus has been shown to be the cause of infectious pustular vulvovaginitis (12, 13), Balanoposthitis (14), abortion (15, 16, 17, 18), and conjunctivitis (19). It may occasionally cause encephalomyelitis in young calves (20), and has been suggested as a cause of mastitis (21), infertility (22), and metritis (23).

The virus was first isolated by Madin in 1956 (24). It was characterized subsequently and classified in the family herpesviridae based on its physical, biochemical, and epidemiological properties (25). Virions consist of a core containing double standed deoxyribonucleic acid (DNA) which is surrounded by an icosahedral protein capsid and a pleomorphic envelope (26). Replication of the viral DNA occurs within the cell nucleus, and acquisition of an envelope follows maturation in the nucleus by budding through the inner lamella of the nuclear membrane (26). The virus produces intranuclear inclusion bodies in vivo and in vitro, and it has the potential to exist in a latent state following primary infection (26, 27).

A polypeptide analysis of BHVI by Misra et al. (28) has shown the virus to contain 25-33 structural (virion) polypeptides. Eleven of

these polypeptides were shown to be glycosylated. It was shown also that BHVI infected cells contained a minimum of fifteen nonstructural (nonvirion) polypeptides (28).

Virus-specific proteins produced in herpes virus-infected cells are given the designation alpha, beta, or gamma based on their temporal order of synthesis, and whether their synthesis is dependent upon certain preceeding functions of infected cells (29). Synthesis of alpha, or immediate early proteins, is not dependent upon the expression of other viral genes (29). If protein synthesis is inhibited in infected cells, alpha gene mRNAs and alpha proteins accumulate (29). The synthesis of beta, or early proteins, is dependent upon the prior synthesis of the alpha proteins (29). If viral DNA synthesis is inhibited, beta proteins accumulate within the infected cell (29). Synthesis of gamma, or late proteins, require the onset of viral DNA synthesis and the prior synthesis of both alpha and beta proteins (29).

Bovine Herpesvirus I-Induced Abortion

Jensen et al. in 1955 (30) were the first to suggest that BHVI was a cause of bovine abortion. They observed that pregnant heifers with current or prior clinical signs of BHVI infection frequently aborted. McKercher and Wada (16) later isolated BHVI from aborted fetuses and experimentally reproduced abortion with this virus. They demonstrated that pregnant heifers inoculated with the virus by the intranasal, intravenous, or intramuscular routes aborted dead fetuses 18-64 days later. Other investigations on experimentally-induced abortion suggested abortion

may occur as early as 8 days (31) and as late as 3 months (32) following exposure to the virus by various routes. Owen et al. (33), and Saunders et al. (31) reported a high percentage of abortions in pregnant heifers inoculated with BHVI by the intramuscular route. Inoculation of BHVI by the intranasal route was less efficient in inducing abortion (31).

Abortion as a result of BHVI infection may occur at any time during gestation but most frequently occurs between the fourth and seventh month of pregnancy (34). Abortion may follow respiratory or conjunctival BHVI infections, but rarely follows genital infections (2). Clinical signs of BHVI infection, preceeding abortion, often are not observed (34).

During maternal infection the virus is believed to infect the placenta, and after a variable period of time, cross the placenta to infect the fetus (35). The fetus is highly susceptible to BHVI and experiences a rapidly fatal, generalized infection with widespread necrosis (7). Expulsion of the dead fetus generally occurs 4 to 7 days after fetal death (7). This delay between fetal death and expulsion results in considerable autolysis of the fetal tissues, and often renders these tissues unsuitable for histopathological examination (6).

Kendrick et al. (35) have suggested that the placenta is a site of BHVI latency following primary infection of the pregnant animal. They reported the isolation of the virus from the placentae of experimentally infected cows which delivered normal, uninfected calves. They suggested that the placental reaction to infection by this virus differed markedly from other tissues since histopathological lesions suggestive of viral infection were not present in the placentae of infected fetuses. The

only histopathological changes present in these placentae consisted of nonspecific postmortem changes and an occasional necrotic trophoblast cell. These histopathological changes were similar in all experimentally infected heifers whether they delivered normal uninfected calves or aborted infected fetuses (35).

Diagnosis of BHVI-Induced Abortion

The laboratory diagnosis of BHVI-induced abortion may be accomplished by several different methods (8). The choice of methods is generally dictated by the specimens submitted and the condition of such specimens. Histopathologic diagnosis based on the detection of focal necrosis, although nonspecific, represents incriminating evidence of BHVI infection (34). Focal necrosis may be detected in the liver, spleen, lung, and kidney of BHVI aborted fetuses (34). The presence of inclusion bodies which would represent more specific evidence of BHVI infection, infrequently are present (34). Problems encountered with histopathologic diagnosis include autolysis of fetal tissues and the similarity of BHVI lesions to those induced by Listeria monocytogenes (6).

Diagnosis based on the detection of specific viral antigen may be performed using fluorescent antibody (FA) techniques (36). Kidney and adrenal gland are the preferred tissues for FA examination since the intensity of fluorescence and the frequency of detection is greatest in these tissues (6). Antigen may also be detectable in the placenta by this technique but autofluorescence often hinders interpretation (6).

Virus isolation may be attempted, but according to Kirkbride (6), BHVI is recovered from fewer than 33% of fetuses aborted as a result of

BHVI infection. The virus may be isolated from virtually any fetal tissue but is most consistently isolated from the placenta (6).

Serology is frequently the method of choice due to the absence of aborted fetal tissue or the poor condition of such tissue. The serological methods currently used to diagnose BHVI infection include serum virus neutralization (SVN), the indirect fluorescent antibody test (IFAT), passive hemagglutination (PHA) and complement fixation (CF), (37, 38, 39, 40). The serum virus neutralization (SVN) test is the most commonly used method for measurement of anti-BHVI antibody (37, 41). However, according to several investigators the sensitivity of this test is relatively low (42). The requirement of approximately 1 week for the completion of the test is an additional disadvantage of this serological method. The sensitivity of the IFAT, PHA, and CF tests is similar to the SVN test for detecting anti-BHVI antibody, and are less expensive and less time consuming (38, 39, 40). The serologic diagnosis of BHVI infection by these methods requires the demonstration of a seroconversion from negative to positive titer, or a fourfold increase in antibody titer in paired serum samples (6). Ideally, paired serum samples consist of a serum sample obtained during the acute phase of infection and a second sample obtained during convalescence (6).

The timing of serum sample collection is also important. A rising antibody level may not be detectable if the acute serum sample is obtained late in the acute phase of infection. The acute phase serum sample must be obtained early, before a significant antibody response occurs (34).

Cattle are infected with BHVI from one to several weeks prior to abortion and therefore these animals usually have significant antibody titers to BHVI at the time of abortion (34). The presence of specific anti-BHVI antibody at the time of abortion may explain the frequent failure to detect an increase in antibody titer at the time of abortion even when other evidence incriminating BHVI is present (18).

The presence of specific antibody at the time of secondary antigenic exposure is inhibitory to antibody formation (43, 44). Uhr and Bauman in 1964 (43) demonstrated that diphtheria antitoxin, passively administered to guinea pigs up to 5 days following immunization with toxoid, inhibited the primary antibody response. They also demonstrated that the secondary antibody response was inhibited by passively administered antitoxin when administered 4 days after a secondary injection of toxoid (44).

Several investigators were unable to detect an increase in serum antibody titers to BHVI after secondary exposure (45, 46). Lejon and Asso (45) reported that antibody titers did not change in a group of calves inoculated by the intranasal route 30 days after the first intranasal inoculation. Similarly, Franck et al. (46) reported that an increase in antibody titer was not detectable, in animals having high serum antibody titers, after secondary BHVI exposure. The secondary exposure occurred 100 days following intranasal BHVI inoculation.

In cases of BHVI-induced abortion, acute phase serum samples are generally not available because the initial infection is often asymptomatic, and may occur several weeks prior to abortion (34). The absence of paired serum samples, in general, preclude the use of the above

serological techniques in the diagnosis of BHVI infection (6). Likewise, serological methods are of little use if the initial serum sample is obtained late during the initial phase of infection (34). For certain human viral infections, serological techniques have been devised to circumvent these problems. These include the detection of specific antiviral IgM antibody and the detection of specific antibody directed against certain virus-specific antigens (47, 48).

The use of IgM antibody detection in the serodiagnosis of human viral infections is well documented (47). Because of the early disappearance of this isotype following viral infection, the detection of specific IgM antibody is useful as a means of determining recent viral infection (47). Secondary exposure to the viruses causing acute human infections such as rubella, influenza, and hepatitis A, does not result in IgM antibody formation (49). This provides a means for differentiating primary and secondary infection (49). The detection of specific IgM antibody represents an immunodiagnostic approach utilizing single serum samples and a defined pattern of isotype formation (47).

There is currently some doubt concerning the applicability of IgM antibody detection in the serodiagnosis of human herpesvirus infections (49). Specific IgM antibody formation occurs following the reactivation of varicella-zoster, herpes simplex, and cytomegalovirus infections (47, 50, 51). As a consequence, the use of IgM antibody detection for differentiating primary from reactivated latent human herpesvirus infection is questioned (49).

Detection of IgM antibody to determine recent BHVI infection was first suggested by Potgieter (52). He demonstrated that IgM antibody, but not IgG antibody, was dependent upon the presence of guinea pig complement for the neutralization of BHVI. Therefore, he suggested that the presence of complement-dependent antibody, in a single serum sample, would indicate recent BHVI infection. The use of IgM antibody detection to differentiate primary from reactivated latent BHVI infection in the bovine has not been examined.

Several techniques have been devised to demonstrate the presence of specific IgM antibody in serum. The techniques most commonly used depend on the physical separation of IgM from IgG by gel filtration (53), or sucrose density gradient centrifugation (54). Less commonly used methods include ultrafiltration (55) and ion exchange chromatography (56). Some are based on the removal of IgG by absorption with Protein A (57), and precipitation of IgG with antibody specific for the Fc portion of IgG (58). These techniques are relatively simple and provide reliable results, but are very time consuming (59).

Radioimmunoassays and enzyme-linked immunosorbent assays have been used to detect IgM antibody (60, 61). These techniques are based on the binding of specific IgM antibody to viral antigen absorbed to a solid phase (60). The bound IgM antibody is then detected by antibody specific for the Fc portion of IgM (anti-u) and labelled with either an enzyme (61), or a radioisotope (60). Problems encountered with these techniques include interference by specific IgG antibody and IgM rheumatoid factor (59).

The presence of specific IgG antibody may compete with IgM antibody in these assays (62). Antibody of low avidity, primarily IgM antibody, would tend to be excluded by antibody of higher avidity, primarily IgG antibody (62). Interference by the presence of specific IgG antibody may result in false negative results using these IgM antibody detection methods (62).

Similarly, the presence of IgM rheumatoid factor, an autoantibody to the Fc portion of IgG, may result in false-positive results (62). This autoantibody has been detected in humans following infection by influenza (64), Epstein-Barr virus (65), cytomegalovirus (66), and rubella (67). It has been detected also in apparently normal individuals (68). In the presence of RF, the binding of specific IgG antibody to the antigen in these assays would conceivably lead to the binding of RF and the detection of this nonspecific IgM antibody (63).

Antibody capture techniques have been devised to detect IgM antibody and circumvent the problems associated with the presence of RF and specific IgG antibody (59, 69, 70). This technique is based on the binding of serum IgM by anti-u antibody absorbed to the solid phase (59). This method indiscriminately binds a portion of serum IgM. Specific antiviral IgM is then detected by the sequential addition of viral antigen and anti-viral antibody labelled with an enzyme (69) or a radioisotope (70). Krech and Wilhelm (71) described an antibody capture technique for rubella virus infection which detected bound IgM antibody using the sequential addition of rubella virus (a hemagglutinating virus), and erythrocytes.

Detection of antibody to early antigens (EA) of certain deoxyribonucleic acid (DNA) viruses has been suggested as a means of determining recent infection in the absence of paired serum samples (72, 73, 74, 75). Early antigens (EA) are virus specific proteins produced in infected cells prior to viral DNA synthesis (29). Late antigens (LA) represent viral structural proteins primarily, and are produced subsequent to viral DNA synthesis in infected cells (29).

Henle et al. in 1971 (72) first suggested the presence of EA antibody as an indication of recent infection. They demonstrated that humans developed serum antibodies to both EA and LA following Epstein-Barr virus (EBV) infection. Antibodies to LA persisted indefinitely, whereas EA antibodies disappeared within a few months after infection. Similar observations were made later with cytomegalovirus (73), varicella-zoster virus (74), and adenovirus (75). Antibodies to EA, however, also were present following reactivation of latent cytomegalovirus infection (76), and in certain instances, persisted following infection (77).

