Characterization of the antibody response of swine to *Mycoplasma hyosynoviae* vaccination

by

Barbara Jean Zimmermann

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INTRODUCTION

Mycoplasma hyosynoviae is a causative agent of polyarthritis in swine. The organism is a common inhabitant of the tonsil and oropharyngeal area of swine and susceptible pigs exposed to these carriers may become infected, with dissemination of the organism via the blood to other parts of the body, especially the joints. Poor joint conformation, heavy muscling, etc., may favor development of arthritis, especially when the pig is under stress.

M. hyosynoviae arthritis occurs more often in heavily muscled breeds and may lead to loss of appetite, slow growth rate, poor breeding ability and condemnation of carcasses at slaughter. The morbidity of M. hyosynoviae arthritis is usually 5 to 15% but in some herds, 50% or more of the swine may be affected, leading to large economic losses.

In addition to concern over economic losses in the swine industry, interest in mycoplasmal arthritis in swine has grown in recent years with the observation of several similarities between the arthritis in swine and rheumatoid arthritis of unknown etiology in man. Knowledge gained about M. hyosynoviae arthritis may also lead to better understanding of the chronic stages of arthritis in man and mycoplasmal arthritides in other species. This has lead to increased interest in the pathogenesis of M. hyosynoviae arthritis, serological characterization of the antibody response and possible use of a vaccine in prevention of the disease.

Because of the septicemic phase of M. hyosynoviae arthritis in swine, it can be speculated that circulating antibody would be important in prevention of the disease. Therefore, one criterion for an effective vaccine

should be ability to elicit a humoral antibody response. This study dealt with evaluation of the potential of a vaccine preparation by characterization of the elicited antibody response and comparison to the response detected in infected pigs. The sequential antibody response of swine vaccinated with one or two doses of sonicated M. hyosynoviae vaccine plus incomplete Freund's adjuvant was evaluated by use of complement fixation, latex agglutination, metabolic inhibition and mycoplasmacidal tests. The nature of the antibody detected with the serological procedures was determined by gel filtration of selected sera and testing the resultant peaks with the four serological tests. The antibody response of sera from infected pigs was also characterized and compared with the response of vaccinated pigs.

LITERATURE REVIEW

Mycoplasma hyosynoviae Arthritis in Swine

Ross and Switzer (1963) described a mycoplasma isolated from swine joint fluids which differed from *Mycoplasma hyorhinis*. This organism was originally designated *Mycoplasma granularum* (Switzer, 1964) but it was later demonstrated that two groups of mycoplasmas were included in this species, differing distinctly by serological, biological and electrophoretic techniques (Ross and Karmon, 1970). Dextrose-utilizing, non-sterol-requiring mycoplasmas with the characteristics of the type strain retained the designation *M. granularum* (now *Acholeplasma granularum*). Isolates belonging to the group of arginine-utilizing, sterol-requiring organisms were named *Mycoplasma hyosynoviae*.

Mycoplasma hyosynoviae is a cause of polyarthritis in 10 week or older swine (Ross, 1973a). The organism may be harbored in the nasopharyngeal region of adult swine and transmitted to susceptible pigs older than 10 weeks of age. Ross and Spear (1973) found that *M. hyosynoviae* was rarely isolated from the pharyngeal secretions of 1- to 6-week-old pigs nursing infected sows, but was commonly isolated from older pigs. Septicemia may develop within 2 days after susceptible pigs have been exposed to virulent *M. hyosynoviae*. The organism may be recovered from the spleen, lymph nodes, joints and other tissues during the course of the infection. By 24 days after exposure the organism may disappear from all tissues and secretions except the pharyngeal secretions and the tonsil, where it persists indefinitely in the adult (Ross, 1973a). Infection of the joints with *M. hyosynoviae* does not always develop into arthritis.

Such traits as poor joint conformation and heavy muscling may be predisposing factors in the development of arthritis. Ross (1973b) has speculated that environmental stress may cause some joint damage and allow the organism to grow to higher titers and in some way further damage the joint tissue.

Mycoplasma hyosynoviae has been isolated from field cases of swine pneumonia (Friis, 1970; Goiš and Taylor-Robinson, 1972). Ross et al. (1971) isolated M. hyosynoviae from 3 of 30 lungs of experimentally infected swine, indicating that the organism may occasionally be disseminated to the lung during the septicemic phase of the disease.

A review of the growth requirements of this organism has been given by Potgieter (1970).

Immunoglobulins

Determination of the class of animal immunoglobulins has been based on antigenic and physicochemical relationship to human immunoglobulins. Therefore, a brief review of human immunoglobulins will be included before discussion of swine immunoglobulins.

For many years there was confusing terminology for the classes of human immunoglobulins until the World Health Organization (1964; 1973) attempted to standardize the nomenclature. Table 1 presents a summary of the current designations for the major human immunoglobulin classes. Two other immunoglobulin classes, IgD (Rowe and Fahey, 1965) and IgE (K. Ishizaka et al., 1966) are present in man in relatively low amounts in normal serum and will not be covered here.

Table 1. Nomenclature of the major human immunoglobulins[a]

Past usage	Present usage
gamma, 7S gamma, 6.6S gamma, gamma-2, gamma-ss, gamma-G	IgG
beta-2A, gamma-1A, gamma-A	IgA
gamma-1M, beta-2M, 19S gamma, gamma-macroglobulin, gamma-M	IgM

[a]Adapted from World Health Organization (1964; 1973).

A review of the early work in antibody characterization has been given by Hogle (1967).

The structure of immunoglobulins was investigated by Edelman (1959) who reduced the disulfide bonds of human IgG with mercaptoethanol in 6M urea. The molecular weight of the products was about one-third of the original IgG, suggesting that the immunoglobulin had a multichain structure. Edelman and Poulik (1961) extended this work to include separation of the reduced components of gamma globulin by cation exchange chromatography in 6M urea, further indicating several polypeptide chains. Fleischman et al. (1962) modified the procedure to retain the biological activity of reduced rabbit immunoglobulins which were separated into two components, light (L or B) chains and heavy (H or A) chains. Based on these data, R. Porter (1962) postulated that the monomeric immunoglobulin unit is composed of two light and two heavy chains held together by disulfide bonds. This four chain structure has a sedimentation coefficient of 7S (Svedberg units).

Porter's model of the immunoglobulin molecule has been further expanded by other researchers. An individual immunoglobulin has either two kappa or two lambda light chains but never one of each (Fahey, 1963). Class identity of immunoglobulins is based on the type of heavy chain (Franklin and Stanworth, 1961). In man, gamma chains belong to IgG, alpha to IgA, mu to IgM, delta to IgD and epsilon to IgE.

Designations of various regions of immunoglobulin structure have been based on R. Porter's work (1959) with papain digestion of rabbit IgG. Three fragments are obtained with papain cleavage, two of which are identical. These two fragments each contain one L chain joined by a disulfide bond to the Fd piece, the amino (N) terminal end of an H chain (Fig. 1).

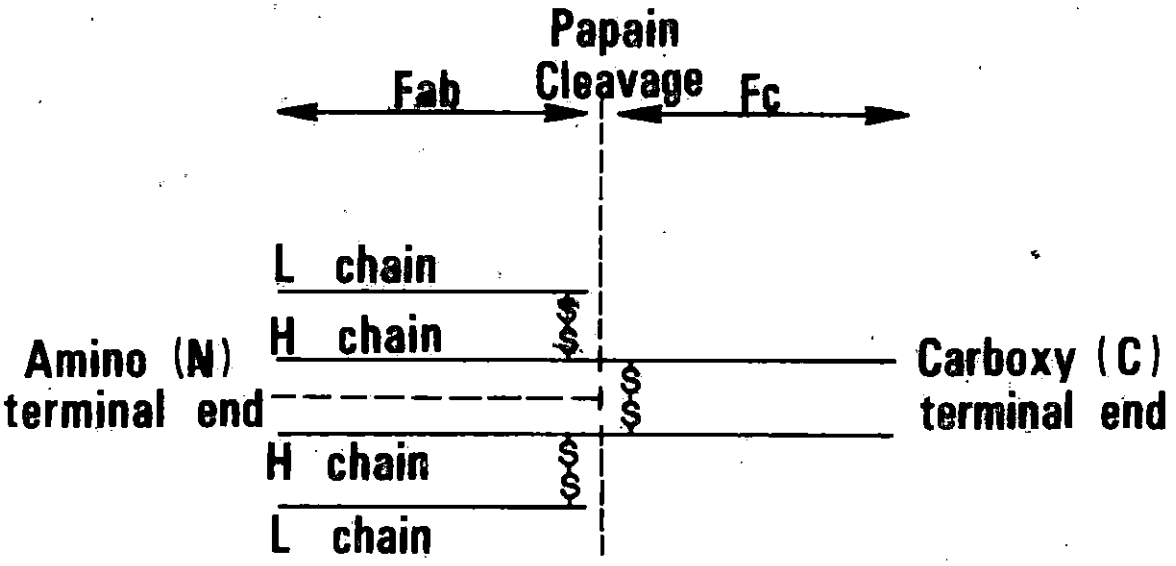

Fig. 1. Schematic diagram of the four chain structure of an IgG molecule. Sites of papain cleavage and resultant fragments, antigen-binding (Fab) and crystallizable (Fc), are indicated. Based on Stanworth and Turner (1973).

These two fragments retain antigen-binding ability and are termed Fab (fragment, antigen-binding). The third fragment does not bind antigen and is designated Fc (fragment, crystallizable). The Fc fragment has many biological functions including fixation of complement (Taranta and Franklin, 1961).

As indicated earlier, the three major immunoglobulin classes are IgG, IgM and IgA. In man, there are four antigenically distinct IgG subclasses, designated IgG1 through IgG4. They vary in electrophoretic mobility (Skvaril and Morell, 1970), complement fixation by the conventional pathway (T. Ishizaka et al., 1967), ability to sensitize guinea pig mast cells for passive anaphylaxis (Terry, 1964) and binding to macrophage receptors (Abramson et al., 1970). A complete summary of the properties of the IgG subclasses is given by Natvig and Kunkel (1973). Human IgG is a 7S monomer, with a molecular weight of about 150,000. All human IgG subclasses may be transferred across the placenta. Human IgG is almost equally distributed between the blood and extravascular fluids and has major antibody activity against bacteria, viruses and toxins (Stanworth and Turner, 1973). IgG is the major antibody in the late response to most antigens (Pike, 1967).

Human IgM is chiefly found as a 19S polymer of five monomeric subunits. Mestecky et al. (1971) have isolated a distinct polypeptide chain termed "J" from human 19S IgM which they speculate may stabilize the pentameric structure. High molecular weight IgM may be dissociated into the 7S monomers by mild reduction with such agents as mercaptoethanol. Naturally occurring 7S IgM has been isolated and Solomon and McLaughlin (1970) have indicated in biosynthetic studies that 7S IgM is formed

directly and at a different rate than 19S IgM. The possibility of two IgM subclasses has been presented (Metzger, 1970; Natvig and Kunkel, 1973). Polymeric IgM has 10 antigen binding sites, but steric hindrance may prevent subunits from binding more than 1 mole of antigen (Metzger, 1970). Human IgM fixes complement, but does not sensitize guinea pig mast cells for passive cutaneous anaphylaxis (Fahey, 1965) or bind directly to macrophages (Huber et al., 1968). Antibodies of the IgM class are mainly intravascular and may have an important role in reacting with particulate antigens in the blood stream (Stanworth and Turner, 1973). The first antibody to be detected in the immune response is usually IgM (Bauer and Stavitsky, 1961) and antibody of this class has some antibacterial and antiviral activity.

Human IgA exists in a variety of forms, with the 7S monomer predominating in serum. Polymers of IgA with sedimentation coefficients of approximately 10S, 13S, 15S and 17-18S are found in lesser amounts in serum (Tomasi and Grey, 1972). As with pentameric IgM, polymeric IgA contains a nonimmunoglobulin polypeptide chain, the "J" chain, which may function in maintaining the structure of the polymer (Halpern and Koshland, 1970). Immunoglobulin A comprises only about 20% of human serum immunoglobulins but is the predominant immunoglobulin in the external secretions of man and many other mammals. Secretory IgA (SIgA) exists mainly as an 11S dimer with a J chain and an additional polypeptide, the secretory component (SC). Monomer IgA and higher polymers of SIgA are also found in secretions. The majority of SIgA is produced locally in plasma cells of the submucosa as a 10S dimer and is complexed with SC as it is transported across the mucosa to the luminal surface (Tomasi, 1972).

Secretory IgA is believed to play a major role in the prevention of colonization of microorganisms at mucosal surfaces (Tomasi and Bienenstock, 1968) and may prevent nonviable antigens such as those in food and milk from gaining access to the circulation (Tomasi, 1972). There are two subclasses of IgA, IgA1 and IgA2. One genetic type of IgA2, Am(2)+, lacks light-heavy disulfide bonds whereas the other IgA molecule has typical light-heavy structure. Serum consists of about 80% IgA1 whereas in secretions there are about equal amounts of the two subclasses (Stanworth and Turner, 1973). Human IgA does not fix complement by the conventional pathway (T. Ishizaka et al., 1966) and its role in promoting phagocytosis is not clear (Tomasi and Grey, 1972). Formation of IgA both early and late in the primary immune response has been reported, with differences possibly due to the type of antigen, the method of antigen administration and the serological techniques employed for detection (Tomasi and Grey, 1972).

Three classes of porcine immunoglobulins have been identified, and, based on their antigenic and physicochemical properties, are analogous to human IgG, IgM and IgA.

Immunoglobulin G is quantitatively the major immunoglobulin of pig serum and colostrum (Bourne, 1971). Metzger and Fougereau (1967) have identified two main subclasses of porcine IgG, IgG1 and IgG2. These subclasses differ in electrophoretic mobility but have identical sedimentation coefficients of approximately 7S and molecular weights of 160,000. A 19S IgG has been found as the initial antibody in the true primary immune response of germfree piglets (Kim et al., 1966a,b). However, Prokešová et al. (1969), using precolostral germfree piglets, detected

only 19S IgM in the primary immune response, and Bourne et al. (1974), using porcine fetuses, found mainly 19S IgM with only small amounts of 19S IgG. P. Porter (1969a,b) detected low levels of 19S IgG in adult serum and colostrum and determined that 7S IgG was the main immunoglobulin absorbed by the neonatal piglet from the colostrum although high molecular weight IgG was absorbed also (P. Porter, 1969a). A low molecular weight (4-5S) substance antigenically related to IgG has been demonstrated in the sera of newborn and germfree piglets (Franěk and Říha, 1964; Prokešová et al., 1969; Bourne, 1974). Franěk and Říha (1964) reported that this low molecular weight material did not contain L chains, but other workers (Prokešová et al., 1969; Bourne, 1974) indicated that this component was actually half an IgG molecule, with one L and one H chain. Bactericidal activity of low molecular weight IgG to Escherichia coli has been demonstrated in precolostral piglet serum (P. Porter and Hill, 1970), sow serum and postcolostral piglet serum (Hill and Porter, 1974). The contribution of the small amount of 4S IgG to protection of the newborn piglet is probably minimal compared to that of the large amount of 7S IgG absorbed from the colostrum in the first hours of life. Wilson (1972) determined that colostral IgG protects the newborn pig from E. coli enteritis by neutralizing enterotoxin and decreasing the multiplication rate of E. coli. In the pig, where immunoglobulin transfer across the placenta does not occur (Kim et al., 1966a; P. Porter, 1969b), colostral immunoglobulins absorbed via the intestinal tract during the first 24-36 hours of life play an important role in protection of the newborn piglet (P. Porter, 1969b).

Porcine IgM has a pentameric structure with a sedimentation coefficient of approximately 18S (P. Porter and Kenworthy, 1969). Although IgM is quantitatively the least abundant immunoglobulin in the pig (Bourne, 1971), it plays an important role in immunity. The importance of IgM as a serologic antibody to gram negative bacteria has been demonstrated by Franěk et al. (1962), P. Porter (1969a), and Rice and L'Ecuyer (1969). The major bactericidal antibody against E. coli in sow colostrum (Knop et al., 1971) and passively acquired from colostrum by newborn piglets (P. Porter and Hill, 1970) was associated with IgM. More recently, Hill and Porter (1974) have indicated that bactericidal activity of sow serum to E. coli was associated with IgM. However, IgM was a minor bactericidal component compared to 7S IgG in sow colostrum and 2-day-old postcolostral piglet sera. P. Porter and Hill (1970) found IgM passive immunity in the postcolostral piglet shortlived, with the IgM serum level declining rapidly after the first day of life and decreasing to very low levels by the end of the first week of life. The serum level of IgM started to increase by about the 10th day after birth, without an associated increase in E. coli antibody activity. The authors speculated that this represented a primary response to other antigens. IgM was the main antibody detected in conventional pigs infected with E. coli (P. Porter and Kenworthy, 1969), but gnotobiotic pigs responded predominantly with 7S IgG (P. Porter and Kenworthy, 1970). The authors indicated that this may reflect a need for pre-experience with an antibody class before its initial synthesis. However Bourne et al. (1974) demonstrated that the major immunoglobulin response of fetal pigs to in utero antigenic stimulation was 19S IgM.

The existence of the IgA class of immunoglobulin in the pig was confirmed by Vaerman et al. (1969) by demonstration of cross reactions with human IgA. Most of the work on swine IgA has centered on its presence in sow colostrum and milk and its role in passive immunity in the newborn pig. Two subclasses of IgA have been recognized in the pig, IgA1 and IgA2. They occur in equal amounts in colostrum and milk, but IgA1 is predominant (4:1) in serum (Bourne, 1971). Immunoglobulin A exists mainly as a 9.3S dimer and 6.4S monomer in pig serum and as a 10.6S secretory molecule in milk. Colostral IgA ranges from 6.4S to 18S with 8.6S and 10.6S forms predominating (P. Porter, 1971). The IgA class constitutes only about 14% of the colostral immunoglobulins (P. Porter, 1969b) but emerges as the major immunoglobulin in porcine milk where the concentrations of IgG and IgM are about one-tenth of their colostral levels and IgA has decreased only two- to threefold from its colostral level (P. Porter et al., 1970). P. Porter (1969a) demonstrated that anti-E. coli activity in sow serum was almost exclusively IgM, but colostral antibody activity was associated with IgA. The IgA serum levels in postcolostral piglets exceeded adult levels, indicating absorption of colostral IgA, but E. coli antibody activity was not detected. It was speculated that SIgA may not be absorbed and that this might be due to the presence of the secretory component. Bourne (1971) disagreed with these findings, indicating that the secretory molecule is absorbed by the newborn piglet but possesses a shorter half-life than the IgA dimer. P. Porter (1973) reported that SIgA represents less than 5% of the total IgA in postcolostral piglet serum and contributes little to circulating passive immunity in the young pig.

After 24 to 36 hours of life, when immunoglobulins are no longer absorbed from the colostrum (Lecce and Morgan, 1962), milk IgA plays an important role in immunity of the young pig. In work with fistulated piglets, P. Porter et al. (1970) demonstrated that after a single feeding, milk IgA continued to pass through the small intestine for longer than the normal feeding intervals of a suckling piglet. This continual presence of milk IgA in the small intestine would seem to provide local defense until the secretory immune system has developed. This has been supported by Bohl and coworkers in research on transmissible gastroenteritis (TGE). They have demonstrated the role of TGE antibodies of the IgA class in providing passive intestinal immunity to piglets during this early period of life (Bohl et al., 1972a,b; Saif et al., 1972; Bohl and Saif, 1975). The onset of intestinal immunocompetence does not occur until after the first week of life and IgM constitutes the initial response, with the IgA class predominating after about 3 weeks (P. Porter et al., 1974). Allen and Porter (1973) found equal amounts of IgM- and IgA- synthesizing cells in intestinal lamina propria of unweaned pigs and speculated that the early IgM response may be important as a second line of defense in the tissues during this stage in the piglets' lives. The biological activity of IgA has been debated, but it now appears that both human (Adinolfi et al., 1966) and swine (Hill and Porter, 1974) SIgA are bactericidal for E. coli in the presence of complement and lysozyme, but lack this activity with complement alone. Burdon (1973) presented evidence that human serum IgA may also be bactericidal. Knop et al. (1971) demonstrated that sow colostral IgA antibody to E. coli was more active in promoting phagocytosis and intracellular killing than IgM or IgG.

Use of Complement Fixation, Latex Agglutination, Metabolic Inhibition and Mycoplasmacidal Tests in Determining the Humoral Response to Mycoplasmas in Species Other than Swine

In this section, only a brief overview will be given of the use of four serological tests in determination of the humoral response to non-swine mycoplasmas. The literature on several of the tests is quite extensive and has been reviewed by Purcell et al. (1969), Hayflick (1969) and Riggs and Sharp (1970).

Complement fixation (CF) test

The CF test has been one of the most extensively used procedures for detection of mycoplasmal antibodies. Schmidt et al. (1966) found that the CF antibodies to Mycoplasma pneumoniae in man consist of both 19S and 7S immunoglobulins. Fernald et al. (1967) and Biberfeld (1968) further indicated that IgM is usually the major early CF antibody, with the relative proportion of IgG increasing with time. Rabbits and hamsters have been used as experimental models and, in rabbits CF antibodies were found in both 19S and 7S fractions (Fernald et al., 1967), while hamsters responded with only IgG CF antibodies (Clyde, 1968; Fernald, 1969). It was also demonstrated that hamsters immunized with killed organisms or parenterally vaccinated with avirulent organisms developed CF antibodies similar to actively infected hamsters, but were unable to resist challenge with virulent M. pneumoniae. Fernald and Clyde (1970) reaffirmed that serum antibody levels did not correlate with protection. Local immunity, either secretory or cellular, was felt to play a role in resistance to M. pneumoniae infection. The use of the CF test for diagnosis of M. pneumoniae infection in man was evaluated by Taylor-Robinson et al. (1966c). The CF

test detected only 86% of the total infections diagnosed by immunofluorescence, indirect hemagglutination or tetrazolium reduction inhibition. Kenny and Grayston (1965) extracted the CF antigen from M. pneumoniae with organic solvents and identified the antigen as lipid or lipid-associated. This antigen has been further identified as a glycolipid (Lemcke et al., 1967) and association of complement fixation with both membrane and soluble antigens has been reported (Pollack et al., 1970).

The complement fixation test has been used with M. mycoides subsp. mycoides (M. mycoides), the causative agent of contagious bovine pleuropneumonia. Barber et al. (1970) demonstrated the sequential change from 19S to 7S CF antibodies in cattle experimentally infected with M. mycoides. Pearson and Lloyd (1972) detected IgM CF antibodies initially and found they persisted throughout the course of the disease. The IgG CF response occurred later in the disease but did not always persist. Onset of CF activity coincided with the onset of symptoms. Gilbert et al. (1970) and Windsor et al. (1972) demonstrated that vaccinated cattle developed a transient CF response with negative titers by 8 to 12 weeks postvaccination. Davies and Hudson (1968) found that complement fixing antibodies, as well as agglutinating and growth inhibiting antibodies, could not be correlated with immunity. Davies (1969b) correlated positive CF titers in vaccinated cattle with the persistence of viable organisms in lymph nodes. The CF antigens of M. mycoides have been studied by several researchers. Dafaalla (1957) determined that the complement fixing antigen was alcohol soluble. Since then, the concept has emerged that there are two or more CF antigens; a major carbohydrate-lipid complex (Buttery, 1972; Stone and Razin, 1973) and at least one minor antigen.

The CF test has been used to characterize the response of rats and mice to M. arthritidis. In rats, Cole et al. (1969) detected CF antibodies by 4 days after intravenous inoculation. A correlation was found between the number of organisms injected and the level of CF antibodies. Mice developed CF antibodies later than rats and the titers were lower (Cole et al., 1971). In mice, only 7S CF antibodies were detected, whereas in rats the 19S, 7S sequence occurred (Cole et al., 1971). Convalescent serum with a high CF titer effectively protected rats when given intravenously. Adsorption of the serum with M. arthritidis significantly lowered the CF titer but did not affect the protective properties of the serum (Cole et al., 1969). Kenny (1967) studied the properties of the CF antigens of 10 different mycoplasmas and found that the CF antigen of M. arthritidis was not extracted by organic solvents, but was heat labile.

Latex agglutination (LA) test

Morton (1966) and Lynn (1967) used the LA procedure to detect antibodies in hyperimmune rabbits. Morton was able to detect antibodies with the LA test sooner than by agar-gel diffusion or growth inhibition on agar. The sensitivity of the LA test was comparable to direct agglutination. Kende (1969) compared the use of LA for detecting the antibody response of guinea pigs to M. pneumoniae vaccines and found a good correlation between the CF and LA responses. However, the CF response declined earlier than the LA titer. Purcell et al. (1969) found the LA test to be as sensitive as CF, but not as sensitive as indirect hemagglutination. Jain et al. (1969) used four procedures, LA, plate agglutination, agar-gel double diffusion and growth inhibition on agar, to analyze sera from normal cows, naturally and experimentally infected cows and vaccinated rabbits for

antibodies against a mycoplasmal strain, probably M. agalactiae subsp. bovis, causing mastitis in cows. Most cows from infected herds had high LA titers whereas the other tests only indicated a few positive sera. Antibodies in experimentally infected cattle were detected earlier and more consistently with the LA test. The latex test proved to be the most sensitive test for use with vaccinated rabbits.

Metabolic inhibition (MI) test

Metabolic inhibition techniques indirectly measure the inhibition of mycoplasmal growth by antibody. Some actively growing mycoplasmas can reduce 2,3,5-triphenyltetrazolium chloride with production of a red color and most mycoplasmas utilize glucose or arginine with a pH change indicated by phenol red. Metabolic inhibiting antibodies can prevent these color changes and this is the basis of several procedures: tetrazolium reduction inhibition (TRI-Jensen, 1964; Senterfit and Jensen, 1966), fermentation inhibition of glucose (FI-Taylor-Robinson et al., 1966a) and inhibition of arginine utilization (Purcell et al., 1966). The mechanisms functioning in the metabolic inhibition and mycoplasmacidal techniques, including the requirement of heat-labile accessory factors for some sera, will be reviewed in the mycoplasmacidal test section.

Mycoplasma pneumoniae reduces tetrazolium and utilizes glucose. Fernald et al. (1967) detected FI antibodies in adults with natural M. pneumoniae infection. The early response was primarily IgM with increasing amounts of IgG FI antibodies in later samples. Fermentation inhibiting antibodies were also detected in IgA fractions. Biberfeld (1968), using the TRI test, detected the 19S, 7S shift in antibodies in adults with M. pneumoniae pneumonia. Fernald et al. (1967) used fluorescent

antibody, CF, TRI and FI techniques to determine the humoral response in rabbits immunized with M. pneumoniae. Tetrazolium reduction inhibiting and FI antibodies were detected later than FA and CF responses and maximum titers were also reached later. Fermentation inhibiting antibodies were detected in both 19S and 7S fractions. Hamsters infected with virulent M. pneumoniae or parenterally vaccinated with an avirulent strain developed FI titers only in the IgG fraction (Fernald, 1969). The protective value of TRI antibodies was examined by Taylor-Robinson et al. (1966b) who found that low levels of TRI antibodies in the preinoculation sera of volunteers correlated with resistance to febrile illness caused by M. pneumoniae. Smith et al. (1967) also correlated the presence of circulating TRI antibodies with protection against M. pneumoniae. However, all of the inoculated volunteers became infected with M. pneumoniae and it was suggested that antibodies in respiratory tract secretions may be more important than circulating antibodies in the prevention of respiratory tract infection. Fernald (1969) found that hamsters parenterally immunized with avirulent M. pneumoniae developed high levels of FI antibodies but were not protected from pneumonia with virulent M. pneumoniae. Taylor-Robinson et al. (1966a) found the FI and TRI tests comparable in sensitivity for detecting antibody rises in adults inoculated with M. pneumoniae. Taylor-Robinson et al. (1966c) compared CF, indirect hemagglutination, TRI and immunofluorescence for detecting M. pneumoniae antibody responses. The TRI test detected 86% of the total serologically demonstrated responses. The specificity of TRI antibodies has been demonstrated to be directed against the serologically active lipids of the cell membrane of M.

pneumoniae (Soběslavský et al., 1966). Williams and Taylor-Robinson (1967) inoculated rabbits with M. pneumoniae whole cells, cell membranes and cell contents and detected FI antibodies against membrane components.