Immunoglobulin Isotype Formation

The chronological sequence of immunoglobulin isotype formation following viral infection was demonstrated initially by Brown and Graves in 1959 (78) using cattle infected with foot and mouth disease virus (FMDV). They showed that the antibody present at 7 days following infection had electrophoretic characteristics of beta globulin whereas that present at 14 days consisted primarily of antibody with a slow gamma globulin mobility. Later studies showed the beta globulin to be

IgM, or 19s antibody, and the slow gamma globulin to be IgG, or 7s antibody (79, 80).

Subsequent studies on the antibody response to poliovirus (81, 82) and bacteriophage (83) confirmed the earlier findings that 19s antibody formation preceded 7s antibody formation. These studies also examined the kinetics of antibody formation and determined a number of factors which influenced antibody formation during the primary and secondary responses. These studies, as well as others (84, 85), demonstrated that the character of immunoglobulin isotype formation following antigenic exposure is influenced by the chemical and physical nature of the antigen, the immunological status of the host, the manner in which antigen is administered, and the dose of antigen.

The mechanism by which B cell precursors are committed to produce a specific antibody isotype is not understood (86). It is currently believed that the chemical and physical structure of the antigen, and the T helper cells involved, are responsible for regulating isotype formation by B cells (86). While certain studies indicate that T cells are essential in regulating isotype formation (87), others have demonstrated that certain IgG subclasses, particularly IgG3, may be formed without T cell involvement, therefore implying a major role for antigen in this process (88). Antigens which have been termed T-independent because they induce B cell responses in the apparent absence of T helper cells, result in the formation of IgM antibody primarily (88).

The four IgG subclasses of the human, IgG1, IgG2, IgG3, and IgG4 constitute approximately 65%, 25%, 6%, and 4% respectively of the total

serum IgG (89). It has become evident that the antibody activity against a number of different antigens is not distributed among these isotypes in the proportion in which they are found in serum (89). For example, anticarbohydrate antibodies are restricted to the IgG2 subclass (90), isoantibodies to erythrocytes are primarily IgG1 and IgG3 (90), and antibody to ragweed pollen in hay fever patients is primarily IgG4 (91).

Recently, Beck (92) reported on the distribution of antibody activity among the human IgG subclasses to three different human viruses, herpes simplex I, poliovirus, and rubella virus. Using serum from donors with high antibody titers to these viruses he determined that the antibody activity to these viruses was primarily present in the IgG3 subclass.

The importance of antigen dose on the character of isotype formation was shown by Svehag and Mandel in 1964 (81). They demonstrated that the induction of antibody formation in the IgM and IgG classes following the intravenous inoculation of rabbits with poliovirus, was dependent upon the antigen dose. A very low dose of antigen induced the synthesis of IgM antibody without subsequently detectable IgG antibody formation. Higher doses of antigen, at least 50 times more, was required to induce IgM antibody formation which was subsequently followed by an IgG antibody response. Very low doses of antigen were shown to induce IgM antibody responses characterized by a rapid rise in IgM antibody titer accompanied by a sudden cessation of IgM antibody synthesis. This sudden cessation of IgM synthesis was believed to be due to a lack of continuing antigenic stimulation. Svehag and Mandel (93) later demonstrated that a second injection of antigen, at the time at which IgM synthesis ceased,

resulted in renewed IgM antibody formation. Additional evidence supporting the idea that continual IgM antibody synthesis requires the persistence of antigen was provided by Finkelstein and Uhr in 1964 (94) and Sahiar and Schwartz in 1964 (95). They demonstrated that the passive administration of specific IgG antibody at the time of immunization markedly inhibited IgM antibody synthesis.

Benacerraf et al. in 1963 (85) showed that the manner in which antigen is administered affected antibody isotype formation. In the guinea pig both IgG1 and IgG2 antibodies were formed following immunization with soluble antigen emulsified in adjuvant. Bovine gamma globulin conjugated to 2, 4 dinitrophenyl and emulsified in complete Freund's adjuvant was administered by the foot pad route. This immunization procedure resulted in the formation of both IgG1 and IgG2 antibodies, with IgG2 antibody formation preceeding IgG1 antibody. Administration of the same antigen without adjuvant, by the intraperitoneal route, resulted in the formation of IgG1 antibody primarily.

The sequential appearance of IgM and IgG antibody formation following primary exposure to viral antigens has been the general observation of numerous investigators (78, 96, 97, 98, 99). This sequence has been documented in several different animal species following natural infection (78, 97, 98) and immunication with virus vaccines (96, 99). The many host-virus systems in which this has been demonstrated include cattle and guinea pigs infected with FMDV (78), guinea pigs immunized with herpes simplex virus (HSV) (96), humans infected with Russian encephalitis virus (97), measles virus-immunized guinea pigs (99), and

Coxsackie virus-infected humans and monkeys (98). From these studies it has been observed that following natural infection, or immunization, IgM antibody appears rapidly and reaches maximal levels in approximately 5 to 10 days (100). The IgM antibody titers then decline to relatively low levels after 30 to 40 days (100). Antibody of the IgG class becomes detectable a few days following IgM antibody and maximal levels are attained in 4 to 12 weeks (100). While IgM antibody is transient, IgG antibody tends to persist indefinitely (100).

The sequential appearance of IgM antibody followed by IgG antibody as reported in these early studies has been questioned recently by Osler (101). He suggested that the earlier appearance of IgM antibody determined in these studies may have been due to methodological errors in measuring the antibody responses. The precedence of IgM antibody in these studies was based on the decrease in serum antibody titers after treatment of serum with 2-mercaptoethanol. They based their observations on the assumption that the destruction of sulfhydryl bonds by 2-mercaptoethanol only affected IgM antibodies. However, as early as 1963, Berlin (102) demonstrated that 2-mercaptoethanol treatment of serum resulted also in a decrease in IgG antibody activity. Osler also noted that antibody assays based on virus neutralization, hemagglutination, and bacterial agglutination preferentially detects IgM antibody over IgG antibody.

Individual variation with respect to the formation of IgM antibody has been noted (84, 103, 104, 105). Svehag (84) reported that IgM antibody persisted in some rabbits for up to six months following immunization with poliovirus. McKercher and Giordano (103) reported that

some swine infected with FMDV also had a persistent IgM antibody response. Similarly, Monath (104) has reported that some humans immunized with a live yellow fever virus vaccine developed an IgM antibody response which persisted for a prolonged period of time. Prolonged IgM antibody responses have been reported also in humans with certain chronic viral infections, such as hepatitis B virus infection (105).

Antibody isotype formation as a result of secondary antigenic exposure has received less attention than that following initial antigenic exposure (47). The acute virus infections of man, such as measles, rubella, and hepatitis A, induce solid, long lasting immunity which makes reinfection uncommon (47). Secondary infections by these viruses, if they occur, are generally subclinical (47). Because of this, the secondary response following reinfection with these viruses has not been extensively studied.

Early studies by Stelos et al. (106), Fink et al. (107), and Uhr et al. (108) indicated that IgM antibody formation did not occur following secondary antigenic exposure of animals to viral antigens using various routes of inoculation. Antibody formation detected during the secondary response in these studies was entirely of the IgG class. However, later studies by Bauer et al. (109) and Svehag and Mandel (93) reported the detection of IgM antibody as well as IgG antibody after secondary exposure to viral antigens using similar routes of exposure. The reasons for the discrepancy between these studies has not been satisfactorily explained. However, in the earlier studies (106, 107, 108) antibody titers were determined by sensitivity to 2-mercaptoethanol, whereas in

later studies (93, 109) antibody titers were determined after separation of the antibody isotypes. Svehag and Mandel (93) determined that the antigen dose did not influence secondary IgM antibody formation. They demonstrated that IgM antibody formation occurred after a second antigenic exposure with both high or low doses of antigen.

The secondary response of humans to rubella and measles virus after subclinical reinfection, or inoculation with attenuated strains of these viruses, has been studied (110, 111). It has been demonstrated that the secondary response to these viruses consisted entirely of IgG antibody. Gonchoroff et al. in 1982 (112) studied the secondary response of mice to homologous and heterologous challenge with various strains of influenza A virus. After homologous challenge, an increase in IgG antibody titer to type and subtype specific antigens occurred but a secondary IgM antibody response to these antigens was not detected. Heterologous challenge resulted in an increase in IgG antibody but not IgM antibody to type specific antigens, whereas both IgM and IgG antibodies were formed against the newly encountered subtype antigens.

In general, the secondary response to viral antigens following reinfection, or immunization, consists of IgG antibody primarily (47). Secondary IgM antibody responses have not generally been observed (47). However, the secondary response of humans to herpesviruses, occurring as a result of the reactivation of latency, differs from this pattern of antibody formation. Specific IgM antibody formation occurs after reactivation of certain herpesvirus infections including varicella-zoster (47), cytomegalovirus (51), and herpes simplex virus (50). Less attention

has been focused on the IgG antibody response following herpesvirus reactivation (47). This is primarily due to the current interest in specific IgM antibody detection and its application to the diagnosis of human viral infections (47). Cradock-Watson et al. (47) reported an increase in specific IgG antibody titers following the reactivation of varicella-zoster infection. In contrast, others have reported that IgG antibody titers did not change following the reactivation of latency (113, 114).

The kinetics of IgG antibody formation following antigenic stimulation has mainly been studied at the level of the immunoglobulin class, not subclass (92). Studies involving the human IgG subclass response following antigenic stimulation have been hampered by a lack of reliable chromatographic methods for separating these immunoglobulin isotypes (92). The few studies which have examined the IgG subclass response following antigenic exposure suggest that distinct patterns of isotype formation occur within these subclasses (96, 115). Tokumara in 1967 (96), reported that guinea pigs inoculated with HSV developed antibody primarily of the IgG2 subclass, whereas IgG1 antibody became the predominant isotype following a second HSV inoculation. Leonard in 1970 (115) demonstrated that humans infected with varicella-zoster virus developed an IgG antibody response which was restricted to an electrophoretically slow IgG subclass. Following the reactivation of latent varicella-zoster infection, both fast and slow IgG subclass antibodies were formed. Leonard proposed that the antibody response following primary antigenic exposure was incomplete and that a second exposure was necessary to complete the response to this virus.

A recent study which examined the formation of antibodies against phospholipase A (PLA), a major component of honey bee venom, demonstrated a distinct pattern of isotype formation following exposure to this antigen (89). Persons with limited exposure to honey bees developed IgG1 subclass antibody to PLA, whereas persons having long term exposure (beekeepers) had primarily IgG4 antibody. Novice beekeepers developed a gradual shift in their antibody response to PLA from a predominant IgG1 antibody response to a predominant IgG4 antibody response. Prolonged immunization with this antigen led to an IgG4 restricted antibody response (89).

The Bovine Humoral Immune System

The nomenclature and physiochemical properties of bovine immunoglobulins are similar to that of other species. Four distinct classes of immunoglobulins have been recognized and characterized: IgG (116, 117), IgM (116, 118), IgA (116, 119), and IgE (120). The IgG class consists of two distinct subclasses, IgG1 and IgG2 (117, 121).

Bovine IgG1 and IgG2 are approximately 7s molecules with molecular weights of approximately 160,000 and 150,000 respectively (117, 122). Immunoglobulin G1 is the predominant immunoglobulin in serum, at a concentration of approximately 11.2 mg/ml (116). This IgG subclass may be differentiated from IgG2 by its faster migration toward the anode in an agar gel electrophoretic field and by the detection of specific antigenic determinants on the gamma heavy chains (116).

Bovine IgG2 makes up slightly less than half of the total serum IgG at a concentration of approximately 9.2 mg/ml (116). It migrates slower

than IgG1 in an electrophoretic field, has a greater net positive charge than IgG1, and may be differentiated antigenically from IgG1 (116). Bovine IgG2 gamma chains carry the A1/A2 allotypes which are not present on IgG1 gamma chains (123).

Mach et al. in 1969 (119) established the existence of an IgA immunoglobulin class in cattle. Bovine serum IgA is an 11s dimeric immunoglobulin of approximately 385,000 to 430,000 daltons and contains a secretary component (119). It is the principal immunoglobulin of the bovine secretary immune system (119), with the exception of the mammary gland (124). This immunoglobulin is present in low concentrations in bovine serum, 0.06 to 1.0 mg/ml (116).

Bovine IgM is a 19s protein with a molecular weight of approximately one million (118). Its concentration in adult serum is approximately 3 mg/ml, but various reports indicate a range of concentrations from 0.6 to 4.3 mg/ml (116).

A bovine immunoglobulin believed to be the homolog of human IgE was described first by Wells and Eyre in 1971 (120) and by Hammer et al. in 1971 (125). This antibody is antigenically distinct from IgG, IgM and IgA (120). Evidence confirming the isotype of this immunoglobulin was presented by Neilson (126) who showed that it could activate mast cells in a similar manner to human IgE.