The correlation between FI antibodies and immunity to M. mycoides in cattle was examined by Davies and Hudson (1968). Only one of four vaccinated cattle developed FI antibodies, although all four were resistant to natural challenge. Nonvaccinated cattle developed FI antibodies after becoming infected. Davies (1969a) studied the fermentation inhibition response in rabbits in relation to the mycoplasmacidal response. Rabbits inoculated subcutaneously with M. mycoides whole cells and Freund's complete adjuvant developed FI antibodies. After additional inoculations with whole M. mycoides intravenously, they detected both FI antibodies as determined when the pH of the controls had changed 0.5 units and mycoplasmacidal antibodies as determined by reading the test when no more color changes had occurred.

Cole and coworkers attempted to detect MI antibodies against M. arthritidis in mice and rats, utilizing the inhibition of arginine metabolism. No or very low MI titers could be detected in rats inoculated intravenously or subcutaneously (Cole et al., 1969; 1970). Nonmurine mycoplasma species were able to induce MI antibodies in the rat. It was hypothesized that M. arthritidis and rat tissue shared certain heterogenetic antigens which prevented a MI response. Mice exhibited a low MI response to both active infection and subcutaneous immunization (Cole et al., 1970; 1971). Cole et al. (1970) suggested a positive correlation between ability of mycoplasmas to produce disease and the lack of MI antibody production in its natural host.

The response of chickens and turkeys to Mycoplasma gallisepticum infection after intratracheal inoculation has been determined by Jordan and Kulasegaram (1968) using three agglutination tests, hemagglutination inhibition, indirect complement fixation, agar-gel double diffusion, and fermentation inhibition. The FI test detected the lowest titers of antibody in both birds and the response was detectable for only 4 months after infection. Hyperimmunized chickens developed only low titers compared to similarly immunized rabbits. Taylor-Robinson and Berry (1969) had comparable results using chickens, turkeys and rabbits, with chickens and turkeys developing much lower FI responses. Williams and Taylor-Robinson (1967) examined the antigenic specificity of the FI response to M. gallisepticum by inoculating rabbits with whole cells, cell membranes and cell contents of M. gallisepticum. The cell membranes, but not cell contents, were capable of eliciting a FI response. Kahane and Razin (1969) further characterized the FI antigen by preparing defatted membranes. These preparations were able to stimulate FI antibodies in rabbits and to adsorb the antibody. Membranes lost their ability to adsorb FI antibodies when heated to 65C for 1 hour, suggesting that the FI antigens are proteins.

Mycoplasmacidal (MC) test

The mechanics of antibody-mediated killing of mycoplasmas in broth have been extensively studied but, due to the cumbersome nature of the technique, the procedure has not been used widely to study immune responses to mycoplasmas. The MC test is a direct way of measuring the immune inhibition of mycoplasmas; the MI test is an indirect method. There appear to be two main mechanisms involved in immune inhibition, one dependent on

a heat-labile accessory factor presumed to be complement or complement components (Riley et al., 1966), and the other not dependent on this factor. Brunner et al. (1971) have demonstrated lysis of M. pneumoniae in the presence of complement and specific antibody. Riggs and Sharp (1970) speculated that antibody not dependent on complement may attach to mycoplasma surface antigen and interfere with a vital metabolic process, leading to slow death. This growth inhibition was first described by Edward and Fitzgerald (1954) with M. hominis.

Coleman and Lynn (1972a,b) were able to demonstrate the multiple mechanisms for immune inhibition of mycoplasmas by testing sera from rabbits immunized with M. pneumoniae. The sera were tested for MI and MC antibodies and for lysis of M. pneumoniae as demonstrated by the release of labelled nucleic acids from the damaged organisms. Early sera, collected up to 30 days after inoculation, required a heat-labile factor for demonstration of MI or MC activity. No lytic activity could be demonstrated in this early sera. The MI activity was found in both IgM and IgG fractions, whereas MC activity was only demonstrated in the IgG fractions. Late sera (173 days postinoculation) had MI and MC activity in the IgG fraction independent of a heat-labile accessory factor. Lytic activity however required this factor. The IgM fraction had a low amount of dependent MI activity but no MC activity. Coleman, Lynn, and Patrick (1974) further characterized the different mechanisms involved in immune inhibition by use of the MI test. The heat-labile accessory factor dependent early sera (9 days) were shown to be dependent on complement components C3, C8 and possibly other components which follow C3 in the complement reaction sequence. Complement components C1, C4 and C2 were not involved.

Late sera (252 days), with activity independent of heat-labile accessory factors, contained two different antibody groups. One group did not require any of the complement components, but the second group required C3 and possibly C5, C6, C7 and C9. Clyde (1968) used the hamster model for M. pneumoniae infection and detected MC as well as CF antibodies by 2 to 3 weeks postinoculation when the pneumonic changes had reached a peak. Mycoplasmacidal activity peaked at 10 weeks and correlated better than CF activity with the disappearance of pneumonia and M. pneumoniae. Hamsters immunized with killed organisms developed similar humoral responses but did not resist challenge with virulent M. pneumoniae. Brunner et al. (1972) compared the MC, CF, MI and immunofluorescence responses in 10 human volunteers infected with M. pneumoniae. In nine of the ten volunteers the preinoculation sera had MC antibody titers of 1:25 or greater when the CF and/or MI tests were negative. Four weeks after infection the MC response was greater than the other serologic responses. Brunner et al. (1973b) compared MC, radioimmunoprecipitation (RIP), CF, MI and immunofluorescence procedures for detecting M. pneumoniae antibodies in experimentally infected adult volunteers. The MC and RIP techniques were considerably more sensitive. Mycoplasmacidal activity was detected in preinoculation sera and those volunteers who did not develop illness had considerably higher MC titers in preinoculation sera than did those who became ill. Purcell et al. (1967) studied the relationship between MI and MC antibodies using antisera to M. pneumoniae, M. hominis, M. fermentans and M. pulmonis. It was speculated that the MC titers could be due to agglutination rather than growth inhibition. However, because of the good agreement between the MC and MI titers, the 10 million-fold decrease in growth

by hyperimmune serum, and the steepness of the inhibition curves, it was felt that the procedure measured MC antibodies and that these antibodies were similar or identical to MI antibodies. Brunner et al. (1971) demonstrated that hyperimmune rabbit serum against M. pneumoniae membrane glycolipids could sensitize M. pneumoniae to the lytic action of the heat-labile accessory factor. Membrane glycolipids could block the action of MC antibodies against M. pneumoniae (Brunner et al., 1972).

Priestley (1952) demonstrated the mycoplasmacidal action of blood from vaccinated or recovered animals for M. mycoides. This reaction was dependent on a specific heat-stable substance (antibody) and a nonspecific heat-labile component (presumably complement). Cottew (1963) was able to detect growth inhibiting antibodies in sera of cattle which had no detectable CF antibodies. He suggested that the reaction may be aggregation of M. mycoides by complement and conglutinin rather than true inhibition. Gourlay and Domermuth (1967) reported that immune inhibition of M. mycoides was not dependent on a heat-labile factor and that the reaction often occurred without agglutination. The mycoplasmacidal test was approximately as sensitive as CF but more sensitive than the slide agglutination test for detection of antibodies in 10 cattle infected with M. mycoides. Davies (1969a) used the fermentation inhibition test to detect both mycoplasmastatic and mycoplasmacidal antibodies in rabbits inoculated with M. mycoides. After subcutaneous inoculation, mycoplasmastatic activity was detected which was dependent on a heat-labile accessory factor. After intravenous boosters, both mycoplasmacidal and mycoplasmastatic antibodies were detected and their activity was enhanced by, but not dependent on, heat-labile factor. Davies and Hudson (1968) found no

correlation between growth inhibition titers as measured by MI or MC tests and immunity in cattle to contagious bovine pleuropneumonia.

As previously mentioned, Cole and coworkers were unable to detect MI antibodies against M. arthritidis in rats and only very low levels in mice. In attempts to demonstrate phagocytosis of M. arthritidis by murine peritoneal macrophages, it was discovered that rat and mouse antisera in the absence of macrophages were mycoplasmacidal for M. arthritidis in Eagle's minimal essential medium (MEM), but when macrophages were included, the antisera had no effect. Because macrophages allowed multiplication of the organisms and MEM by itself did not, it was speculated that the antibody in the mouse and rat antisera was inhibitory to resting mycoplasmas, but not to actively growing organisms (Cole and Ward, 1973c). Using MEM as the base medium, Cole and Ward (1973a) were able to detect complement-dependent mycoplasmacidal activity in rats as early as 2 days after inoculation of M. arthritidis and the response persisted at least through 300 days. Five day MC antibody was detected in the IgM fraction, whereas 42 day antibody was IgG. Mice developed MC antibodies by 3 days postinoculation and titers persisted through 487 days. Five day mouse serum had IgM MC activity. Mycoplasmacidal activity in rats peaked at 7 days and circulating mycoplasmas could be detected in the peripheral circulation through 5 days post infection. It was suggested that the rat mycoplasmacidal antibody against M. arthritidis may be directed towards minor antigenic membrane components, while the major antigen may be nonantigenic due to its antigenic similarity to rat tissue antigens. An antibody which is inefficient against actively growing organisms may play no role in immunity but may play a role in the disease process.

Matsumoto and Yamamoto (1972; 1973) have investigated the mycoplasmacidal action of rabbit immune sera on Mycoplasma meleagridis. Two systems were identified, a complement-dependent system which resulted in a rapid decrease in colony forming units, and a complement-independent system which suppressed the multiplication of the organism and resulted in a slower killing. The dependent titers in rabbit sera were very high at 7, 21 and 90 days whereas the independent titers were relatively low. The activity for both systems was with IgM in the early sera and IgG in the later sera.

Barker and Patt (1967) found that immune inactivation of M. gallisepticum required complement. Taylor-Robinson and Berry (1969) noted deposits of organisms in wells of FI plates where there were high dilutions of antiserum. They found a close correlation between the FI titers and the deposit inhibition titers, and therefore presumably growth inhibiting titers. Woode and McMartin (1973) concluded that the antigens of M. gallisepticum associated with the inhibition of growth were physiologically active receptors on the cell membrane.

Serologic Response of Swine to Mycoplasmas

Mycoplasma hyosynoviae

Antibody to Mycoplasma hyosynoviae in swine has been studied most often with complement fixation techniques. Ross (1973b) detected CF antibodies to M. hyosynoviae within 10 days after intranasal inoculation of a virulent strain. Two pigs infected with a noninvasive strain did not have detectable CF antibodies by 1 month postinoculation, although M. hyosynoviae could be isolated from nasopharyngeal secretions. Ross and Spear (1973)

were able to detect CF antibodies in the sera of many infected sows and their 1-week-old nursing piglets. Complement fixing antibodies were not usually detected in the piglets at 4 weeks of age, but often were detectable at 7, 8 and 12 weeks of age, probably indicating actively produced antibodies. Metabolic inhibiting antibodies were not detectable in the sows. Potgieter and Ross (1972) detected MI and latex agglutinating activity in hyperimmune swine, rabbit and rooster antisera against M. hyosynoviae. Latex agglutinating titers were comparable for all the sera, but rabbit antisera had high MI titers, pig sera moderate titers and rooster and turkey sera low titers. The occurrence of macroglobulin-like, warm anti-human O and anti-rabbit gamma globulin antibodies in naturally occurring M. hyosynoviae arthritis was reported by Aho et al. (1966).

Mycoplasma hyorhinis

The indirect hemagglutination test (IHA) was used by Ross and Switzer (1963) in attempts to detect antibodies in natural and experimental M. hyorhinis infections. Low levels of antibody were detected in two of seven naturally infected pigs, whereas sera collected from 18 pigs 7 to 21 days after experimental infection did not have detectable IHA antibodies. The MI test (either inhibition of glucose fermentation or tetrazolium reduction) has been used most often with M. hyorhinis infections. Pigs infected intraperitoneally (Barden and Decker, 1971; Ross et al., 1973), intranasally and by exposure to infected animals (Goiš, 1968) developed detectable MI antibodies. Barden and Decker detected a response by 2 weeks postinoculation, while Ross et al. were unable to detect MI activity until at least 6 weeks after infection. Both groups of workers found MI antibodies in synovial fluid at greater levels than in serum. Goiš et al.

(1972) infected 6-day-old gnotobiotic piglets with M. hyorhinis and detected LA activity, but not MI, from 14 to 35 days postinfection. The LA activity was chiefly IgM. Ross et al. (1973) detected CF antibodies by 2 weeks after intraperitoneal infection of pigs with M. hyorhinis, as well as MI antibodies at 6 weeks after infection. Complement fixing and MI antibodies were still detectable at moderate levels in two pigs at 1 year. Serum CF and MI antibodies were shown to be principally IgG. The level of CF antibodies in synovial fluid from infected pigs was usually higher than in serum. Metabolic inhibiting antibodies correlated better with active arthritis lesions than did CF antibodies. Sera by 2 weeks postinoculation contained low amounts of agglutinins for sheep red blood cells sensitized with rabbit or swine antibody and for tanned sheep red blood cells sensitized with swine globulin. Agglutinins for globulin-sensitized latex particles were found in synovial fluid from arthritic joints but not in sera. Goiš et al. (1974) examined the protective value of intraperitoneally injected hyperimmune pig serum, IgG and IgM against M. hyorhinis administered intranasally to gnotobiotic piglets. Control infected piglets and those receiving nonspecific IgG and IgM showed dissemination of M. hyorhinis from the upper and lower respiratory tract. Piglets treated with specific whole serum, IgG or IgM had colonization of M. hyorhinis in the respiratory tract but no dissemination of the organisms. The MI test detected primarily IgG whereas LA and IHA tests detected chiefly IgM. Barden and Prescott (1973) examined the antigens of M. hyorhinis by using complement fixation and blocking of tetrazolium reduction inhibition to test lipid, carbohydrate and protein fractions of M. hyorhinis. They

determined that the major antigenic component of M. hyorhinis was protein and that this could be found in the cell membrane and in soluble cell contents.

Mycoplasma hyopneumoniae

The test used most often for studying the humoral response of swine to Mycoplasma hyopneumoniae, the causative agent of a chronic pneumonia of swine, has been complement fixation. In both experimental and natural infection, CF antibodies were detected 2 to 3 weeks after infection and could be demonstrated as long as 267 days postinfection (Boulanger and L'Ecuyer, 1968; Takatori et al., 1968; Hodges and Betts, 1969; Slavik and Switzer, 1972). Fairly good correlation was reported between development of lesions and presence of CF antibodies (Boulanger and L'Ecuyer, 1968; Takatori et al., 1968; Slavik and Switzer, 1972). Slavik and Switzer also detected CF antibodies as early as 5 weeks after pigs were contact-exposed to M. hyopneumoniae. The CF antibodies were probably 7S immunoglobulins. The indirect hemagglutination test was used by Lam and Switzer (1972) to characterize the immune response of experimentally infected pigs and IHA titers were detected 2 to 3 weeks postinfection and peaked by 8 to 11 weeks after infection. These levels were maintained through 28 weeks postinoculation when titers declined. Antibodies were still detectable at 47 weeks postinoculation. Contact infected pigs developed detectable IHA titers 7 to 8 weeks after exposure. High titers were reached by 13 to 19 weeks and maintained through 28 weeks after contact. Holmgren (1974b) used the IHA test to characterize the immune response of pigs to intranasal inoculation with M. hyopneumoniae. Serum IHA antibodies were detected at 2 to 4 weeks after infection and reached peak titers at 8 to 9

weeks postinfection. The IHA antibodies of one pig were characterized as IgG and IgA. Indirect hemagglutinating antibodies were found in tracheobronchial secretions at 2 and 4 weeks after infection and persisted through 13 weeks postinfection in one pig. The antibody activity of the secretions was mainly associated with IgA. Lam and Switzer (1971) and Holmgren (1974a) demonstrated a good correlation between pneumonic lesions and IHA antibodies. Goodwin et al. (1969a) utilized MI, IHA and CF tests to detect humoral responses in pigs experimentally infected with M. hyopneumoniae and later challenged with the same organism. Previous infection provided strong immunity to reinfection. The MI test detected nonspecific inhibitory substances in pre- and postinfection sera. Complement fixing titers were detected earlier and declined more rapidly than IHA titers. Comparable titers were detected in pigs vaccinated with formalinized antigen but not resistant to intranasal challenge (Goodwin et al., 1969b). A pregnant sow was given nonformalinized vaccine and developed high levels of IHA and CF antibodies in the colostrum which were absorbed by the suckling piglets. Neither the sow nor piglets resisted challenge infection.

MATERIALS AND METHODS

Mycoplasma Strain

The Mycoplasma hyosynoviae strain used throughout this study was prototype strain S16 which had been characterized previously (Ross and Karmon, 1970).

Growth of Mycoplasma hyosynoviae and Antigen Production

One mycoplasmal medium (D-TS) used for propagation of M. hyosynoviae consisted of Bacto PPLO broth[1], 15% (v/v) heated turkey serum and 0.5% (w/v) Bacto bacteriological mucin[1] (Ross and Karmon, 1970). Medium D-HS consisted of PPLO broth, 15% (v/v) unheated horse serum and 5% (v/v) yeast extract (Cole and Ward, 1973a). A medium (D-SF) modified from Morton (1966) consisted of PPLO broth, 1% (v/v) Bacto PPLO serum fraction[1], 10% (v/v) fresh yeast extract and 0.5% (w/v) bacteriological mucin. Two agar media were used for determination of colony forming units (CFU); PPLO agar[1] with 15% (v/v) heated turkey serum and 0.5% (w/v) bacteriological mucin (D-TS agar) or beef heart infusion, 1% (w/v) Noble agar, 15% (v/v) heated turkey serum and 1% (w/v) peptone[1] (BHI-TS agar) (Ross and Karmon, 1970). Agar media and D-TS used for growth of mycoplasmas for vaccines and complement fixing antigen contained 1:4000 thallium acetate as a bacterial inhibitor.

Mycoplasmal cells for vaccine preparation were grown in D-TS for 48 hours at 37C, examined microscopically for bacterial contamination and

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[1]Difco Laboratories, Detroit, Michigan.

harvested at 16,000 x g for 1 hour in a refrigerated, angle head centrifuge.[1] The harvest was resuspended to approximately 0.5% of the original volume in Hank's balanced salt solution[2] (HBSS-1x) and the number of colony forming units present were determined before freezing using BHI-TS agar. Aliquots of the culture were stored at -70C. For disruption, aliquots of cells were thawed, diluted in HBSS-1x to contain 10 billion cells/ml and sonicated while in an ice bath for 30 minutes with a Biosonik II sonicator[3] at a 50% setting. The sonicated preparations were heated for 20 minutes at 56C and stored at -70C.

Cells for CF antigen were grown in D-TS for 24 hours, harvested at 23,000 x g for 1 hour in a refrigerated angle head centrifuge and washed two times in 0.85% NaCl solution (saline) at 27,000 x g. The cells were resuspended in saline and heated for 30 minutes at 56C before use.

Culture for the latex agglutination test was grown in D-SF for 41 hours, harvested at 16,000 x g for 1 hour and washed five times at 27,000 x g for 15 minutes in saline prepared with deionized water to lessen spontaneous agglutination of the cells. The antigen was resuspended in saline and stored at -20C.

Mycoplasma culture for the metabolic inhibition and mycoplasmacidal tests was grown in D-TS and D-HS for 24 hours and aliquots were stored at -70C. Colony counts were performed on thawed aliquots as described previously.

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[1]Ivan Sorvall Inc., Newtown, Connecticut.

[2]Grand Island Biological Company, Grand Island, New York.

[3]Bronwill Scientific, Rochester, New York.

Experimental Pigs

The swine used in this study were obtained from a herd maintained at the Veterinary Medical Research Institute, Iowa State University. They were housed in isolation units with concrete floors and fed 16% protein swine grower rations without added antibiotics or other growth stimulants. Pharyngeal and nasal swabs were collected prior to the beginning of the experiment and were found to be negative for mycoplasmas (Ross and Spear, 1973). Six Yorkshire swine 16 weeks of age were used; two pigs (7541B and 7550G) were vaccinated intramuscularly with 4 ml of sonicated antigen + 4 ml of incomplete Freund's adjuvant[1] (ICF) on day 0. Two additional pigs (7550B and 7570G) were vaccinated with the same preparation on day 0 and day 6. Two control pigs (7570B and 7552G) received one injection of 4 ml of HBSS-1x + 4 ml of ICF on day 0. All pigs were bled prior to receiving antigen on day 0 and on days 3, 6, 9, 12, 15, 21, and weeks 4, 6, 8, 10, 12, 16, 20 and 24 after vaccination. Sera were heated at 56C for 30 minutes prior to serological testing.

For comparative purposes, sera from swine which had been actively infected with M. hyosynoviae in a previous experiment were tested with the different serological procedures. One Yorkshire (Y8-2B) and two Hampshire pigs (H9-3B and H9-4B) had been infected intranasally at 5 to 6 months of age with 1 ml of a 22 hour D-TS culture containing 2.2 x 10^9 CFU/ml. Sera were collected on days 0, 3, 6, 9, 12, 15, 21, and weeks 4, 6, 8, 10, 12, 18, and 22 after infection. Pig H9-4B was bled on week 26 and at necropsy

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[1]Difco Laboratories, Detroit, Michigan.

on week 34. Pigs Y8-2B and H9-3B were bled at necropsy, 24 and 25 weeks postinfection, respectively.

Serological Procedures

Complement fixation

The complement fixation procedure used, a modification of a microtiter technique (Laboratory Branch of the Communicable Disease Center, 1965), was that of Slavik and Switzer (1972). Lyophilized guinea pig complement[1] was reconstituted with normal unheated swine serum from a 6 to 8 week old pig to circumvent the procomplementary nature of the serum. Five 50% hemolytic units of complement were used and the optimal concentration of antigen was determined by block titration. End points were determined as the highest serum dilutions which fixed 70% or more of the complement. Antibody titers were expressed as reciprocals of the serum dilutions after addition of all test reagents. Slavik and Switzer (1972) demonstrated the specificity of the CF test for homologous antibodies from pigs infected with M. hyosynoviae, M. hyorhinis or M. hyopneumoniae.

Latex agglutination

The latex agglutination test was basically the procedure of Morton (1966). The thawed antigen was homogenized with a glass Ten Broeck tissue grinder[2] because of its tendency towards spontaneous agglutination, especially after freeze-thawing. It was then diluted in glycine buffered saline (pH 8.2) with 0.2% bovine serum albumin (GBS-BSA) to an optical

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[1]Grand Island Biological Company, Grand Island, New York.

[2]Fisher Scientific Company, Chicago, Illinois.

density of 0.35 in a Coleman Junior spectrophotometer[1] at 420 nm. The organisms were further dispersed by a Biosonik II sonicator[2] for 20 seconds at 100%. Latex particles[3] (0.81 μ) were sensitized following the procedure of Morton (1966). For the tests, serial twofold dilutions of serum were made in 0.25 ml of GBS-BSA in 13 x 100 mm glass tubes. An equal volume of antigen-sensitized latex particles was added to each tube and they were incubated in a 42C waterbath for 1 hour. Control tubes were set up containing unsensitized latex plus serum, unsensitized latex plus buffer and sensitized latex plus buffer. After incubation, the tubes were centrifuged for 10 minutes at 2000 rpm in an International No. 2 centrifuge.[4] Test reactions were graded as 2+ (strong agglutination), 1+ (moderate agglutination) and ± (slight agglutination). The highest dilution of antiserum with a 1+ reaction was considered the end point. Antibody titers were expressed as reciprocals of the serum dilutions after addition of all test reagents. The specificity of the M. hyosynoviae latex procedure was determined by testing heterologous sera from swine infected with Mycoplasma hyorhinis and Mycoplasma hyopneumoniae and rabbits immunized with deoxycholate (DOC) extracts (Ross and Karmon, 1970) of M. hyorhinis, M. hyopneumoniae and Acholeplasma laidlawii.

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[1]Coleman Instrument Corporation, Maywood, Illinois.

[2]Bronwill Scientific, Rochester, New York.

[3]Difco Laboratories, Detroit, Michigan.

[4]International Equipment Company, Boston, Massachusetts.

Metabolic inhibition

Metabolic inhibiting activity was detected by a modification of the microtiter technique of Purcell et al. (1966). The diluent for the test was D-HS supplemented with 1% (w/v) L-arginine HCl and 0.002% phenol red and adjusted to pH 7.2. It was found necessary to add 1% unheated normal rabbit serum to the medium for optimal swine antibody activity (see Results section). Serial twofold dilutions of serum were made in 0.025 ml diluent in microtiter plates.[1] Thawed culture diluted 1:10 was added (0.05 ml) to all wells except the medium control wells. Unheated guinea pig serum, which had been stored at -70C, was diluted 1:10 and 0.125 ml was added to each well to enhance the metabolic inhibiting activity of the sera. The plates were sealed[2] and incubated at 37C for 24 to 30 hours. The metabolic inhibiting titer was the highest serum dilution which prevented a pH change when the organism control had changed approximately 0.5 pH units. Antibody titers were expressed as reciprocals of serum dilutions after addition of all reagents. Specificity of the metabolic inhibition test for M. hyosynoviae was determined by testing heterologous sera from swine infected with M. hyorhinis and M. hyopneumoniae and rabbits inoculated with DOC extracts of M. hyorhinis, M. hyopneumoniae and A. laidlawii.

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[1]Model IS-MVC-96, Linbro Chemical Company, New Haven, Connecticut.

[2]Microtiter plate sealers, Cooke Engineering Company, Alexandria, Virginia.

Mycoplasmacidal test

Mycoplasmacidal activity was determined with a modification of the microtiter technique of Cole and Ward (1973a).[1] The base medium consisted of Eagle's minimum essential medium[2] (MEM) supplemented to a final concentration of 1% (v/v) with a 200 mM L-glutamine solution, 10% (v/v) unheated fetal calf serum[2] (FCS) and 1000 units/ml of penicillin. The pH was adjusted to 7.2 with 1N sodium hydroxide. Serial twofold dilutions of serum were made in 0.025 ml media in microtiter plates.[3] Mycoplasma culture diluted 10^{-4} was added (0.15 ml) to all wells. Guinea pig complement[2] was diluted 1:9 and 0.025 ml was added to all wells. Organism control wells contained culture and complement, but no test serum. The microtiter plates were sealed and incubated 24 hours at 37C. After incubation, aliquots from all wells were diluted 1:9 in medium, and 0.05 ml from all undiluted and 1:9 wells were inoculated on D-TS agar. After 5 to 7 days incubation at 37C, colonies on all plates were counted. The mycoplasmacidal end point was the highest serum dilution that resulted in 75% or greater reduction in the number of viable organisms as compared to the organism controls. Antibody titers were expressed as reciprocals of serum dilutions after addition of all reagents. Specificity of the M. hyosynoviae mycoplasmacidal test for M. hyosynoviae was determined by testing heterologous sera from swine infected with M. hyorhinis and M. hyopneumoniae

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[1]Laura Golightly-Rowland, University of Utah Medical Center, Salt Lake City, Utah. Personal communication. 1973.

[2]Grand Island Biological Company, Grand Island, New York.