In addition to the two well described bovine IgG subclasses, IgG1 and IgG2, the existence of an additional subclass has been suggested. In 1968 Kickhofen et al (117) described an IgG subclass which eluted from anion exchange columns between IgG1 and IgG2. They described these IgG

subclasses as gamma 1, gamma 2, and gamma G2, which they later revised to "IgG2a", "IgG2b", and IgG1, respectively (127). The quotation marks have been added here in accordance with the 1978 World Health Organization report dealing with the nomenclature of immunoglobulins of animals (116). The subclass Kickhofen et al. (127) described as "IgG2b" is the IgG subclass eluted from anion exchange columns between IgG1 and IgG2. Their "IgG2a" subclass is described by other investigators (116, 128, 129), and in this review, as IgG2. Convincing evidence that "IgG2b" is a distinct IgG subclass has not been presented (116). To obtain isotypic status an immunoglobulin must be shown to have a unique heavy chain amino acid sequence and unique heavy chain antigenic determinants (116).

Certain biological activities of bovine immunoglobulins have been well characterized (129, 130, 131, 132). The ability of bovine immunoglobulins to fix complement by the classical pathway has been demonstrated for both IgM and IgG antibodies (130). Murphy et al. (130) demonstrated that following experimental Anaplasma infection in cattle, complement fixing (CF) antibodies of the IgM class appeared by day 10, followed 4-5 days later by IgG CF antibodies. Bovine IgG1 and IgG2 fix complement by the classical pathway (129). However, IgG1 fixed guinea pig complement more efficiently than IgG2 (129), and high levels of IgG2 antibody inhibited complement fixation by IgG1 antibody (131).

The opsonic activities of bovine immunoglobulins have been examined (129). McGuire et al. (129) demonstrated that IgG2, but not IgG1, is cytophilic for freshly isolated neutrophils and monocytes. Freshly isolated neutrophils and monocytes phagocytosed IgG2 coated, but not

IgG1 coated, erythrocytes. However, when monocytes were cultured in vitro for 5 days they developed the ability to phagocytize erythrocytes coated by IgG1 as well (129). Freshly isolated monocytes lacked Fc receptors for bovine IgM (129).

The relative neutralizing efficiencies of bovine immunoglobulins were examined by Mukkur et al. (132). Neutralization kinetics indicated that bovine IgM antibody was much more efficient in neutralizing BHVI than was IgG antibody. This observation was in accord with an earlier report by Blank et al. (133) who demonstrated that polymeric antibodies, in general, were more effective in neutralizing virus despite their relatively lower affinity. The relative efficiency of the bovine IgG subclasses in neutralizing virus has not been examined.

The influence of complement on viral neutralization by bovine immunoglobulins was investigated by Potgieter in 1974 (52). Neutralization kinetics indicated that IgM antibody was dependent upon complement for the neutralization of BHVI whereas IgG antibody did not require, nor was it enhanced, by the addition of complement. Contrary to these findings, Rossi and Kiesel in 1976 (134) reported that IgG antibody titers, measured by serum virus neutralization, were enhanced by the addition of complement.

The antibody response, in terms of specific isotype formation, has not been extensively studied in the bovine. Previous studies primarily have examined antibody formation at the level of the class rather than the subclass.

Sanderson in 1968 (135) examined the antibody response of young calves following the subcutaneous inoculation of Murray Valley

encephalitis virus. The IgG and IgM antibody titers were determined by hemagglutination inhibition. Antibody activity was first detected in the IgM class at 7-9 days post inoculation. Antibody activity within the IgG class was detectable soon thereafter, 7-11 days post inoculation. Maximal IgG antibody titers occurred 10-20 days post inoculation, and by day 25 IgM antibody was no longer detectable. Following a second subcutaneious inoculation of this virus, IgG antibody was the predominant isotype formed but a minimal IgM antibody response occurred. Maximal IgG and IgM antibody responses were present by the sixth day following the second inoculation.

Rossi and Kiesel in 1976 (134) examined the neutralizing antibody response of six-month-old calves to BHVI administered intramuscularly. Their results were similar to those reported by Sanderson (135). At 9 days post inoculation maximal IgM antibody titers were detected, but IgG titers though detectable at this time, were minimal. At 32 days post inoculation IgM antibody titers had declined to low levels and IgG antibody became the predominant isotype. Following a second intramuscular inoculation on day 196, an anamnestic IgG antibody response and a minimal IgM antibody response were observed. Potgieter in 1974 (52) also reported the formation of IgM antibody following secondary BHVI inoculation by the intramuscular route.

Hammer et al. in 1968 (121) studied the bovine humoral immune response to protein antigens administered with and without adjuvant, by various routes. They measured antibody activity in terms of antigen binding capacity using a modification of the Farr technique (136).

Soluble egg albumin administered by the intravenous route, and precipitated egg albumin administered subcutaneously evoked similar antibody responses. Both IgG and IgM were detectable by day 7 post inoculation. The antigen binding capacity of IgG antibody exceeded that of IgM antibody at all sampling times. The IgM antibody activity decreased rapidly and was not detectable 2 weeks post inoculation. The IgG antibody titers reached peak levels between 5 and 6 weeks post inoculation. The kinetics of antibody formation during a secondary response were studied by inoculating cattle intramuscularly with egg albumin in Freund's adjuvant on days 1, 7, 14, 21 and 28. This was followed by a series of intramuscular inoculations of egg albumin without adjuvant at 31, 34 and 37 weeks after the start of the initial series of inoculations. Both IgM and IgG antibody were produced anamnestically as a result of the second series of inoculations.

Hammer et al. in 1970 (137) studied the IgG subclass antibody response of cattle which were hyperimmunized with either egg albumin or rabbit serum albumin in incomplete Freund's adjuvant. These antigens were inoculated by either the intramuscular or subcutaneous routes. Following the initial series of inoculations, antibody activity was present within the IgG1 subclass but virtually no activity was present in the IgG2 or "IgG2b" subclasses. After subsequent booster inoculations, antibody activity was detected in the IgG1 and IgG2 subclasses but little activity was present in the "IgG2b" fraction. They also reported a change in antigen association rates, a measure of antigen binding activity (138), of the IgG1 and IgG2 subclasses following repeated antigenic exposure.

There was a marked increase in the association rate of IgG2 antibody, whereas the association rate of IgG1 antibody declined following repeated antigenic exposure.

Reactivation of Latent BHVI Infection

In common with other herpesviruses (139), BHVI frequently induces latent infection in its host following natural exposure or vaccination with live virus vaccines (140). Stevens (140) has defined latency as a unique host-virus relationship in which initial infection is followed by the continual presence of the virus within host tissues, often for the lifetime of the host. The reactivation of latency may periodically occur following the establishment of latent infection and clinical signs of infection are seen again (141). Virus may be recovered only during these periods (141). In persistent viral infections, as opposed to latent infection, virus may be recovered continuously, often in the presence of specific antibody and in the absence of overt disease (141).

The sites of herpesvirus latency are not well defined (142). Human herpesviruses may remain latent in various neural ganglia including those of the heart (142). Narita et al. (143) demonstrated the presence of BHVI in the trigeminal ganglia of cattle following intranasal inoculation of the virus. The virus also has been isolated from the trigeminal ganglia of cattle at slaughter (144).

The mechanisms responsible for the reactivation of latent herpesvirus infections are poorly understood (145). In the bovine the reactivation of latent BHVI infection is believed to occur as a result of stresses such as disease, overcrowding, transport, estrus, and parturition (145).

Reactivation may be experimentally induced by the administration of corticosteroids, adrenocortical hormones, and methyl indole (146, 147, 148).

During reactivation the virus multiplies at the initial sites of infection and is excreted (147). Kahrs (140) suggested that virus shedding during reactivation may be responsible for new outbreaks of infection among susceptible cattle.

Humoral antibody to BHVI is believed to persist for the lifetime of the animal following primary infection (140). Persistence of specific antibody may be due to the periodic reactivation of latency with the subsequent reexposure of the humoral immune system to viral antigens (140). However, the nature of the antibody response occurring as a consequence of the reactivation of latent herpesvirus infection in the human or the bovine has received little attention.

McKercher et al. in 1958 (146) followed the antibody response to BHVI in a group of cattle following intranasal BHVI inoculation. Over a 14 month period they observed fluctuations in the antibody titers of certain of these animals. The reason for these fluctuations were unexplained. Snowden in 1965 (149) suggested that the changes in antibody titers observed by McKercher et al. (146) were due to the periodic reactivation of latent BHVI infection in these animals. In support of this idea Snowden (149) reported the reactivation of BHVI infection following parturition, and the subsequent rise in specific anti-BHVI antibody.

III. MATERIALS AND METHODS

Cells

Secondary bovine turbinate cells (BTU), passaged 60 to 80 times, were grown as monolayers in plastic cell culture vessels under a growth medium consisting of Dulbecco's modified Eagle Medium,[a] 0.185% $NaHCO_3$, 25mM HEPES buffer,[b] and 5% fetal calf serum (FCS).[c]

Viruses

The Jensal strain of Bovine herpesvirus I (BHVI) was obtained from Jensen-Salsbery Laboratories, Kansas City, Missouri. The Cooper strain of BHVI was obtained from P.C. Smith, Auburn University, Auburn, Alabama. The strain of BHVI herein referred to as BHVI [299] was isolated from an aborted fetus by L.N.D. Potgieter.

Virus Assay

Virus titers were determined by the plaque method. Assays were done on BTU cells grown to confluency in 12-well cell culture plates.[d] Virus dilutions were made in a maintenance medium (MM) consisting of Dulbecco's Modified Eagle Medium, 0.185% $NaHCO_3$, 25mM HEPES buffer, and 100 mg/ml gentamicin.[e] Serial 10-fold dilutions were made and 0.25 ml amounts of

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[a] K. C. Biological, Lenexa, KA.

[b] Calbiochem-Behring Corp, LaJolla, CA.

[c] Grand Island Biological Co, Grand Island, NY.

[d] Costar, Cambridge, MA.

[e] Schering Corp, Kenilworth, NJ.

each were applied to cell monolayers previously rinsed twice with MM. The inoculum was replaced with MM containing 2% specific anti-BHVI rabbit serum after a 1 h incubation at 37° C. Cells were incubated for 3 days in a humidified CO_2 incubator. The cells then were fixed for 5 minutes by the addition of 10% formalin to each well. After 5 minutes of fixation the formalin and medium were decanted from the cells, and the cells were stained by adding approximately 0.5 ml of a 0.25% solution of crystal violet in 70% ethanol to each well. The stain was rinsed from the wells with distilled water after 5 minutes and the plates were dried at room temperature. The plaques then were counted and virus titers were determined as plaque forming units (pfu) per ml.

Virus Isolation

The presence of BHVI in nasal secretions and in fetal tissues was determined by attempts at virus isolation in cell culture. Nasal swabs were placed in 2 ml MM containing 1% FCS and antibiotics [penicillin[f] (1000 units/ml), and amphotericin B[g] (5 mg/ml)]. Fetal tissue was prepared as a 10% suspension in MM containing antibiotics, homogenized, and clarified by low speed centrifugation. These fluids then were inoculated onto BTU cells previously rinsed twice with MM. Cells were incubated at 37°C in a humidified CO_2 incubator. The identity of BHVI in infected cells was verified by the indirect fluorescent antibody test (IFAT).

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[f]E.R. Squibb, Princeton, NJ.

[g]Fungizone, Flow Laboratories, McLean, VA.

Virus Propagation

Confluent monolayers of BTU cells were grown in 850 cm^2 plastic roller bottles,[h] using a roller bottle apparatus,[i] and a speed of 0.5 revolutions per minute. The approximate number of BTU cells per roller bottle was determined using a cell counter.[j] Cells were infected at a multiplicity of infection of approximately 1 pfu per cell. After a 1 h absorption at 37°C the inoculum was decanted and 50 ml of MM was added to each roller bottle. Incubation was continued for 24 to 36 h.

Viral Antisera

Virus (BHVI, Cooper strain) used for immunization of rabbits was grown in 3 roller bottles and partially purified by differential centrifugation. The virus was harvested from infected cells, and cell supernatant fluids, following a series of 3 freeze-thaw cycles to rupture the infected cells. The virus preparation was clarified by centrifugation for 30 minutes at 2000 xg at 4°C, followed by centrifugation of the supernate at 50,000 xg for 60 minutes. The pellet obtained was resuspended in phosphate buffered saline (PBS). Two additional cycles of low and high speed centrifugation were performed. The final pellet constituted the partially purified virus and was resuspended in 3 ml of PBS and stored at -70°C until used.

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[h]Corning Glass Works, Corning, NY.