[3]Model IS-MRC-96, Linbro Chemical Company, New Haven, Connecticut.

and rabbits inoculated with DOC extracts of M. hyorhinis, M. hyopneumoniae and A. laidlawii.

Serum Fractionation

Fifteen selected, unheated serum samples were fractionated on Sephadex G200[1] in a 1.5 x 90 cm column[1] using upward flow with a 0.05M Tris, 0.1M NaCl, pH 8.0 buffer (Ross et al., 1973). The samples were applied in 0.5 ml amounts and collected in 3 ml fractions at an elution rate of 13 ml/hour. The optical densities of the fractions were analyzed at 280 nm using a Gilford 2400 spectrophotometer.[2] Three fractions from each of the three peaks obtained for each sample were pooled, concentrated approximately 10-fold by pervaporation and then dialyzed against four changes of Tris buffer prior to serological evaluation. Each pool was tested unheated in all four serological tests.

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[1]Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey.

[2]Gilford Instrument Laboratories, Inc., Oberlin, Ohio.

RESULTS

Complement Fixation Test

CF antibody response in vaccinated pigs

Complement fixing antibodies to Mycoplasma hyosynoviae were first detected in the four vaccinated pigs at 9 days postinoculation (PI) and were still detectable at 24 weeks PI. The two pigs (7550B and 7570G) which received two doses of vaccine developed slightly higher titers than the two pigs (7541B and 7550G) which received one dose. The individual antibody responses are given in Table 2. The geometric mean CF titers for the different groups of pigs are presented graphically (Fig. 2).

CF antibody response in infected pigs

The complement fixing antibody response of the three infected pigs, which had been determined prior to the beginning of this project, was first detected 9 days after infection and persisted through 34 weeks PI in one pig (Table 2). The response of the one infected Yorkshire, Y8-2B, paralleled the response of the vaccinated Yorkshires, with peak titers persisting from 8 weeks PI through the end of the study. The two infected Hampshires reached an early CF peak and began to decline by 6 weeks after infection.

CF antibody response in control pigs

Complement fixing antibodies were not detected in any of the sera from the control pigs.

Table 2. Complement fixing antibody titers[a] in swine vaccinated or infected with Mycoplasma hyosynoviae

Group	Pig No.	Days postinoculation						
		0	3	6	9	12	15	21
Vaccinated-1 dose[b]	7541B	<10	<10	<10	40	160	320	640
	7550G	<10	<10	<10	320	320	320	640
Vaccinated-2 doses[b]	7550B	<10	<10	<10	160	320	640	1280
	7570G	<10	<10	<10	160	640	1280	1280
Infected[c]	Y8-2B	<10	<10	<10	160	640	640	640
	H9-3B	<10	<10	<10	640	2560	2560	2560
	H9-4B	<10	<10	<10	640	2560	5120	5120
Control[d]	7570B	<10	<10	<10	<10	<10	<10	<10
	7552G	<10	<10	<10	<10	<10	<10	<10

[a]CF titer expressed as reciprocal of highest serum dilution which fixed 70% or more of the complement. Serum dilutions determined after addition of all reagents.

[b]Vaccinated pigs received 4 ml of sonicated M. hyosynoviae + 4 ml of incomplete Freund's adjuvant intramuscularly. The first dose was given on day 0 and the second on day 6.

[c]Infected pigs received broth culture of M. hyosynoviae intranasally on day 0.

[d]Control pigs received 4 ml of Hank's balanced salt solution + 4 ml of incomplete Freund's adjuvant intramuscularly on day 0.

Table 2. (Continued)

Group	Pig No.	Weeks postinoculation												
		4	6	8	10	12	16	18	20	22	24	25	26	34
Vaccinated 1 dose	7541B	640	1280	2560	2560	2560	2560	-[e]	2560	-	2560	-	-	-
	7550G	1280	640	1280	1280	1280	1280	-	1280	-	640	-	-	-
Vaccinated 2 doses	7550B	1280	1280	2560	2560	2560	2560	-	2560	-	1280	-	-	-
	7570G	1280	2560	2560	2560	2560	5120	-	2560	-	1280	-	-	-
Infected	Y8-2B	640	2560	5120	5120	5120	-	-	-	5120	5120	-	-	-
	H9-3B	2560	1280	1280	1280	640	-	640	-	320	-	320	-	-
	H9-4B	2560	1280	1280	640	640	-	640	-	640	-	-	640	640
Control	7570B	<10	<10	<10	<10	<10	<10	-	-	-	-	-	-	-
	7552G	<10	<10	<10	<10	<10	<10	-	<10	-	<10	-	-	-

[e]No sample or not tested.

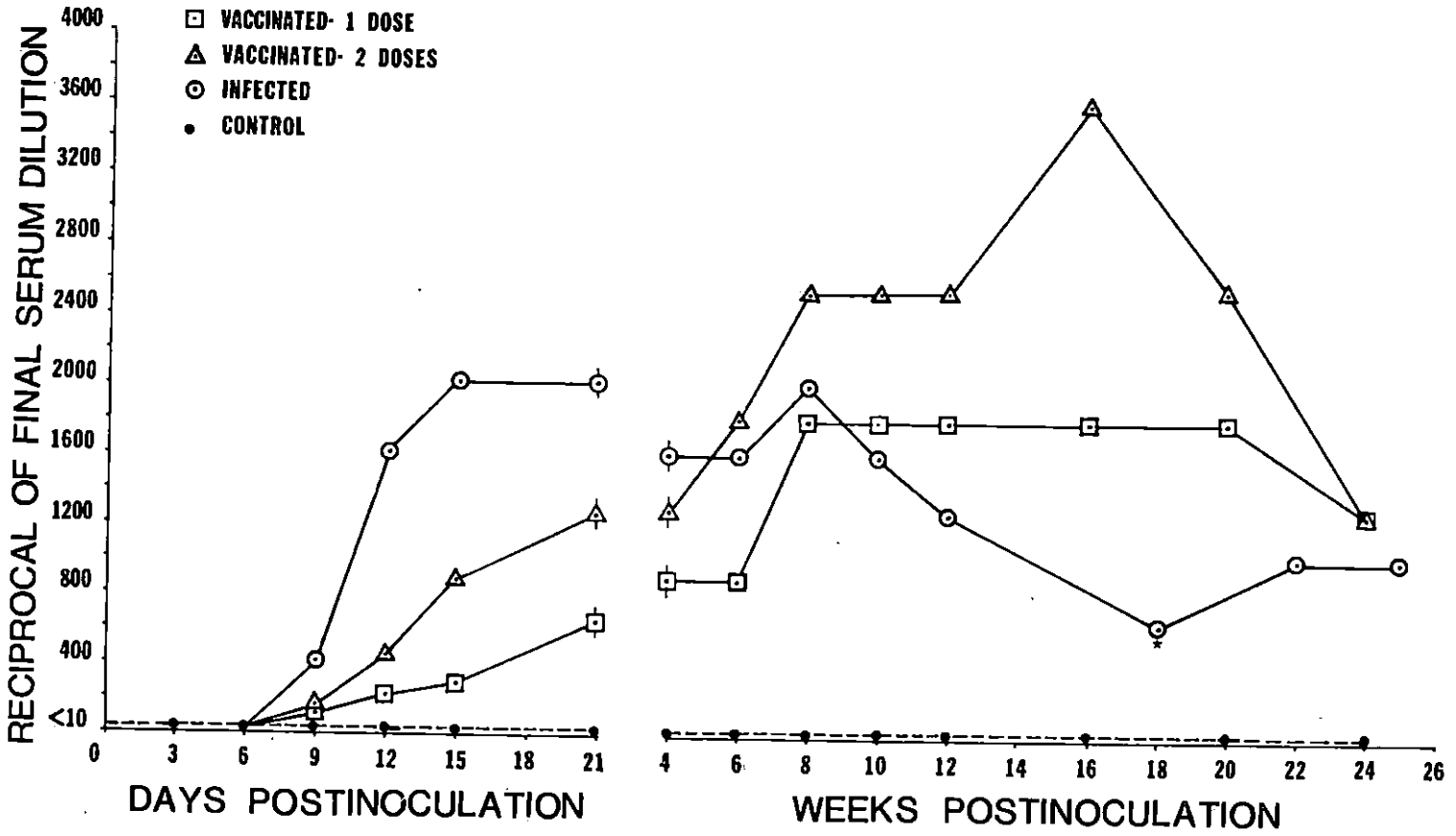

Fig. 2. Complement fixing antibody response of swine to vaccination or infection with Mycoplasma hyosynoviae. The values represent geometric mean titers for two pigs receiving one dose of vaccine, two pigs receiving two doses, three infected pigs and two control pigs. The * denotes geometric mean titer of only two samples.

Latex Agglutination Test

LA antigen production

Initially, M. hyosynoviae antigen for the LA test was grown in D-TS, however, antigen prepared from such cells tended to autoagglutinate when mixed with latex. Likewise, similar problems were encountered with antigen grown in D-HS medium. Cells grown in PPLO medium with 1% serum fraction (D-SF) yielded a smoother antigen preparation, but it was still necessary to homogenize and sonicate the cells, especially after freeze-thawing, to prevent autoagglutination.

Reproducibility of the LA test

Utilizing the procedure of Morton (1966) and D-SF antigen, serum from hyperimmunized swine 7-4 had an LA titer of 20 in 10 of 11 tests. In one test, a titer of 40 was obtained.

Specificity of the LA test

There were no cross reactions detected with the LA test between M. hyosynoviae antigen and convalescent sera from pigs infected with M. hyorhinis or M. hyopneumoniae or rabbit antisera against M. hyorhinis, M. hyopneumoniae or A. laidlawii.

LA antibody response in vaccinated pigs

Low titers of latex agglutinating antibodies were detected at 6 days postvaccination in both groups of vaccinated pigs (Table 3). A peak geometric mean titer of 113 was found at both 12 and 15 days postvaccination (Fig. 3). Little difference was found between the two different vaccination procedures. Sera from both groups of pigs reacted very weakly in the LA test with many 1+ and ± reactions.

Table 3. Latex agglutinating antibody titers[a] in swine vaccinated or infected with Mycoplasma hyosynoviae

Group	Pig No.	Days postinoculation						
		0	3	6	9	12	15	21
Vaccinated-1 dose[b]	7541B	<2	<2	40	40	80	80	80
	7550G	<2	<2	40	80	160	160	80
Vaccinated-2 doses[b]	7550B	<2	<2	20	20	80	80	40
	7570G	<2	<2	20	160	160	160	160
Infected[c]	Y8-2B	2	2	40	640	1280	2560	1280
	H9-3B	2	2	20	320	1280	1280	1280
	H9-4B	10	10	80	640	2560	2560	2560
Control[d]	7570B	<2	-[e]	-	<2	-	-	-
	7552G	<2	-	-	<2	-	-	-

[a]LA titer expressed as reciprocal of highest serum dilution giving a 1+ agglutination. Serum dilutions determined after the addition of all reagents.

[b]Vaccinated pigs received 4 ml of sonicated M. hyosynoviae + 4 ml of incomplete Freund's adjuvant intramuscularly. The first dose was given on day 0 and the second on day 6.

[c]Infected pigs received broth culture of M. hyosynoviae intranasally on day 0.

[d]Control pigs received 4 ml of Hank's balanced salt solution + 4 ml of incomplete Freund's adjuvant intramuscularly on day 0.

[e]No sample or not tested.

Table 3. (Continued)

Group	Pig No.	Weeks postinoculation												
		4	6	8	10	12	16	18	20	22	24	25	26	34
Vaccinated 1 dose	7541B	80	80	40	20	40	40	-	20	-	20	-	-	-
	7550G	80	40	40	20	10	20	-	20	-	20	-	-	-
Vaccinated 2 doses	7550B	40	40	20	20	20	20	-	10	-	10	-	-	-
	7570G	160	160	160	80	160	80	-	80	-	20	-	-	-
Infected	Y8-2B	640	640	640	640	320	-	-	-	320	160	-	-	-
	H9-3B	1280	640	640	320	320	-	160	-	160	-	160	-	-
	H9-4B	2560	640	640	640	640	-	320	-	160	-	-	160	80
Control	7570B	-	<2	-	-	<2	<2	-	-	-	-	-	-	-
	7552G	-	<2	-	-	<2	-	-	-	-	<2	-	-	-