[i]Bellco Glass, Inc., Vineland, NY.

[j]Coulter Counter, Coulter Electronics, Hialeah, Fl.

A New Zealand white rabbit was inoculated simultaneously at an intravenous site and a subcutaneous site; one-half ml was inoculated at each site. Three and six weeks later these inoculations were repeated.

The rabbit was bled by cardiac puncture 10 days after the last inoculations. The serum was stored at -70°C.

Gel Filtration Chromatography

Bovine IgM and IgG were prepared by filtering bovine sera through a molecular exclusion medium.[k] The medium was equilibrated in a 0.1M, pH 7.8 Tris-HCl buffer containing 1M NaCl and 0.2% NaN_3, degassed under vacuum, and then packed in a 2.5 x 90 cm glass chromatography tube with an adjustable plunger.[l] Four ml serum samples were run into the lower end of the column and buffer was run upwards through the column at a rate of approximately 12 ml per h using a peristaltic pump.[m] The optical density (OD) of the eluate at 280nm was determined using an absorbance monitor[n] and fractions of approximately 4 ml were collected using a fraction collector.[o] The contents of the tubes corresponding to the absorbance peaks registered on the chart recorder[p] were pooled.

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[k]Ultrogel AcA 34, LKB Instruments, Inc, Durham, NC.

[l]Glencoe Scientific, Inc, Houston, TX.

[m]Varioperpex II, LKB Instruments, Inc, Durham, NC.

[n]Model UA-5, Instrument Specialties Co, Inc, Lincoln, NE.

[o]Model 1200, Instrument Specialties Co, Inc, Lincoln, NE.

[p]Model 613, Instrument Specialties Co, Inc, Lincoln, NE.

Four ml of 1% bovine serum albumin (BSA)[q] was added to each IgM preparation to prevent aggregation of IgM proteins. The pooled fractions were concentrated to 4 ml by ultra-filtration,[r] using a filter with a 100,000 molecular exclusion rating,[s] at a pressure of 15 pounds per square inch. The immunoglobulin preparations then were dialyzed for 3 days against 3 changes of Hank's balanced salt solution (HBSS) at 4°C. Immunoglobulin fractions were stored at -70°C until used.

Ion Exchange Chromatography

Bovine IgG subclasses were separated by ion exchange chromatography using a modification of a previously described procedure (150). Diethylaminoethyl (DEAE) Sephadex[t] was equilibrated with starting buffer consisting of 0.1M Tris-HCl pH 8.3 with 0.05 M NaCl, degassed under vacuum, and poured into a 2.5 x 40 cm glass chromatography tube.[u] The column was rinsed once with starting buffer.

Two ml of IgG-rich preparation, obtained by gel filtration and dialyzed 3 days against 3 changes of starting buffer at 4°C, was placed on top of the column. The IgG subclasses were eluted differentially from the column using 0.1 M Tris-HCl pH 8.3 with stepwise increases in

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[q]Difco Laboratories, Detroit, MI.

[r]Model 613, Instrument Specialties Co, Inc, Lincoln, NE.

[s]Diaflo Ultrafiltration Membranes, XM100A, Amicon Corp, Lexington, MA.

[t]DEAE-Sephadex A-50, Pharmacia Fine Chemicals, Piscataway, NJ.

[u]Glencoe Scientific, Inc, Houston, TX.

NaCl concentrations (0.1M, 0.2M, and 0.3M). Absorbance peaks were determined, and fractions corresponding to the peaks collected. The pooled protein fractions were concentrated to 2 ml by ultrafiltration. The immunoglobulin preparations were then dialyzed for 3 days against 3 changes of HBSS at 4°C. Immunoglobulin preparations were stored at -70°C until used.

Immunoelectrophoresis

Bovine immunoglobulin preparations were tested by immunoelectrophoresis. Clean 7 x 7 cm glass slides were placed on a level surface and 6 ml of 1% agarose[V] in 0.05 M, pH 8.2 barbital buffer (140) was carefully layered onto each slide. An immunoelectrophoresis pattern consisting of 2.5 mm diameter wells and 1.0 x 40 mm antiserum channels were cut into the agarose-coated slides. The agar was removed from the 2.5 mm diameter wells and 5 ul samples were placed in the wells. The slides were electrophoresed for approximately 1.5 h using a current of 35 amps and a potential of 100 volts. The agar was then removed from the antiserum channels and filled with rabbit antisera specific for bovine IgG1, IgG2 or IgM.[W] The slides were incubated at room temperature in a humid chamber for 18h and then examined.

[V]Sigma Chemical Co, St. Louis, MO.

[W]Miles Laboratories, Inc, Elkhart, IN.

Radial Immunodiffusion

Immunoglobulin concentrations were measured by single radial immunodiffusion using commercially prepared plates.[x] The measurements were performed according to the manufacturer's instructions.

Indirect Fluorescent Antibody Test

The indirect fluorescent antibody test (IFAT) was performed as previously described (151).

Plaque Reduction Neutralization Assay

Antibody titers to BHVI were determined by a virus plaque reduction neutralization assay (PRA) using a 50% plaque reduction end point. Serial two-fold dilutions of the immunoglobulin preparations were made in MM containing 2% FCS. One-half ml of MM containing 2% FCS, 400 pfu BHVI [Jensal], and 6% fresh guinea pig serum (GPS) was added to an equal volume of each of the diluted immunoglobulin fractions. These mixtures were agitated well and incubated at 37°C for 30 minutes. The growth medium was decanted from monolayers of BTU cells in 12-well tissue culture plates. The monolayers were rinsed once with MM, and 0.25 ml of the above mixtures were placed on the monolayers. Following a 1 h incubation at 37°C in a humidified CO_2 incubator, the inoculum was replaced with 1 ml of MM containing 2% specific anti-BHVI rabbit serum. The cells then were fixed and stained as described above after 3 days of incubation at 37°C in a humidified CO_2 incubator. The reciprocal of

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[x]Veterinary Medical Research, Pullman, WA.

the antiserum dilution, expressed as log 2, which resulted in a 50% reduction of the plaque count was considered to be the antibody titer.

Reagents added to immunoglobulin preparations during purification (BSA), and those used in the assay of specific anti-BHVI antibody activity by PRA (FCS, GPS), were tested at a dilution of 1:2 for the presence of anti-BHVI activity.

Experimental Animals

Group I animals consisted of six, nonpregnant Holstein-Friesan heifers. These heifers did not have antibody to BHVI, determined by PRA and IFAT, and were inoculated intranasally on day 0 and day 90 of the experiment with BHVI [299]. A total virus dose of approximately 1×10^8 pfu per heifer was administered by squirting 4 ml of inoculum into each nostril.

Group II animals consisted of six Holstein-Friesan heifers in the second trimester of pregnancy and free of antibody to BHVI. These heifers were inoculated intranasally on day 0 of the experiment with approximately 1×10^8 pfu of BHVI [299] per heifer, administered as described above. Those heifers which did not abort by day 77 were inoculated intraamniotically with approximately 1×10^7 pfu of BHVI [299] as previously described (152).

Group III animals consisted of six, nonpregnant Holstein-Friesan heifers. They had antibody to BHVI and therefore were presumed to be latently infected with this virus. These heifers were given corticosteroids, 30 mg dexamethasone[y] intraveneously for 3 consecutive days,

[y]Azium, Schering Corp, Kenilworth, NJ.

to reactivate latent infection. Reactivation of latency was assessed by determining the presence of BHVI in nasal secretions following dexamethasone treatment.

Serum was collected at weekly intervals and stored at -70°C until used.

Enhancement of Neutralizing Antibody by Complement

The enhancement of virus neutralizing activity of IgM and IgG antibody by complement was determined by assaying their neutralizing effect with varying concentrations of complement.

An IgM and an IgG preparation, obtained from an animal in Group I on day 14 and day 35 respectively, were chromatographed twice by gel filtration to ensure the purity of these preparations. Antibody titers in these preparations were determined by PRA using excess (10%) fresh GPS. The dilution of these preparations used in this experiment was the highest dilution at which these preparations resulted in 100% plaque reduction in the presence of excess complement. The IgM preparation was diluted 1:100 and the IgG preparation 1:40 for the experiment.

Serial two-fold dilutions of fresh GPS, heat inactivated GPS, fresh bovine serum, and heat inactivated bovine serum were prepared in duplicate in MM. Sera were heat inactivated at 56°C for 30 minutes. One-fourth ml of the dilutions of each serum was added to one-fourth ml of the IgM preparation diluted 1:25 in MM (final dilution 1:100). One-fourth ml of the duplicate dilutions of each werum was added to one-fourth ml of the IgG preparation diluted 1:10 in MM (final dilution 1:40). These mixtures were then added to one-half ml of MM containing

4% FCS and 400 pfu of BHVI [Jensal], mixed well, and incubated at 37°C for 30 minutes. The mixtures were assayed for residual virus by placing 0.25 ml of each mixture onto BTU cell monolayers in 12-well tissue culture plates as described before.

The results of each assay were expressed as the percent of plaque reduction compared to controls in which antibody and complement were absent.

IV. RESULTS

Purification of Antibody Classes

The 280nm optical density (OD) profile of bovine serum proteins eluted from a gel filtration column is depicted in Fig. 1. Figure 1 also illustrates that bovine IgG and IgM are clearly separated by gel filtration. Bovine IgM, with a molecular weight (MW) of approximately one million (118), was eluted from the column in the ascending limb of the first 280nm-OD peak. Bovine IgG, with a MW of 150,000 - 160,000 (117), was eluted from the column within the second 280nm-OD peak.

The efficacy of this chromatographic method for separating bovine IgM and IgG was determined by assessing the presence of these immunoglobulin isotypes in the eluted fractions by radial immunodiffusion assays (Fig. 1) and immunoelectrophoresis. The first and second 280nm-OD peaks contained IgM and IgG, respectively. The IgM-rich fractions were not contaminated by IgG and vice versa. Bovine serum IgA, with a MW of approximately 420,000 (119), was eluted from the column just prior to the elution of IgG in the second 280nm-OD peak. The protein eluted in the third 280nm-OD peak was primarily serum albumin. Following these preliminary determinations, IgM and IgG preparations were routinely obtained by pooling the eluted fractions of the respective 280nm-OD peaks.

Purification of IgG Subclasses

Bovine IgG preparations obtained by gel filtration, were dialyzed against "starting" buffer and placed on an anion exchange column (DEAE-Sephadex). The bovine IgG subclasses were separated by eluting

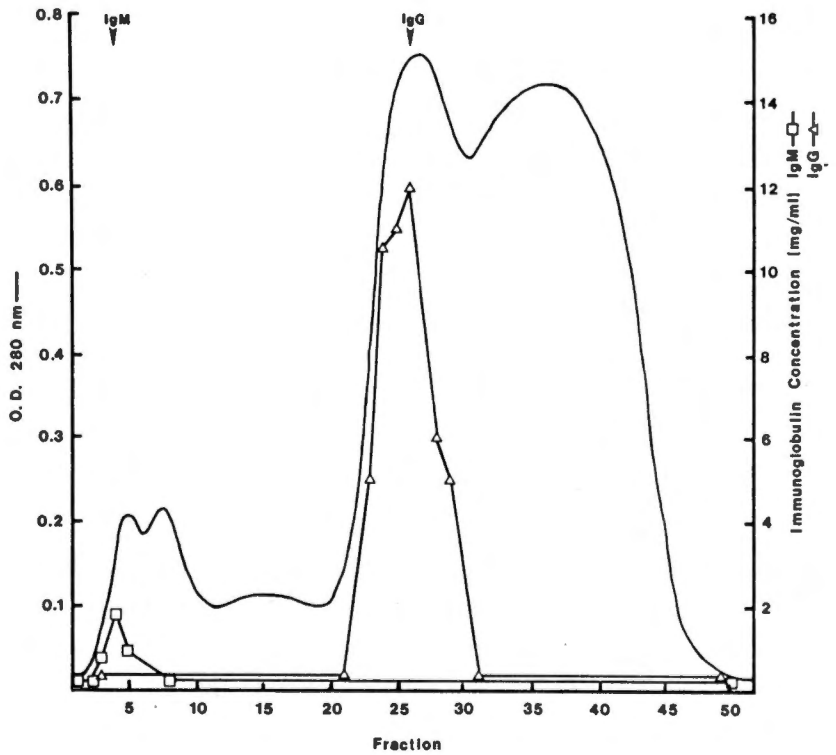

Fig. 1. Preparation of bovine IgM and IgG by gel filtration chromatography as described in the Materials and Methods. The optical density (OD) of the eluate at 280nm was determined using an absorbance monitor. The distribution of IgM and IgG within the eluted fractions was determined by radial immunodiffusion.

them stepwise from the column with buffers of increasing NaCl concentrations (Fig. 2). The 280nm-OD profile of bovine IgG subclasses separated by anion exchange chromatography is depicted in Fig. 2.

The separation of proteins by this chromatographic method is based on the differential charge characteristics of the proteins being separated. Bovine IgG2 has a mean isoelectric pH (pI) of approximately 7.7 (116). The pH and ionic strength of the "starting" buffer allowed IgG2 to be eluted in the "fall-through" eluate. Bovine IgG1 with a pI of approximately 6.5 (116), was eluted from the column by increasing the NaCl concentration of the elution buffer to 0.2M (Fig. 2).

The efficacy of this chromatographic method for separating bovine IgG subclasses was determined by assessing the presence of these immunoglobulin isotypes in the eluted fractions by radial immunodiffusion (Fig. 2) and immunoelectrophoresis. The bovine IgG subclasses were eluted from the column in four distinct 280nm-OD peaks. Bovine IgG2, in agreement with the findings of others (128,137), was eluted in the first 280nm OD peak ("fall-through" peak). Bovine IgG1 was eluted from the column in the third 280nm OD peak. A small amount of protein, designated "IgG2b", was eluted in a peak just prior to IgG1 (Fig. 2). This is in agreement with the findings of Kickhofen et al (117) who described this protein as a distinct IgG subclass which eluted between IgG1 and IgG2 in anion exchange chromatography. Elution of "IgG2b" overlapped slightly with the elution of IgG1. A small amount of IgG1 was eluted in the fourth 280nm-OD peak. Following these preliminary determinations, bovine IgG subclasses were obtained by pooling the eluted fractions of the respective 280nm-OD peaks.

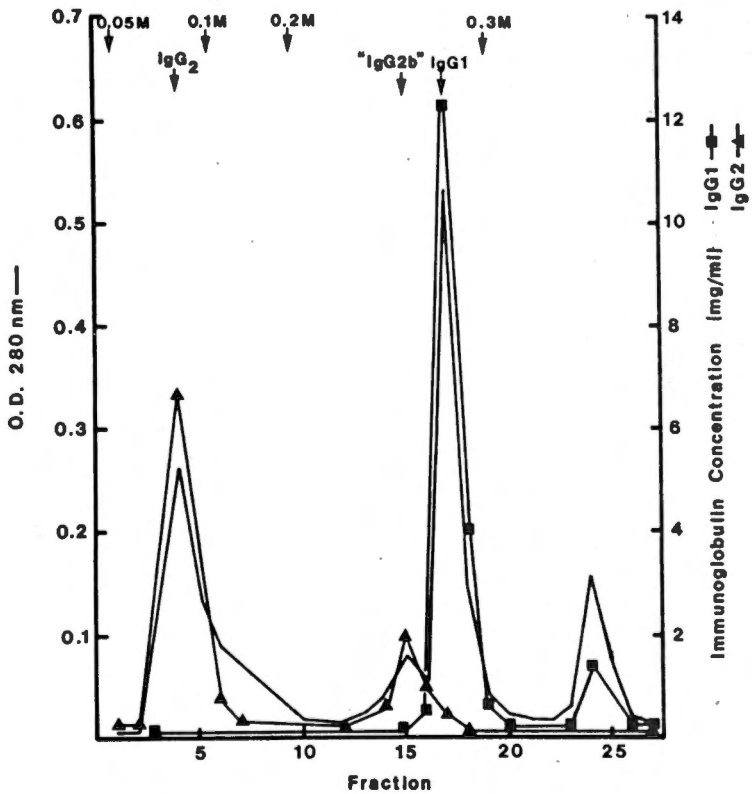

Fig. 2. Preparation of bovine IgG subclasses by anion exchange chromatography as described in the Materials and Methods.

Contaminating subclass proteins were not found in the IgG2 and "IgG2b" preparations by radial immunodiffusion (Fig. 2) and immunoelectrophoresis. However, the IgG1 preparations contained small concentrations of IgG2 ("IgG2b").

Enhancement of Neutralizing Antibody by Complement

The influence of complement in the neutralization of BHVI by bovine immunoglobulins was examined (Figs. 3 and 4). The enhancement of IgM and IgG neutralizing antibody activity by complement, was determined by assaying their virus neutralizing effect with varying concentrations of complement. The quality of fresh bovine serum (BS), and fresh guinea pig serum (GPS), as sources of complement, was also determined.

The effect of varying concentrations of complement on the neutralization of BHVI by IgG antibody is shown in Fig. 3. The addition of fresh GPS to the IgG preparation at concentrations of 0.6% and greater enhanced virus neutralization. Maximal enhancement of virus neutralization occurred in the presence of 5% fresh GPS. At this concentration, 98% reduction of virus plaques occurred whereas 84% reduction occurred in the presence of 5% heat-inactivated GPS. Virus neutralization by IgG antibody was enhanced by both fresh and heat-inactivated BS at all concentrations tested (Fig. 3). However, enhancement of virus neutralization attributable to the presence of complement, did not occur at concentrations of fresh BS less than 5% because below this concentration fresh and heat-inactivated BS had a similar effect. At a 5% concentration, fresh BS reduced virus plaques by 92% whereas heat-inactivated BS resulted in 86% reduction of plaques.

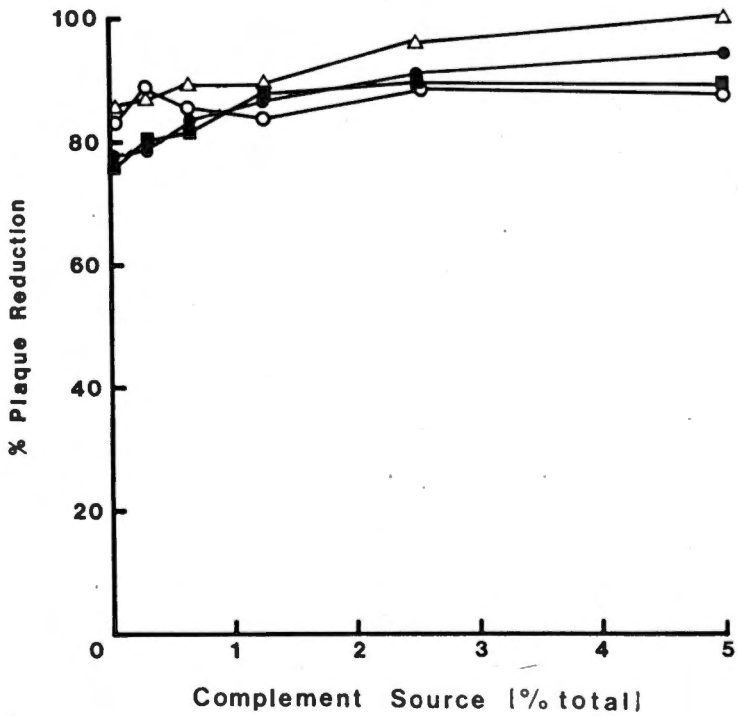

Fig. 3. Enhancement of neutralizing IgG antibody activity by complement. Constant amounts of bovine anti-BHVI IgG antibody were mixed with varying concentrations of fresh guinea pig serum (△), heat inactivated guinea pig serum (○), fresh bovine serum (●), and heat inactivated bovine serum (■). The effect of complement, from these sources, on the neutralizing antibody activity of bovine IgG was determined as described in the Materials and Methods.

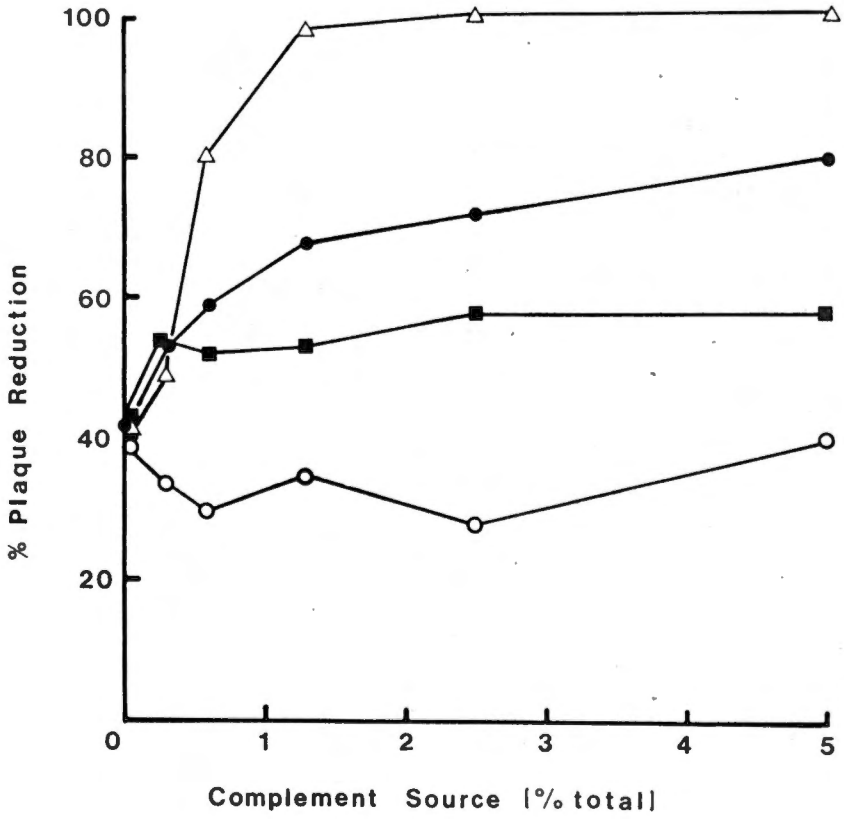

Fig. 4. Enhancement of neutralizing IgM antibody activity by complement. Constant amounts of bovine anti-BHVI IgM antibody were mixed with varying concentrations of fresh guinea pig serum (△), heat inactivated guinea pig serum (○), fresh bovine serum (●), and heat inactivated bovine serum (■). The effect of complement from these sources, on the neutralizing antibody activity of bovine IgM antibody was determined as described in the Materials and Methods.

The effect of varying concentrations of complement on the neutralization of BHVI by IgM antibody is depicted in Fig. 4. Virus neutralization of BHVI by IgM antibody was enhanced by fresh GPS and fresh BS. Enhancement of IgM virus neutralization was detectable even in the presence of less than 1% fresh GPS, but maximal enhancement occurred at concentrations between 1% and 2%. Fresh GPS at a concentration of 1.3% in the IgM preparation resulted in 98% reduction of virus plaques. Heat-inactivated GPS did not enhance virus plaque reduction. Enhancement of virus neutralizing IgM antibody activity with fresh BS, at concentrations less than 1%, was detectable also. However, fresh GPS enhanced virus neutralization by IgM to a greater degree than fresh BS. Maximal enhancement of virus neutralization by IgM and fresh BS occurred at a concentration of 5%. At the latter concentration of fresh BS in the IgM preparation, 80% reduction of virus plaques occurred, whereas 58% reduction occurred when the fresh BS was substituted by an equal concentration of heat-inactivated BS.

Enhancement of IgM virus neutralization occurred in the presence of low concentrations (0.3%) of fresh and heat-inactivated BS. At this concentration, fresh and heat-inactivated BS in the IgM preparation caused 54% and 53% virus plaque reduction respectively; whereas 41% reduction occurred in the absence of BS (Fig. 4). This apparent enhancement of IgM antibody activity by low concentrations of both fresh and heat-inactivated BS remains unexplained.

Primary Antibody Response to BHVI in Nonpregnant Heifers

The kinetics of IgM and IgG antibody formation following primary BHVI infection in nonpregnant, seronegative heifers (Group I) is shown in Fig. 5. Following intranasal inoculation of BHVI, both IgM and IgG antibodies were detectable, but at low titer, 7 days post inoculation (pi). Both IgG and IgM antibody titers increased rapidly between the seventh and fourteenth day pi. Antibody of the IgG class increased from a 1.3 log 2 mean titer (LMT) to 8.3 LMT from the seventh to the fourteenth day pi. Antibody of the IgM class increased from 1.3 LMT to 12.2 LMT over the same period. Maximal IgM antibody titers were observed 14 days pi, after which IgM titers declined rapidly and by 90 days pi relatively low levels of IgM antibody (2.7 LMT) remained.

Maximal IgG antibody titers occurred by the thirty-fifth day pi (9.2 LMT), after which IgG antibody titers slowly declined and by 65 days pi the IgG antibody titer (8.2 LMT) was approximately equal to that measured on the fourteenth day pi (8.3 LMT). By 90 days pi IgG antibody titers had declined to 7.2 LMT.

The distribution of neutralizing antibody activity among serum IgG subclasses following primary BHVI infection in Group I heifers is given in Table 1. At 14 days pi of the primary immune response, when IgG antibody titers were approaching maximal levels (Fig. 5), the IgG antibody response was restricted to the IgG1 subclass. At this time, antibody activity within the IgG1 subclass was 6.3 LMT, whereas the antibody activity within the IgG2 and "IgG2b" subclasses was not detectable.