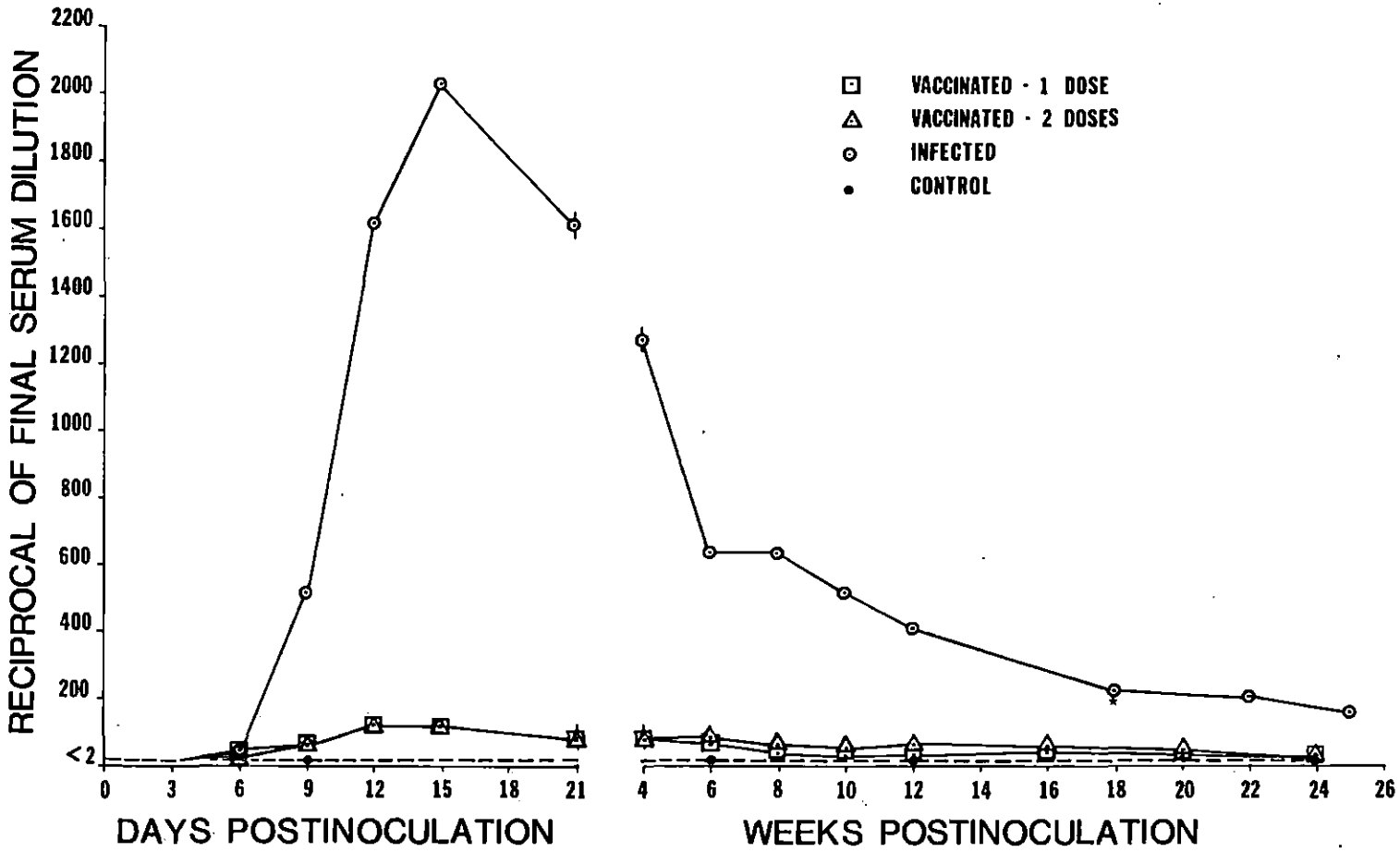

Fig. 3. Latex agglutinating antibody response of swine to vaccination or infection with Mycoplasma hyosynoviae. The values represent geometric mean titers for two pigs receiving one dose of vaccine, two pigs receiving two doses, three infected pigs and two control pigs. The * denotes geometric mean of only two samples.

LA antibody response in infected pigs

Latex agglutinating antibodies to M. hyosynoviae were initially detected in infected pigs at 6 days postinoculation. They increased rapidly to a geometric mean titer of 2032 at 15 days PI (Fig. 3). Titers then began to drop but were still detectable at 34 weeks PI at higher levels than in the vaccinated pigs (Table 3). The LA response of infected pigs was strong with many 2+ reactions.

LA antibody response in control pigs

Latex agglutinating antibodies were not detected in any of the sera from control pigs.

Metabolic Inhibition Test

Development of a modified MI procedure

Initially the MI test was performed with M. hyosynoviae culture grown in D-TS medium and utilizing D-TS-1% arginine with and without 10% yeast as the test diluent. Only a few swine sera had detectable MI titers with this system and these results were not consistently reproducible. Hyperimmune rabbit serum R112, however, gave consistently high titers each time the test was done. Another laboratory was able to detect MI antibodies in some of the swine sera using antigen grown in D-HS and performing the test in a D-HS-1% arginine medium.[1] Utilizing this system, some sera from infected pigs had MI titers, but the results of sequentially collected sera from the same pigs were sometimes inconsistent; i.e., infected pig H9-4B

[1]Dr. B. C. Cole, University of Utah Medical Center, Salt Lake City, Utah. Personal communication. 1973.

had no MI titer through 12 days PI, a weak reaction at 15 days PI, negative results at 3 and 4 weeks PI and some positive or weak reactions at 6 weeks PI and thereafter. Throughout all of these various procedures, the titers in rabbit antisera remained relatively constant. With this observation it was decided to add various concentrations of unheated normal rabbit serum (NRS) to the D-HS-arginine medium in the hope that NRS would provide some additional factor(s) required by swine sera and not provided by the unheated horse serum in the medium or the unheated guinea pig serum (GPS) added to the microtiter test system. Without NRS, no or low titers were obtained with the two swine sera tested, but the rabbit antiserum had a high MI titer. Use of 0.2% NRS resulted in only ± reactions with one swine serum tested, whereas concentrations of 0.5%, 1.0% and 2.0% NRS yielded moderately high titers with the same serum (Table 4). The other swine serum had relatively high levels of MI antibodies with all concentrations of NRS. The rabbit antiserum had about the same titers with or without NRS. For subsequent tests, 1% NRS was used as supplement in the medium. With this modification, certain swine sera which had no or low titers in the original procedure demonstrated reproducible moderate to high MI titers (Table 5).

The nature of the factor(s) supplied by NRS was examined. Whether the NRS could provide the necessary factors without added GPS was also determined. Four different test systems were used, all of which had D-HS-arginine as the base medium. System 1 was the original MI procedure with unheated GPS added to the test system. System 2 was the modified MI procedure with 1% unheated NRS added to the base medium and unheated GPS in the test system. System 3 utilized 1% heated NRS (56C for 30 minutes) and

Table 4. Effect of unheated normal rabbit serum (NRS) on the metabolic inhibiting activity[a] of Mycoplasma hyosynoviae antibody: comparison of different concentrations

Serum	Concentration of supplemental[b] unheated NRS				
	0%	0.2%	0.5%	1.0%	2.0%
H9-4B 34 weeks infected pig	<16	±64[c]	128	128	128
Y8-2B 24 weeks infected pig	16	512	1024	1024	1024
R112 hyperimmune rabbit	4096	4096	8192	8192	8192
Normal pig	<16	<16	<16	<16	<16

[a]MI titer expressed as reciprocal of highest serum dilution which prevented a pH change when the organism control had changed approximately 0.5 pH units. Serum dilutions determined after the addition of all reagents.

[b]Base medium consisted of D-HS with 1% arginine and 0.002% phenol red.

[c]± indicates weak reaction.

unheated GPS. System 4 had 1% unheated NRS but no GPS. Four sera tested for MI antibodies against M. hyosynoviae included those from an infected pig, a normal pig, a hyperimmune horse[1], and a hyperimmune rabbit. As can be seen by the results in Table 6, MI antibodies in hyperimmune rabbit serum required unheated GPS but not the supplemental unheated NRS. Metabolic inhibiting antibodies in hyperimmune horse serum were active regardless of whether NRS was heated or not. The swine serum collected 34 weeks postinfection required both unheated NRS and unheated GPS for its activity.

[1]Kindly supplied by Dr. O. Stalheim, National Animal Disease Center, Ames, Iowa.

Table 5. Effect of unheated normal rabbit serum (NRS) on the metabolic inhibiting activity[a] of Mycoplasma hyosynoviae antibody: comparison of serum collected from pig H9-4B at various stages following infection using medium with and without NRS[b]

Time postinoculation	Medium[c]	
	Without 1% NRS	With 1% NRS
0 day	<16	<16
3 day	<16	<16
6 day	<16	<16
9 day	<16	256(128)[d]
12 day	<16	8192(8192)
15 day	±64[e]	8192(4096)
21 day	<16	8192(4096)
4 week	<16	2048(2048)
6 week	32(32;±16)	1024
8 week	16(16;±32)	256
10 week	16(32;16)	512
12 week	±16(±32)	256
18 week	16(32;32)	256
22 week	32(16)	256
26 week	32(32)	256
34 week	32(32;<16)	256(256;256)

[a]MI titer expressed as reciprocal of highest serum dilution which prevented a pH change when the organism control had changed approximately 0.5 pH units. Serum dilutions determined after the addition of all reagents.

[b]The results were determined on different days.

[c]Base medium consisted of D-HS with 1% arginine and 0.002% phenol red.

[d]Results of replicate tests are given in parentheses.

[e]± indicates weak reaction.

Table 6. Comparison of the effect of several supplemental serum preparations on the Mycoplasma hyosynoviae metabolic inhibiting titer[a] of sera from different animals

Serum	Supplemental normal sera added to MI test			
	10% unheated GPS[b]	10% unheated GPS + 1% unheated NRS[c]	10% unheated GPS + 1% heated NRS[d]	1% unheated NRS
H9-4B 34 week infected pig	<16	128	<16	<16
NADC hyper-immune horse[e]	-[f]	4096	2048	-
R112 hyper-immune rabbit	8192	8192	8192	16
Normal pig	<16	<16	<16	<16

[a]MI titer expressed as reciprocal of highest serum dilution which prevented pH change when the organism control had changed approximately 0.5 pH units. Serum dilutions determined after addition of all reagents.

[b]Guinea pig serum added to the microtiter test system.

[c]Normal rabbit serum incorporated into the MI base medium consisting of D-HS with 1% arginine and 0.002% phenol red.

[d]Heated at 56C for 30 minutes.

[e]Kindly supplied by Dr. O. Stalheim, National Animal Disease Center, Ames, Iowa.

[f]Not done.

Heating the NRS or use of unheated NRS or unheated GPS alone resulted in no MI activity in the swine serum.

The modified MI procedure was developed with M. hyosynoviae grown in D-HS medium, but since medium constituents have been shown to alter the antigenic properties of mycoplasmas (Smith et al., 1966), culture grown in D-TS was compared to that grown in D-HS. The results (Table 7) indicate little difference between activity of the two cultures in the modified MI procedure.

Table 7. Effect of M. hyosynoviae growth medium composition on metabolic inhibiting antibody titer[a]

Serum	Medium used for culture preparation	
	D-HS[b]	D-TS[c]
H9-4B 15 day infected pig	4096	4096
H9-4B 3 weeks infected pig	4096	4096
H9-4B 34 weeks infected pig	256	128
Y8-2B 24 weeks infected pig	1024	1024
R112 hyperimmune rabbit	4096	2048
Normal pig	<16	<16

[a]MI titer expressed as reciprocal of highest serum dilution which prevented pH change when the organism control had changed 0.5 pH units. Serum dilutions determined after addition of all reagents.

[b]PPLO broth, 15% unheated horse serum and 5% yeast extract.

[c]PPLO broth, 15% heated turkey serum and 0.5% bacteriological mucin.

Reproducibility of the modified MI procedure

Use of the modified MI procedure with 1% unheated normal rabbit serum and D-HS antigen resulted in reproducible MI titers which never varied more than one twofold dilution for both swine and rabbit serum. Pig serum Y8-2B (24 weeks PI) had MI titers of 1024 in seven tests and a titer of 512 in one test. Serum from pig H9-4B (34 weeks PI) had MI titers of 128 five times and titers of 256 four times. Hyperimmune rabbit R112 had MI titers of 8192 in six tests and 4096 in three tests.

Specificity of the MI procedure

There were no cross reactions detected with the modified MI procedure between M. hyosynoviae culture and convalescent sera from pigs infected with M. hyorhinis or M. hyopneumoniae or rabbit antisera against M. hyorhinis, M. hyopneumoniae or A. laidlawii.

MI antibody response in vaccinated pigs

The metabolic inhibiting antibody response in the two pigs which received one dose of antigen was minimal. Pig 7541B had a low response at 9 and 12 days after vaccination whereas pig 7550G had low titers of MI antibodies at 3 and 4 weeks PI (Table 8). Metabolic inhibiting antibodies were not detected in sera from these two pigs at any other time. The pigs which received two doses of vaccine developed a greater MI response (Fig. 4). Responses in pigs 7550B and 7570G were first detected 9 days PI with moderate levels of MI antibodies 12 days PI. At 4 weeks postvaccination, both animals had no detectable MI antibodies. Pig 7550B had moderate levels of MI antibodies 6 weeks postvaccination which persisted through 10 weeks PI. No MI antibodies were detected in this pig after 10

Table 8. Metabolic inhibiting antibody titers[a] in swine vaccinated or infected with Mycoplasma hyosynoviae

Group	Pig No.	Days postinoculation						
		0	3	6	9	12	15	21
Vaccinated-1 dose[b]	7541B	<16	<16	<16	16	32	<16	<16
	7550G	<16	<16	<16	<16	<16	<16	16
Vaccinated-2 doses[b]	7550B	<16	<16	<16	64	256	256	256
	7570G	<16	<16	<16	32	128	64	64
Infected[c]	Y8-2B	<16	<16	<16	128	1024	512	512
	H9-3B	<16	<16	<16	<16	512	1024	1024
	H9-4B	<16	<16	<16	256	8192	8192	8192
Control[d]	7570B	<16	-[e]	-	<16	-	-	-
	7552G	<16	-	-	<16	-	-	-

[a]MI titer expressed as reciprocal of highest serum dilution which prevented a pH change when the organism control had changed approximately 0.5 pH units. Serum dilutions determined after the addition of all reagents.

[b]Vaccinated pigs received 4 ml of sonicated M. hyosynoviae + 4 ml of incomplete Freund's adjuvant intramuscularly. The first dose was given on day 0 and the second on day 6.

[c]Infected pigs received broth culture of M. hyosynoviae intranasally on day 0.

[d]Control pigs received 4 ml of Hank's balanced salt solution + 4 ml of incomplete Freund's adjuvant intramuscularly on day 0.

[e]No sample or not tested.

Table 8. (Continued)

Group	Pig No.	Weeks postinoculation												
		4	6	8	10	12	16	18	20	22	24	25	26	34
Vaccinated 1 dose	7541B	<16	<16	<16	<16	<16	<16	-	<16	-	<16	-	-	-
	7550G	32	<16	<16	<16	<16	<16	-	<16	-	<16	-	-	-
Vaccinated 2 doses	7550B	<16	128	64	32	<16	<16	-	<16	-	<16	-	-	-
	7570G	<16	<16	<16	32	<16	<16	-	<16	-	<16	-	-	-
Infected	Y8-2B	256	512	512	512	512	-	-	-	2048	1024	-	-	-
	H9-3B	512	256	256	256	256	-	512	-	256	-	128	-	-
	H9-4B	2048	1024	256	512	256	-	256	-	256	-	-	256	128
Control	7570B	-	<16	-	-	<16	-	-	-	-	-	-	-	-
	7552G	-	<16	-	-	<16	-	-	-	-	<16	-	-	-

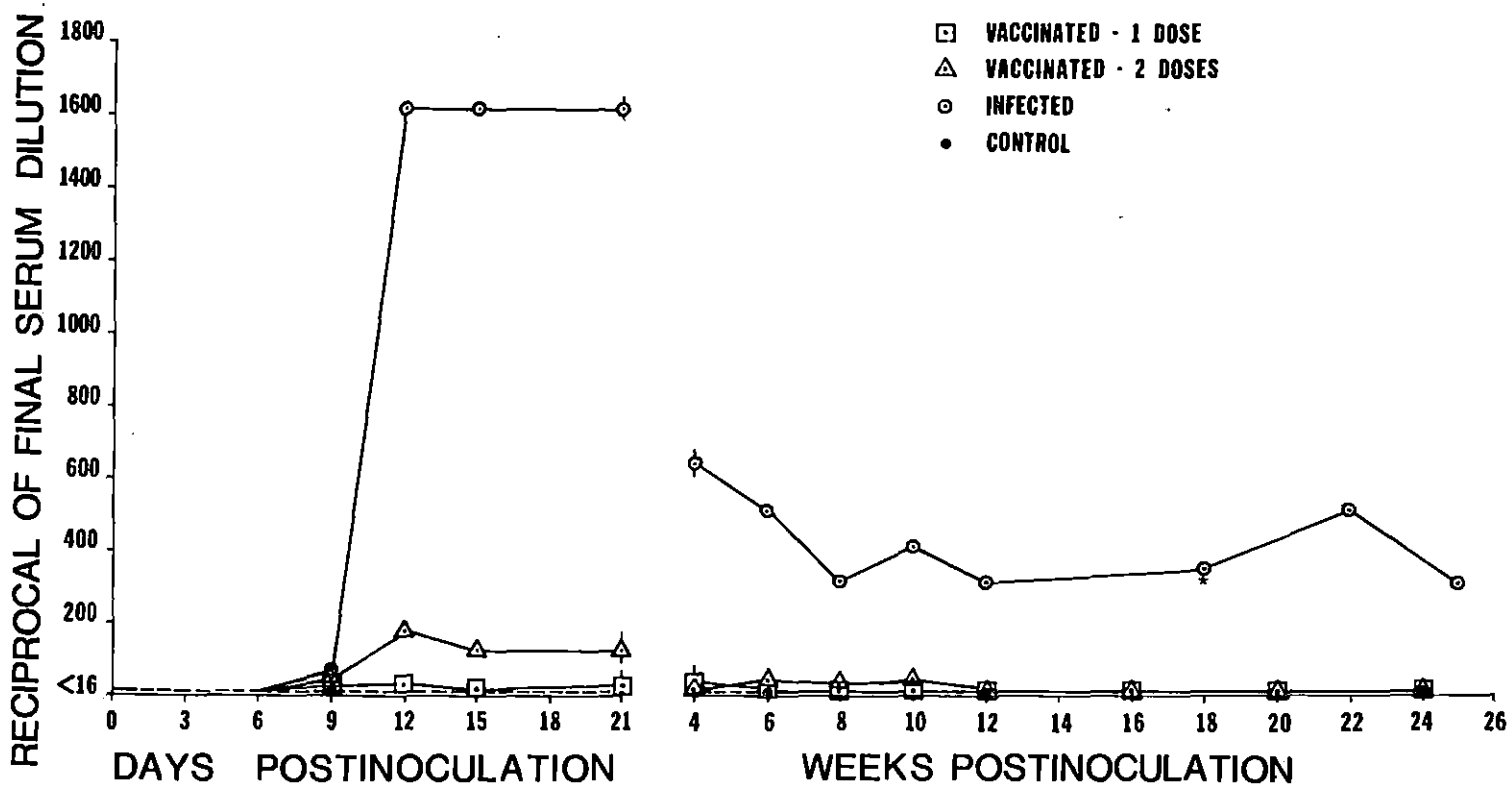

Fig. 4. Metabolic inhibiting antibody response of swine to vaccination or infection with Mycoplasma hyosynoviae. The values represent geometric mean titers for two pigs receiving one dose of vaccine, two pigs receiving two doses, three infected pigs and two control pigs. The * denotes geometric mean of only two samples.

weeks PI. Pig 7570G did not have detectable MI antibodies from 4 to 8 weeks PI and no antibody was detected after 10 weeks PI.

MI antibody response in infected pigs

The metabolic inhibiting antibody response in the three infected pigs was much greater than in the vaccinated pigs (Fig. 4). Two infected pigs (Y8-2B and H9-4B) had detectable MI antibodies by 9 days PI, whereas pig H9-3B had a response by 12 days PI (Table 8). High titers were reached by 12 to 15 days PI and moderate titers were maintained through the end of the study with the exception of Y8-2B which had an increase in titer after 12 weeks PI.

MI antibody response in control pigs

Metabolic inhibiting antibodies were not detected in any of the sera from the control pigs.

Mycoplasmacidal Test

Adaptation of the MC technique

In adapting the mycoplasmacidal technique to a swine system, various components of the technique were studied. Comparisons were made between use of medium MEM-FCS and medium D-TS as test diluent. Utilizing D-TS, which allowed active multiplication of M. hyosynoviae, sera from infected pigs did not have MC titers after 3 hours incubation of the mycoplasmas with the serum, but had low titers when 24 hours incubation was used. With medium MEM, however, swine sera had titers after both 3 and 24 hour incubation periods, but 24 hours incubation gave results which were more consistent and higher than with the D-TS system. The subsequent tests

were performed utilizing the MEM-FCS system with a 24 hour incubation period.

Because of the known tendency for M. hyosynoviae cells to clump, the culture was tested before and after filtration through a 0.45 μ filter. However, filtration appeared to have no effect on the reproducibility of the test. In another experiment, M. hyosynoviae culture was preincubated with antiserum for 1 hour before guinea pig complement was added to the test system. This preincubation period did not appear to increase the cidal activity of sera from infected swine. Comparisons between culture grown in D-HS and D-TS revealed little difference, but to be consistent with the MI procedure, D-HS grown culture was used in the MC test. Variable MC results were obtained with different lots of fetal calf serum in the medium and only one lot tested gave the best cidal results with swine serum. Certain swine sera tested had consistently good MC titers whereas with other sera, erratic results were obtained, with high titers one day and no detectable antibodies the next. Each test was set up with six to twelve identical antigen controls and the resulting colony counts of these controls were averaged before determination of the count equivalent to 75% reduction in the number of viable organisms in the test. A wide range of colony counts was often seen with these antigen controls. Another problem was encountered with certain sera which at low dilutions often had no cidal activity, but at higher dilutions these same sera had strong cidal activity.

Reproducibility of the MC test

Swine serum H9-4B (34 weeks PI) was tested with the MC procedure nine times; five of the nine times a MC titer of 160 was obtained and four

times a titer of 320. Serum H9-4B (22 weeks PI) had a titer of 320 three times and Y8-2B (24 weeks PI) had titers of 320 once and 640 twice.

Specificity of the MC test

The MC test was shown to be specific for antibodies against M. hyosynoviae and did not cross-react with convalescent sera from pigs infected with M. hyorhinis or M. hyopneumoniae or rabbit antisera against M. hyorhinis, M. hyopneumoniae or A. laidlawii

MC antibody response in vaccinated and control pigs

Mycoplasmacidal antibodies against M. hyosynoviae were not detected in any of the sera from vaccinated or control pigs at the lower limit of the test (1:40).

MC antibody response in infected pigs

Mycoplasmacidal antibodies were detected in two infected pigs (Y8-2B and H9-3B) 6 weeks PI (Table 9). Pig H9-4B had detectable MC antibodies by 8 weeks PI and all three pigs had MC antibodies which persisted through the end of the study and even appeared to be increasing through 25 weeks PI (Fig. 5).

Serum Fractionation

Serological activity of fractionated sera from vaccinated pigs

Complement fixing antibodies of two vaccinated pigs (7541B and 7550B) were found principally in the second peak (B) of Sephadex G200 fractions of sera from 9 days, 6 weeks and 24 weeks postvaccination (Table 10). Weak latex agglutinating activity was found in the first peak (A) of the samples from these same pigs. Metabolic inhibiting activity was minimal in pigs receiving one dose of vaccine and therefore was not found in any of

Table 9. Mycoplasmacidal antibody titers[a] in swine vaccinated or infected with Mycoplasma hyosynoviae

Group	Pig No.	Days postinoculation						
		0	3	6	9	12	15	21
Vaccinated-1 dose[b]	7541B	<40	<40	<40	<40	<40	<40	<40
	7550G	<40	<40	<40	<40	<40	<40	<40
Vaccinated-2 doses[b]	7550B	<40	<40	<40	<40	<40	<40	<40
	7570G	<40	<40	<40	<40	<40	<40	<40
Infected[c]	Y8-2B	<40	<40	<40	<40	<40	<40	<40
	H9-3B	<40	<40	<40	<40	<40	<40	<40
	H9-4B	<40	<40	<40	<40	<40	<40	<40
Control[d]	7570B	<40	-[e]	-	<40	-	-	-
	7552G	<40	-	-	<40	-	-	-

[a]MC titer expressed as reciprocal of highest serum dilution that resulted in 75% or greater reduction in the number of viable organisms as compared to the organism controls. Serum dilutions determined after addition of all reagents.

[b]Vaccinated pigs received 4 ml of sonicated M. hyosynoviae + 4 ml of incomplete Freund's adjuvant intramuscularly. The first dose was given on day 0 and the second on day 6.

[c]Infected pigs received broth culture of M. hyosynoviae intranasally on day 0.

[d]Control pigs received 4 ml of Hank's balanced salt solution + 4 ml of incomplete Freund's adjuvant intramuscularly on day 0.

[e]No sample or not tested.

Table 9. (Continued)

Group	Pig No.	Weeks postinoculation												
		4	6	8	10	12	16	18	20	22	24	25	26	34
Vaccinated 1 dose	7541B	<40	<40	<40	<40	<40	<40	-	<40	-	<40	-	-	-
	7550G	<40	<40	<40	<40	<40	<40	-	<40	-	<40	-	-	-
Vaccinated 2 doses	7550B	<40	<40	<40	<40	<40	<40	-	<40	-	<40	-	-	-
	7570G	<40	<40	<40	<40	<40	<40	-	<40	-	<40	-	-	-
Infected	Y8-2B	<40	160	80	80	160	-	-	-	640	640	-	-	-
	H9-3B	<40	80	160	160	320	-	320	-	160	-	160	-	-
	H9-4B	<40	<40	40	80	160	-	160	-	320	-	-	640	320
Control	7570B	-	<40	-	-	<40	-	-	-	-	-	-	-	-
	7552G	-	<40	-	-	<40	-	-	-	-	<40	-	-	-

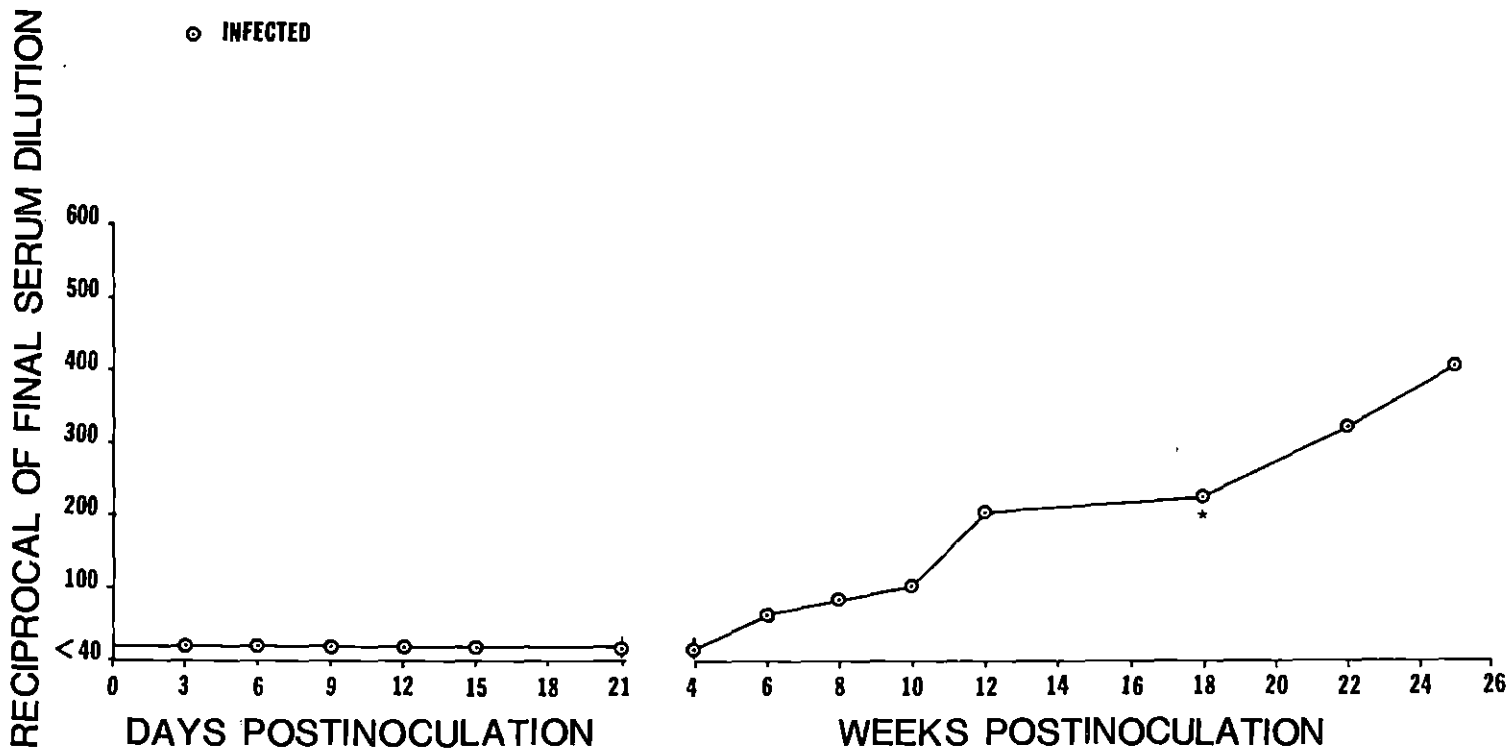

Fig. 5. Mycoplasmacidal antibody response of three swine to infection with Mycoplasma hyosynoviae. Two pigs receiving one dose of vaccine, two pigs receiving two doses and two control pigs had no detectable MC antibody at the limit of the test (1:40). The * denotes geometric mean titer of only two samples.