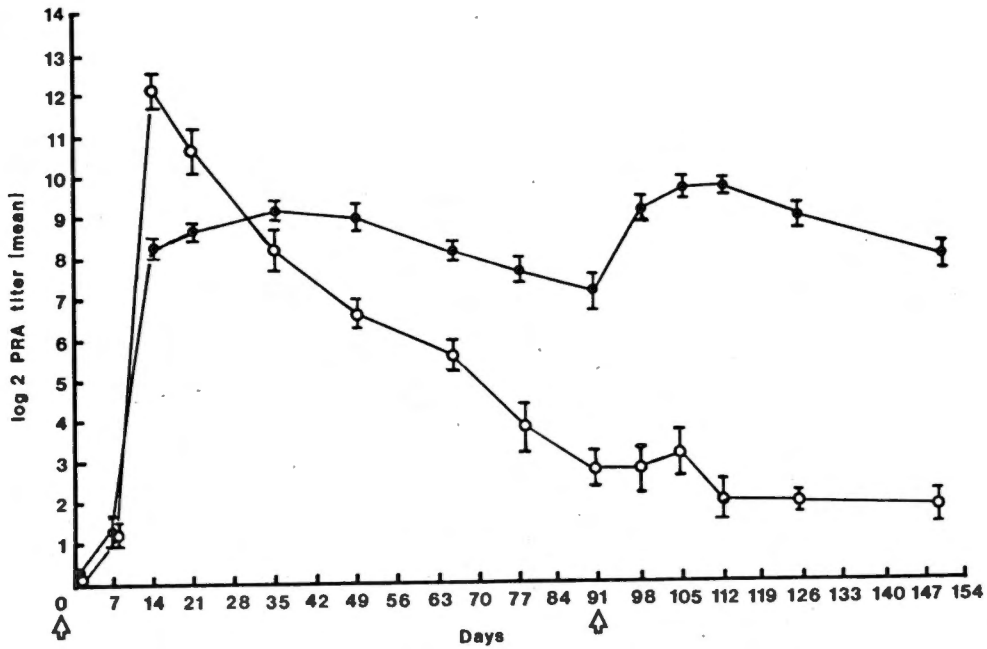

Fig. 5. Kinetics of the IgM (○) and IgG (●) neutralizing antibody responses following primary and secondary (exogenous) BHVI exposure. Heifers (n = 6) were inoculated with BHVI (299) by the intranasal route on day 0 (⇧) and day 90 (⇧). Antibody activity was determined by a plaque reduction neutralization assay (PRA), using a 50% endpoint, and expressed as log 2 mean titers ± the standard error of the mean (SEM).

TABLE 1

DISTRIBUTION OF SPECIFIC ANTIBODY ACTIVITY AMONG SERUM IgG SUBCLASSES FOLLOWING PRIMARY AND SECONDARY BHVI INFECTION IN NONPREGNANT HEIFERS (GROUP I)

	Log 2 Mean Titer ± SEM (n=6)		
DAY[A]	IgG1	IgG2	"IgG2b"
PRIMARY RESPONSE:			
14	6.3 ± 0.29	< 2	< 2
35	8.0 ± 0.39	4.3 ± 0.32	2.0 ± 0.48
SECONDARY RESPONSE:			
105 (14[B])	7.7 ± 0.45	7.0 ± 0.48	4.0 ± 0.58
125 (34)	7.0 ± 0.32	7.3 ± 0.39	ND[C]

[A]Days after initial BHVI intranasal inoculation.

[B]Days after second BHVI intranasal inoculation on day 91.

[C]Not determined.

Secondary Antibody Response in Nonpregnant Heifers

Immunoglobulin G antibody to BHVI increased markedly in heifers inoculated intranasally a second time, 91 days after the first intranasal BHVI inoculation (Fig. 5). Antibody activity within the IgG class increased rapidly during the first 7 days following secondary exposure from 7.2 LMT at 91 days pi, to 9.2 LMT at 98 days pi. Maximal IgG antibody titers (9.7 LMT) were present approximately 14 days following reexposure (105 days pi), and remained at this titer until the twenty-first day (112 days pi).

A slight increase in IgM antibody titer occurred as a result of reexposure by the intranasal route. Antibody activity within the IgM class increased from 2.7 LMT at the time of secondary BHVI exposure (91 days pi), to 3.2 LMT, 14 days after secondary exposure (105 days pi).

Antibody activity was detected in all three IgG subclasses after secondary intranasal BHVI exposure but the increase in antibody activity occurred primarily in the IgG2 subclass (Table 1). Antibody activity within the IgG2 subclass 14 days after secondary exposure (105 days pi) was 7.0 LMT which was approximately equal to the antibody activity present in the IgG1 subclass (7.7 LMT). Antibody activity within the IgG1 subclass 34 days after secondary exposure was less than that determined at 35 days of the primary response.

Primary Antibody Response in Pregnant Heifers

The kinetics of antibody formation following the intranasal inoculation of BHVI in pregnant heifers, seronegative to BHVI (Group II),

is shown in Figs. 6 and 7. Only one animal in this group (heifer #104) aborted as a result of intranasal BHVI inoculation. The antibody response in this animal is shown in Fig. 6. Both IgG and IgM antibody to BHVI were detectable, but at low titer, in heifer #104 at 7 days pi. Between 7 days pi and 14 days pi, a rapid increase in IgM and IgG antibody activity occurred. Antibody activity within the IgG class increased from a titer of 2 log 2 to 11 log 2 during this period. Likewise, the IgM antibody titer increased from 2 log 2 to 12 log 2 during this period. Maximal IgG and IgM antibody was measured at 14 days pi, after which antibody activity in both of these immunoglobulin classes declined. Antibody activity within the IgM class decreased rapidly after the fourteenth day pi and was at low titer (2 log 2) at 70 days pi.

The distribution of IgG antibody activity following primary BHVI infection in heifer #104 is shown in Table 2. At 14 days pi the IgG response was restricted to the IgG1 subclass. Antibody activity within the IgG1 subclass was 8 log 2, while antibody activity within the IgG2 subclass was not detected (<2 log 2).

The remainder of Group II heifers aborted after intraamniotic (IA) inoculation of BHVI 77 days after intranasal exposure. The antibody response in these heifers is given in Fig. 7. Both IgG and IgM antibody to BHVI were detectable by 7 days after intranasal inoculation. The antibody activity within both of these immunoglobulin classes increased rapidly between 7 and 14 days pi. Antibody activity within the IgG class increased from 4 LMT at 7 days pi, to 10 LMT at 14 days pi. Similarly, IgM antibody activity increased from 2.8 LMT to 8.6 LMT during this

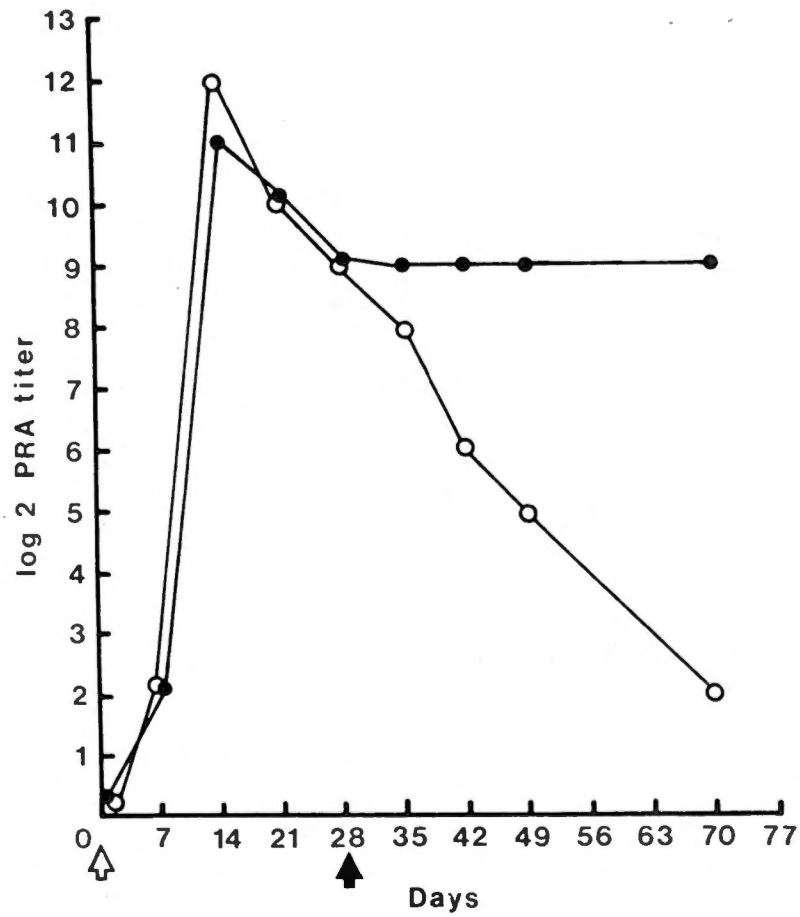

Fig. 6. Kinetics of the IgM (○) and IgG (●) neutralizing antibody responses following primary BHVI exposure and abortion in heifer #104, Group II. This heifer was inoculated with BHVI (299) on day 0 (⇧), and abortion occurred on day 28 (▲). The antibody was determined by PRA and expresses as log 2 titer.

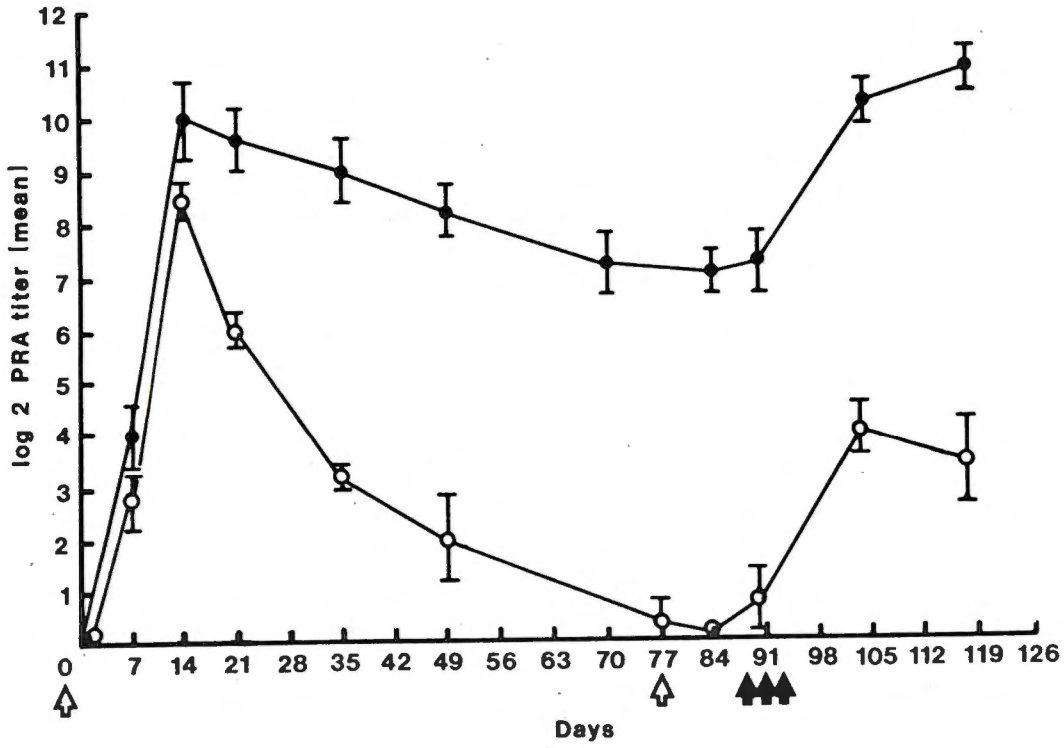

Fig. 7. Kinetics of the IgM (○) and IgG (●) neutralizing antibody responses following primary BHVI infection and abortion. Heifers (n = 5) were inoculated with BHVI (299) by the intranasal route on day 0 (⇧) and by the intraamniotic route on day 77 (⇧). Abortions (▲) occurred on days 89, 90, and 92. The antibody activity was determined by PRA and expressed as log 2 mean titer ± SEM.

TABLE 2

DISTRIBUTION OF SPECIFIC ANTIBODY ACTIVITY
AMONG SERUM IgG SUBCLASSES FOLLOWING
PRIMARY BHVI INFECTION IN
HEIFER #104 (GROUP II)

	Log 2 Titer		
DAY[A]	IgG1	IgG2	"IgG2b"
14	8	< 2	NB[B]
21	8	3	ND

[A]Days after initial BHVI intranasal inoculation.

[B]Not determined.

period. Maximal antibody activity in both immunoglobulin classes occurred at 14 days pi. Antibody activity within the IgM class declined rapidly after 14 days pi, whereas IgG antibody activity declined at a relatively slow rate. Immunoglobulin M antibody activity declined from 8.6 LMT at 14 days pi, to 2.0 LMT at 49 days pi, whereas IgG antibody titers declined from 10.0 LMT to 8.2 LMT during this period.

The IgG subclass antibody response following primary BHVI infection in pregnant heifers (Group II) is shown in Table 3. During the primary immune response IgG antibody activity, determined at 14 days pi, was restricted to the IgG1 subclass. At this time, antibody activity within the IgG1 subclass was 9.4 LMT, while antibody activity within the IgG2 and "IgG2b" subclasses was not detectable (<2 LMT).

Secondary Antibody Response to BHVI in Pregnant Heifers

Abortion occurred in heifer #104 at 28 days pi. A secondary increase in IgG and IgM antibody activity was not observed as a result of abortion, but the decline in IgG antibody occurring from the fourteenth to the twenty-eighth day pi ended at the time of abortion, and IgG antibody titers remained at constant levels until the seventieth day pi, the last day in which antibody titers were determined (Fig. 6). Abortion, occurring at 28 days pi, did not appear to influence the decline in IgM antibody titers.

Abortion in heifer #104, 28 days pi, did not result in a secondary increase in antibody activity within the IgG subclasses (Table 4). Antibody activity within the IgG1 subclass declined between 28 and 42 days pi. However, no change in IgG2 subclass titers occurred during this period.

TABLE 3

DISTRIBUTION OF SPECIFIC ANTIBODY ACTIVITY AMONG SERUM IgG SUBCLASSES FOLLOWING PRIMARY BHVI INFECTION IN PREGNANT HEIFERS (GROUP II)

	Log 2 Mean Titer ± SEM (n=5		
DAY[A]	IgG1	IgG2	"IgG2b"
14	9.4 ± 0.33	< 2	< 2

[A]Days after BHVI intranasal inoculation.

TABLE 4

DISTRIBUTION OF SPECIFIC PRA ANTIBODY ACTIVITY AMONG SERUM IgG SUBCLASSES FOLLOWING ABORTION IN HEIFER #104 (GROUP II)

	Log 2 Titer		
DAY [A]	IgG1	IgG2	"IgG2b"
28	9	5	ND[B]
42	7	5	ND

[A]Days after BHVI intranasal inoculation (pi). Abortion occurred 28 days pi. BHVI was recovered from fetál tissues.

[B]Not determined.

The IA inoculation of BHVI, 77 days pi, resulted in abortions 12-15 days later. Appreciable changes in IgM and IgG antibody titers did not occur between the time of IA inoculation (77 days pi) and 90 days pi, approximately the time at which abortions occurred (Fig. 7). Antibody activity within both immunoglobulin classes increased rapidly following abortion. Immunoglobulin G antibody activity increased from 7.4 LMT at 90 days pi to 10.4 LMT, 14 days following abortion (104 days pi). Similarly, IgM antibody activity increased from <2 LMT to 4.0 LMT during this period. Maximal IgM antibody titers occurred 14 days after abortion (4.0 LMT), after which antibody activity within this immunoglobulin class declined. Antibody activity within the IgM class 28 days after abortion (118 days pi), was 3.4 LMT. The last measurement of IgG antibody activity was done at 28 days after abortion (118 days pi) at which time the IgG levels appeared to have peaked (Fig. 7).

The distribution of IgG subclass antibody activity following BHVI IA-induced abortion is given in Table 5. Following abortion IgG antibody activity was determined to be distributed among the three IgG subclasses. Antibody activity within the IgG1, IgG2, and "IgG2b" subclasses 14 days after abortion (104 days pi) was 9.0, 6.2 and 2.0 LMT, respectively. An increase in IgG subclass antibody activity occurred after abortion, primarily in the IgG2 subclass. Antibody activity within the IgG1 subclass 14 days after abortion (9.0) was lower than that determined at 14 days of the primary response (9.4 LMT) (Tables 3 and 5).

Heifers #163 and #172, and heifers #164 and #184, expelled dead fetuses 12 and 13 days, respectively, after IA inoculation. Heifer #123

TABLE 5

DISTRIBUTION OF SPECIFIC ANTIBODY ACTIVITY AMONG SERUM IgG SUBCLASSES FOLLOWING ABORTION INDUCED BY INTRAAMNIOTIC BHVI INOCULATION

	Log 2 Mean Titer ± SEM (n=5)		
DAY[A]	IgG1	IgG2	"IgG2b"
104 (14[B])	9.0 ± 0.38	6.2 ± 0.41	2.0 ± 0.69

[A]Days after BHVI intranasal inoculation.

[B]Days following abortion induced by the intraamniotic inoculation of approximately 1 x 10^7 pfu BHVI using a previously described procedure (137). Intraamniotic inoculation was done on day 77, abortions occurred 12-15 days later.

was the last heifer to abort, expelling a dead fetus on the fifteenth day after IA inoculation of BHVI. Bovine herpesvirus I was isolated from most of the fetal tissues examined and from maternal uterine exudate (Table 6). Virus was not recovered from the fetus aborted by heifer #123. Despite the inability to confirm BHVI-induced abortion in this heifer by virus isolation, the antibody response by this animal was similar to that of the other heifers after abortion induced by IA virus inoculation. Antibody activity within the IgG class in serum from heifer #123 increased from 6 log 2, at the time abortion occurred (90 days pi), to 9 log 2, 14 days later. Similarly, IgM antibody activity increased from <2 log 2 to 5 log 2 during this period. The failure to isolate BHVI from the fetal tissues of this heifer was attributed to the longer delay between fetal death and expulsion than that which occurred in the other heifers. Herpesviruses are heat labile and sensitive to inactivation by certain proteolytic enzymes (8), conditions which would be present in utero prior to the expulsion of the dead fetus.

Antibody Response Following the Reactivation of Latency

Antibody formation as a result of the reactivation of latent BHVI infection was determined. Heifers with antibody to BHVI, and therefore presumed to be latently infected, were given a series of corticosteroid treatments to induce reactivation. The IgG and IgM antibody response to BHVI by these heifers (Group III) following reactivation of latent BHVI infection is shown in Fig. 8. Antibody activity within the IgM class at the start of the experiment (day 0) was <2 LMT, whereas the IgG antibody

TABLE 6

RECOVERY OF BHVI FROM FETAL TISSUE AND MATERNAL UTERINE EXUDATE FOLLOWING ABORTION

HEIFER[A]	PLACENTA	LUNG	LIVER	SPLEEN	UTERINE EXUDATE
104	+	+	NA[B]	NA	+
123	NA	-	-	-	-
163	+	-	-	+	-
164	+	+	+	+	+
172	+	+	+	+	+
184	+	+	+	+	-

[A]Heifer #104 aborted on day 28 following intranasal inoculation. Other heifers aborted 12-15 days following intraamniotic BHVI inoculation.

[B]Tissue not available.

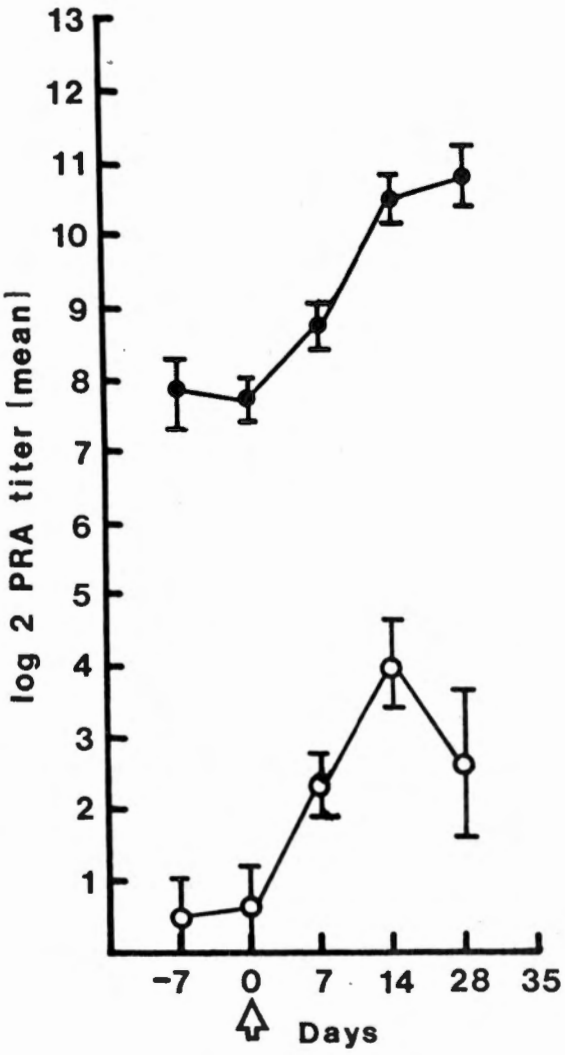

Fig. 8. Kinetics of the IgM (○) and IgG (●) neutralizing antibody responses following the reactivation of latent BHVI infection. The reactivation of latency was induced by the intravenous (IV) administration of corticosteroid (dexamethasone). Heifers (n = 6) were given IV injections of dexamethasone (30mg) on 3 consecutive days beginning on day 0 (⇧). The antibody activity was determined by PRA and expressed as log 2 mean titer ± SEM.

titer was 7.7 LMT. Both IgM and IgG antibody titers increased following the reactivation of latent BHVI infection. Antibody activity within the IgG class increased to 10.5 LMT by the fourteenth day of the experiment, while IgM antibody activity increased to 4.0 LMT. Maximal IgM antibody activity occurred by the fourteenth day of the experiment and declined rapidly to 2.6 LMT by the twenty-eighth day. Antibody activity within the IgG class appeared to be at maximal levels (10.8 LMT) by the twenty-eighth day (Fig. 8) after corticosteroid treatment.

The reactivation of latent BHVI infection by corticosteroid treatment was demonstrated by the recovery of BHVI from the nasal passages of these heifers (Table 7). Five of the 6 heifers were shedding BHVI from the nasal passages by the fifth day following the start of corticosteroid treatment. Although virus was not recovered from one animal, heifer #164, reactivation probably occurred since this animal had a similar rise in IgG and IgM antibody titers to the other heifers in the experiment. Antibody activity within the IgG class in heifer #164 increased from 7 log 2, at the start of corticosteroid treatment, to 11 log 2, 14 days later. Similarly, IgM antibody activity increased from <2 log 2 to 3 log 2 during this period.

The distribution of antibody activity among serum IgG subclasses following the reactivation of latent BHVI infection (Group III) is given in Table 8. At the beginning of the experiment, day 0, antibody activity was distributed relatively equally among the IgG1 and IgG2 subclasses. At this time, antibody activity within the IgG1 subclass was 7.3 LMT, while IgG2 antibody activity was 6.2 LMT. Following reactivation, an

TABLE 7

RECOVERY OF BHVI FOLLOWING
CORTICOSTEROID TREATMENT

	Days Post Corticosteroid Treatment[A]		
HEIFER	0	3	5
104	-	-	+
123	-	+	+
163	-	+	+
164	-	-	-
172	-	+	-
184	-	+	+

[A]Dexamethasone, 30 mg, was administered intravenously on days 0, 1, 2.

TABLE 8

DISTRIBUTION OF SPECIFIC ANTIBODY ACTIVITY AMONG SERUM IgG SUBCLASSES FOLLOWING REACTIVATION OF LATENT BHVI INFECTION (GROUP III)

	Log 2 Mean Titer ± SEM (n=5)		
DAY	IgG1	IgG2	"IgG2b"
0[A]	7.3 ± 0.42	6.2 ± 0.50	ND[B]
14	9.0 ± 0.54	7.0 ± 0.54	4.6 ± 0.68
28	9.2 ± 0.30	7.2 ± 0.30	4.4 ± 0.33

[A]Dexamethasone, 30 mg, administered intravenously on days 0, 1, 2.

[B]Not determined.

increase in antibody titer occurred in both subclasses; however the greatest increase in neutralizing antibody activity occurred in the IgG1 subclass. Antibody activity within the IgG1 subclass increased from 7.3 LMT to 9.0 LMT and IgG2 antibody activity increased from 6.2 LMT to 7.0 LMT, by the fourteenth day following the start of corticosteroid treatment.

Ratios of IgG1 to IgG2 Antibody Activity

Ratios of antibody activity within the IgG1 and IgG2 subclasses, following various primary and secondary antigenic exposures, were determined (Table 9). Ratios of IgG1 to IgG2 antibody activity, 14 days after primary intranasal exposure in Groups I and II, were 6.3 and 9.4, respectively. Ratios of antibody activity 14 days after secondary exposure by the intranasal route (Group I), abortion induced by IA virus inoculation (Group II), and reactivation of latency (Group III), were 1.10, 1.45, and 1.29, respectively.

TABLE 9

RATIOS OF ANTIBODY ACTIVITY WITHIN IgG1 AND IgG2 SUBCLASSES 14 DAYS AFTER ANTIGENIC EXPOSURE

Experiment	Log 2 Mean Titer IgGI:IgG2	Ratio
PRIMARY RESPONSE		
Group I[A]	6.3 : <2	> 6.3
Group II[A]	9.4 : <2	> 9.4
SECONDARY RESPONSE		
Group I[A]	7.7 : 7.0	1.10
Group II[B]	9.0 : 6.2	1.45
Group III[C]	9.0 : 7.0	1.29

[A]Intranasal BHVI exposure.

[B]Abortion induced by BHVI IA inoculation.

[C]Reactivation of latency induced by corticosteroid treatment.

V. DISCUSSION

A virus plaque reduction neutralization assay (PRA) was chosen for this study for quantifying antibody because it is a highly sensitive, virus-specific, antibody detection method (153, 154). The PRA is a highly sensitive method for assessing anti-viral antibody activity (153). Virus neutralization assays, in general, are virus-specific since antibodies directed against virion surface proteins only are detected by this method (154).

The requirement for complement in the neutralization of certain viruses by human and bovine sera has been reported (52, 134, 155, 156). The neutralizing activity of antibodies produced early in a primary immune response in these species, is usually enhanced by the presence of complement (157). This enhancement has been related to the relative levels of certain specific immunoglobulin antibody classes present early in the primary response (158). In studies involving human infection with cytomegalovirus (158), and the bovine immunized with BHVI, neutralizing IgM antibody was much more dependent upon the presence of complement than was IgG antibody. Since significant complement enhancement is associated with IgM antibody primarily, complement enhancement of antibody, early in a primary immune response, reflects the predominance of this immunoglobulin isotype during this phase of the immune response. However, complement enhanced antibody may be produced in the late phase of antibody formation of a primary immune response (159). An example of this is the late formation of complement-enhanced antibody elicited by equine viral arteritis virus (159).

As reported by Potgieter (52) previously, the neutralizing antibody activity of bovine anti-BHVI IgM antibody was enhanced significantly by the presence of complement (Fig. 4, p. 49), whereas antibody of the IgG class was only slightly enhanced by the presence of complement (Fig. 3, p. 48). These results, however, differ from those reported by Rossi and Kiesel (134). The latter reported that the neutralizing antibody activity of bovine anti-BHVI IgM and IgG antibodies both were markedly enhanced by complement. Reasons for these discrepancies are not apparent. Possible explanations include the presence of neutralizing antibody in the complement sources used, different routes of inoculation for antibody production, and the particular antibody detection methods employed. All batches of complement, in the studies reported here, were checked to exclude those containing anti-BHVI neutralizing antibody activity. Potgieter (52), and Rossi and Kiesel (134), studied the effect of complement on the neutralization of BHVI by antibody from intramuscularly inoculated calves, while the study reported here was done using sera from adult animals inoculated intranasally with BHVI. Rossi and Keisel (134) employed a virus neutralization assay similar to the one in the study reported here whereas Potgieter (52) used neutralization kinetics.

Guinea pig serum appeared to be a better source of complement than bovine serum which is consistent with the findings of Rossi and Kiesel (134). Fresh bovine serum did enhance the neutralizing activity of IgM antibody, but to a lesser extent than an equivalent amount of fresh guinea pig serum.

Maximal enhancement of IgM neutralizing antibody activity was obtained with less than 2% fresh guinea pig serum (Fig. 4). A similar

peak in IgG neutralizing activity, with concentrations of guinea pig serum used in this study, did not occur (Fig. 3, p. 48). However, the increase in IgG neutralizing activity with concentrations of 2.5 to 5% guinea pig serum was not appreciable. Subsequent to these findings, all the immunoglobulin preparations tested by PRA were supplemented with 3% fresh guinea pig serum because this concentration provided sufficient complement to maximize the antibody assay.

The findings of this study indicated that the nature of BHVI antigenic exposure in the bovine can be determined by assessing the relative distribution of anti-BHVI antibody activity among the bovine serum immunoglobulin isotypes, IgM, IgG1, and IgG2. These findings are summarized in Table 10. The presence of IgM antibody indicated recent BHVI exposure, with the exception of secondary intranasal exposure. Antibody activity restricted to the IgG1 subclass, distinguished primary immune responses, and the presence of significant IgG2 antibody activity distinguished secondary immune responses. Based on the distribution of anti-BHVI antibody activity among these isotypes, a primary immune response was differentiated from a secondary immune response. In addition, a variety of secondary immune responses were distinguished according to the nature of the antigenic stimulus. This study demonstrates that the serologic diagnosis of BHVI is possible without the acute and convalescent phase, paired serum samples, which traditionally have been used to demonstrate a rise in antibody titer coincident with disease. Paired samples are frequently not available under field conditions, and if they are, the timing of their collection is frequently incorrect.

TABLE 10

DISTRIBUTION OF ANTIBODY ACTIVITY AMONG SERUM IMMUNOGLOBULIN ISOTYPES AS A MEANS OF DETERMINING THE NATURE OF BHVI ANTIGENIC EXPOSURE

IgM - recent antigenic exposure (secondary intranasal exposure an exception)
IgG1 (IgG2 absent) - primary immune response
IgG2 - secondary immune response
IgM, IgG1 - primary immune response
IgG1, IgG2 - secondary immune response (secondary intranasal exposure)
IgM, IgG1, IgG2 - secondary immune response (reactivation of latency and abortion)

Primary immune responses were characterized by the formation of IgM antibody (Figs. 5, 6, and 7, pp. 52, 56, and 57) and IgG antibody localized to the IgG1 subclass primarily (Tables 1, 2, and 3, pp. 53, 58, and 60).

The primary IgM and IgG antibody response to BHVI infection was similar in all the animals tested in this study. Both IgM and IgG antibodies were detectable by 7 days pi, after which the titers in these immunoglobulin classes rapidly increased. Maximal IgG antibody titers, of similar magnitude, were observed to occur at 35 days pi in nonpregnant heifers (9.2 LMT), and at 14 days pi in pregnant heifers (10.0 LMT). The antibody activity within this class declined at a slow rate following the attainment of peak levels.

Maximal IgM antibody titers were consistently detected at 14 days pi (Figs. 5, 6, and 7). Antibody activity in this class declined rapidly after the fourteenth day pi, and were at low, but detectable levels by 90 days after initial BHVI exposure. Antibody activity in the IgM class was much higher during primary immune responses than during secondary responses.

Primary immune responses were also characterized by antibody activity in the IgG1 subclass and the relative absence of antibody activity in the IgG2 subclass (Tables 1, 2, and 3). Bovine anti-BHVI IgG2 antibody was formed late during a primary response and then only in low concentration.

Secondary immune responses were characterized by anamnestic IgG antibody responses (Figs. 5, 7, and 8, pp. 52, 57, and 65), and the formation of appreciable concentrations of IgG2 antibody (Tables 1 and 5,

pp. 53 and 62). Secondary immune responses resulting from the reactivation of latent BHVI (RLI), and abortion induced by IA virus inoculation, were characterized also by substantial increases in IGM antibody. The immune response resulting from secondary BHVI exposure by the intranasal route did not result in secondary IgM antibody formation (Fig. 5, p. 52).

The absence of secondary IgM antibody formation following a second intranasal BHVI exposure may be useful in differentiating this form of secondary antigenic exposure from RLI and BHVI-induced abortion. Reexposure to BHVI in this manner could occur as a result of contact with BHVI-shedding herdmates. Such exposure is believed to have little pathological significance (160), but it may be an important means of maintaining serum antibody concentrations at relatively high levels for the lifetime of the animal.

The absence of a secondary IgM antibody response following reexposure by the intranasal route is similar to the response in humans following secondary exposure to the viruses causing acute infections. Immunoglobulin M antibody formation does not occur, or at least is undetectable, following secondary exposure to rubella virus and hepatitis A virus (110, 111). The reasons for this have not been established. However, antigen dose and the presence of specific antibody may be determining factors. The antigen dose, conceivably, would be low after secondary exposure since virus amplification as a result of replication, would be inhibited by specific immunity developed as a result of primary infection. Reactivation of latency and abortion induced by IA virus inoculation may present the immune system with concentrations of viral antigen which are

high enough to stimulate secondary IgM antibody formation. In contrast to intranasal exposure, the antigen dose as a result of abortion may not be limited by previously established maternal immunity. The virus may replicate to very high titers in tissues of the fetus which may then be released to stimulate the maternal immune system.

The possible mechanisms by which viral antigen within the virus-infected fetus could stimulate the maternal immune system are speculative. Viral antigen could cross the placenta prior to abortion either in a physiological manner or as a result of pathological damage to the placenta. Cells and soluble antigens cross the human placenta (161); however, differences in placental structure suggest that transfers of this type may not occur in the bovine (162). Alternatively, the passage of viral antigens across the bovine placenta may occur as a result of virus-induced pathological damage. In the present study, increases in IgM and IgG antibody activity did not occur until after abortion (Fig. 7, p. 57). Therefore, antigenic exposure prior to abortion seems unlikely since an increase in IgG antibody activity occurred immediately following secondary antigenic exposure in nonpregnant (Group I) animals (Fig. 5, p. 52). Other means by which abortion may elicit a secondary maternal immune response include exposure to viral antigens in fetal fluids which remain in the uterus following abortion, development of uterine epithelial infection by virus remaining in these fluids, and reactivation of latent infection as a result of abortion-associated stress. The increase in antibody titers in heifers soon after abortion suggest that one of these means of antigenic exposure occurred. The virus was recovered from maternal uterine exudate (Table 6, p. 64) after abortion, which supports

the possibility of antigenic exposure by antigen-laden fetal fluids and/or uterine infection, as a means of stimulating a secondary maternal immune response.

Heifer #104 (Group II) aborted as a consequence of intranasal BHVI exposure. Abortion in this animal failed to elicit a secondary increase in IgM and IgG antibody titers (Fig. 6, p. 64). This was attributed to the presence of high levels of specific antibody at the time abortion occurred. Abortion occurred 28 days after BHVI intranasal exposure, 14 days after the attainment of maximal IgM and IgG antibody titers. Previous studies have indicated that the presence of specific antibody at the time of secondary antigenic exposure is inhibitory to the formation of additional specific antibody (43, 44). This concept was supported in the present study by the observation that abortions occurring after anti-BHVI antibody titers had declined, elicited secondary increases in IgM and IgG antibodies (Fig. 7, p. 57).

The failure to detect secondary antibody formation, as a result of abortion occurring within a few weeks of primary infection (heifer #104, Fig. 6), underscores the problems associated with the serological diagnosis of bovine abortion. Serological analysis of paired serum samples, obtained from this heifer at the time of abortion and 10-14 days later, did not demonstrate an increase in antibody titer. These results emphasize the need for an alternative approach to the serological diagnosis of bovine abortion. The data generated from the present study suggest that serological diagnosis may be more efficiently performed by assessing the distribution of antibody activity, among serum immunoglobulin isotypes, within single serum samples.

The decline in IgG1 antibody titer after abortion in heifer #104 (Group II) from 9 log 2 to 7 log 2 (Table 4, p. 60) may be attributed to the selective transport of this immunoglobulin isotype into lacteal secretions during the formation of colostrum (124). It has been shown previously that IgG1 levels decline during the periparturient period (163). Recently, Burridge et al. (164) described a decline in antibody titer to bovine leukosis virus in infected cows during this period, which they attributed to selective transport of IgG1 into lacteal secretions.

The distribution of antibody activity among serum immunoglobulin isotypes allowed distinction of primary and secondary BHVI infection. Additionally, such information also discriminated between secondary intranasal exposure, and secondary responses resulting from RLI and late abortion. Late abortion was defined as abortion occurring several weeks after primary BHVI infection, when antibody titers had dropped well below maximal levels. The inability to distinguish late abortion from RLI using this approach was apparent. However, since bovine abortion, due to BHVI infection, occurs several weeks to months following primary infection, the possibility that BHVI-induced abortion is in fact a form of RLI must be considered. The secondary maternal immune response observed after abortion may be due to RLI and not to antigenic exposure as a result of abortion.

Primary and secondary immune responses also were differentiated by comparing the ratio of antibody activity within the IgG1 and IgG2 subclasses, 14 days after BHVI exposure (Table 9, p. 70). Primary immune responses were characterized by high IgG1 to IgG2 antibody activity ratios, whereas secondary responses were relatively low.

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