Table 10. Serological titers of selected whole sera and their Sephadex G200 fractions as determined by complement fixation, latex agglutination, metabolic inhibition and mycoplasmacidal tests

Group	Pig No.	Time Postinoculation	CF titer[a] Whole serum	Peak[b] A	Peak[b] B	Peak[b] C
Vaccinated 1 dose[c]	7541B	9 days	40	<10	40	<10
		6 weeks	1280	<10	320	<10
		24 weeks	2560	<10	320	20
Vaccinated 2 doses[c]	7550B	9 days	160	20	80	10
		6 weeks	1280	<10	320	20
		24 weeks	1280	<10	320	<10
Infected[f]	Y8-2B	9 days	160	<10	20	<10
		6 weeks	2560	<10	160	<10
		24 weeks	5120	<10	640	<10
Infected[f]	H9-4B	9 days	640	<10	40	<10
		6 weeks	1280	<10	80	<10
		26 weeks	640	<10	80	<10
Control[g]	7552G	9 days	<10	<10	<10	<10
		6 weeks	<10	<10	<10	<10
		24 weeks	<10	<10	<10	<10

[a]Titers are expressed as reciprocals of the highest serum dilutions giving positive reactions.

[b]Three fractions from each peak were pooled, concentrated approximately 10x and dialyzed prior to serological testing. Peak A was the first peak off the Sephadex G200 column, peak B the second and peak C the third.

[c]Vaccinated pigs received 4 ml of sonicated M. hyosynoviae + 4 ml of incomplete Freund's adjuvant intramuscularly. The first dose was given on day 0 and the second on day 6.

[d]± indicates weak reaction.

[e]Not done.

[f]Infected pigs received broth culture of M. hyosynoviae intranasally on day 0.

[g]Control pigs received 4 ml of Hank's balanced salt solution + 4 ml of incomplete Freund's adjuvant intramuscularly on day 0.

LA titer[a]				MI titer[a]				MC titer[a]			
Whole serum	Peak A	Peak B	Peak C	Whole serum	Peak A	Peak B	Peak C	Whole serum	Peak A	Peak B	Peak C
40	±2[d]	<2	<2	16	<16	<16	<16	<40	-[e]	-	-
80	±2	<2	<2	<16	<16	<16	<16	<40	-	-	-
20	±10	<2	<2	<16	<16	<16	<16	<40	-	-	-
20	±2	<2	<2	64	16	<16	<16	<40	-	-	-
40	±4	<2	<2	128	<16	16	<16	<40	-	-	-
10	±10	<2	<2	<16	<16	<16	<16	<40	-	-	-
640	10	<2	<2	128	64	<16	<16	<40	-	-	-
640	20	40	<2	512	<16	128	<16	160	<16	16	<16
160	±10	2	<2	1024	<16	128	±16	640	<16	64	<16
640	40	<2	<2	256	64	<16	<16	<40	-	-	-
640	40	20	±10	1024	64	32	<16	<40	<16	<16	<16
160	20	<2	<2	256	<16	32	<16	640	<16	64	<16
<2	<2	<2	<2	<16	<16	<16	<16	<40	-	-	-
<2	<2	<2	<2	<16	<16	<16	<16	<40	-	-	-
<2	<2	<2	<2	<16	<16	<16	<16	<40	-	-	-

the fractions from pig 7541B. The pigs receiving two doses of vaccine had greater MI responses and slight activity was found in peak B from pig 7550B (6 weeks PI). No mycoplasmacidal activity was found in the unfractionated sera from vaccinated pigs so the fractions were not tested by this procedure.

Serological activity of fractionated sera from infected pigs

Complement fixing activity of sera from infected pigs Y8-2B and H9-4B was in the second peak of all samples examined, 9 days and 6 weeks PI for both pigs, 24 weeks PI for Y8-2B and 26 weeks PI for H9-4B (Table 10). Pig Y8-2B had latex agglutinating activity in the first peak (A) at 9 days and 24 weeks PI and in peaks A and B at 6 weeks PI. The LA activity of pig H9-4B was mainly in the first peak, with some additional activity in peaks B and C at 6 weeks PI. The 9 day PI samples from both pigs had metabolic inhibiting activity in peak A. By 6 weeks PI, the MI activity of pig Y8-2B was present only in peak B and at 24 weeks PI minimal activity was also demonstrated in peak C. Pig H9-4B had MI activity in peaks A and B at 6 weeks PI and in peak B at 26 weeks PI. Mycoplasmacidal activity in sera from pig Y8-2B was found only in peak B at both 6 and 24 weeks PI. The unfractionated serum from Y8-2B at 9 days PI had no MC activity. Likewise, no MC activity was detected in unfractionated serum from H9-4B at 9 days and 6 weeks PI. At 26 weeks PI, H9-4B had MC activity in peak B.

DISCUSSION

In the present study, swine vaccinated or infected with Mycoplasma hyosynoviae were found to develop antibody responses measurable by four serologic techniques: complement fixation, latex agglutination, metabolic inhibition and mycoplasmacidal tests. Evidence obtained indicated that the response in infected pigs was more complex than in vaccinated pigs. Understanding the immune response in infected pigs may help to understand the pathogenesis of arthritis caused by the organism and characterization of the immune response induced with the vaccine may help in developing a protective immunogen for use in control of the naturally occurring disease. Vaccine prepared with incomplete Freund's adjuvant cannot be used in meat producing animals but results obtained with vaccine combined with this adjuvant may also help in evaluating more acceptable adjuvants. In fact, preliminary findings indicate that vaccines prepared with M. hyosynoviae and incomplete Freund's adjuvant do induce appreciable protective immunity against the organism.[1]

Differences were detected between the responses of the M. hyosynoviae vaccinated and infected pigs by use of LA, MI and MC tests, but the CF antibody responses were similar in the time of first detection, magnitude of peak titers and persistence of the response. Differences were, however, detected in the CF responses of the two different breeds of pigs. Infected Hampshires had early peak CF titers which started to decline by 4 weeks PI. The one infected Yorkshire studied and the vaccinated

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[1]Dr. R. F. Ross, Iowa State University, Ames, Iowa. Personal communication. 1972.

Yorkshires developed peak titers at about 8 weeks PI which persisted through at least 24 weeks PI. The relationship between the greater susceptibility of Hampshires to M. hyosynoviae arthritis (Ross et al., 1971; Ross, 1973b) and the more marked initial CF antibody response is not understood. Differences between the breeds of pigs were not detected with the LA test, although infected pigs developed much higher levels of LA antibodies than vaccinated pigs. There was no difference in onset of the response or occurrence of the peak titers and essentially no differences were detected in the LA responses of the pigs receiving different doses of vaccine. With the MI test, however, antibody titers appeared to be dependent on the dose of antigen. Pigs receiving two doses of vaccine had much higher and more persistent MI antibodies than did pigs receiving one dose of vaccine. Infected pigs had still higher levels of MI antibodies which persisted through the end of the study. Onset of the MI response in each group of pigs occurred at about the same time except for the delayed response in one pig which received one dose of antigen. The early detection of MI antibodies in this study agrees with the results of Barden and Decker (1971) who were able to detect MI antibodies 2 weeks after intraperitoneal inoculation of pigs with M. hyorhinis. In contrast, Ross et al. (1973) and Goiš et al. (1972) did not detect MI antibodies in sera from pigs infected with M. hyorhinis until 6 weeks PI. In the present study, MC antibodies were not detected in any of the sera from the pigs vaccinated with M. hyosynoviae but were detected in the infected pigs by 6 to 8 weeks PI and persisted through the end of the study. In contrast, Cole and Ward (1973a) were able to detect MC antibodies in rats by 2 days after infection with M. arthritidis and by 3 days PI in mice.

Sephadex G200 gel filtration of early and late sera from swine infected and vaccinated with M. hyosynoviae resulted in three peaks. The first peak from gel filtration of swine serum has been characterized as mainly IgM and the second peak as mainly IgG (Saif et al., 1972; Ross et al., 1973; Holmgren, 1974b). Holmgren also detected a small amount of high molecular weight IgG in the first peak. Immunoglobulin A was detected at the end of the first peak and the beginning of the second. As expected, CF antibodies in the M. hyosynoviae vaccinated and infected pigs were detected chiefly in the second peak at all intervals examined (9 days, 6 weeks, and 24 or 26 weeks PI). One vaccinated pig also had small amounts of CF antibodies in the first peak at 9 days PI. It was shown previously that CF antibody responses in two other mycoplasmal diseases of swine, M. hyorhinis polyserositis and arthritis and M. hyopneumoniae pneumonia, consist mainly of IgG (Ross et al., 1973; Slavik and Switzer, 1972). The finding in the current study that LA antibody activity was mainly associated with the first Sephadex peak agrees well with the work of Goiš and coworkers in which it was shown that LA activity against M. hyorhinis was in the IgM fractions of serum from gnotobiotic piglets infected with that organism (Goiš et al., 1972) and serum from hyperimmunized swine (Goiš et al., 1974). Results obtained in the present study indicate that the early MI response to M. hyosynoviae in both infected and vaccinated pigs is IgM and that later in the response, IgG is the main antibody. These results agree well with Ross et al. (1973) who found mainly IgG as the late MI antibody response in pigs infected with M. hyorhinis. Mycoplasmacidal antibody against M. arthritidis was found in the IgG fraction in late sera of rats (Cole and Ward, 1973a), and in the

present work, IgG was the immunoglobulin class with MC activity in the late immune sera from the M. hyosynoviae infected pigs.

Vaccinated and infected pigs, therefore, had essentially no differences in the immunoglobulin classes reactive in the serological procedures, but differences were noted in the time of detection, magnitude and duration of the responses. There are several factors which may be involved in the differing responses of the infected and vaccinated pigs. Microorganisms, such as M. hyosynoviae, which have a septicemic phase would be expected to produce higher levels of circulating antibodies than organisms inducing local infections. There is a more sustained antigenic exposure to M. hyosynoviae in the circulation when septicemia occurs than would be found following intramuscular vaccination with killed mycoplasmas. Goiš et al. (1972) presented evidence that in gnotobiotic piglets infected with M. hyorhinis, animals with generalized infection had considerably higher LA titers than did pigs showing no evidence of dissemination of the organism. In addition to route of exposure, the quantity of the antigen probably also plays an important role in the type and magnitude of the response. A live culture which is able to multiply in the host will ultimately provide a much greater antigenic stimulus than can be achieved with most killed vaccines (Edsall, 1966). This dose effect was especially evident with the MI test; infected pigs had a greater and more sustained response than the vaccinated pigs and pigs receiving two doses of vaccine had a better response than did pigs receiving one dose. Differences in the antibody responses may also be due to the type of antigen preparations used. Preparation of the vaccine included sonication and heating. Sonication effectively ruptures the mycoplasmal

cells, but may result in many minute membrane fragments and altered antigens. Heating may also alter certain antigens. Small membrane fragments may not be able to interact effectively with antigen processing cells to initiate antibody formation. The antibodies produced against many of the membrane fragments may have high affinity for the isolated antigen but steric hindrance from neighboring antigenic components on the whole organism may alter the antigen-antibody reactivity. This may affect the ability of the antibody to cause agglutination, fix complement, etc.

Complement fixing antibodies to several mycoplasmas have been shown to be directed against both membrane and soluble antigens (Hollingdale and Lemcke, 1969; Pollack et al., 1970). However, in the present study, whole mycoplasmas were washed and heated prior to use as antigen in the CF test. With such preparations, antibodies detected are directed primarily against surface membrane antigens.

The CF test measures fixation of complement components, but does not indicate the completeness of the fixation. No distinction would be made between ability of antigen-antibody complex to fix only the initial complement components or the entire complement sequence. The possibility may exist that the CF antibody populations of the infected and vaccinated pigs are completely different. As Six et al. (1973) have indicated, antibodies of higher affinity for the antigen are more efficient at causing immune lysis of biological membranes. Antibodies produced against infecting M. hyosynoviae may have higher affinity for the corresponding CF antigens than antibodies produced against fragments of that organism. The higher affinity antibodies in the infected pigs may promote lysis of the mycoplasmas whereas many of the poorer fitting antibodies of the vaccinated

pigs may fix only the initial components of the complement sequence. This difference in fixation would not be distinguished by the CF test.

Many workers have attempted to evaluate the significance of CF antibodies and most have concluded that there is no correlation between CF antibody levels and protection against active infection. In local infections, such as pneumonia caused by M. pneumoniae or M. hyopneumoniae, vaccination with killed or avirulent organisms induces CF titers comparable to those found with active infection, but there is no resistance to challenge (Clyde, 1968; Goodwin et al., 1969b). In research with M. arthritidis, which has a septicemic phase comparable to M. hyosynoviae, it was found that serum with high CF antibody levels from convalescent rats significantly protected previously noninfected rats from intravenous challenge with M. arthritidis (Cole et al., 1969). However, the protective effect of the serum was essentially unchanged when the CF antibodies were removed by adsorption with M. arthritidis cells.

Onset of circulating CF antibody responses has often coincided with onset of clinical symptoms of some mycoplasmal diseases. Development of CF antibodies in M. hyopneumoniae pneumonia of swine has been correlated with development of pneumonic lesions (Boulanger and L'Ecuyer, 1968; Takatori et al., 1968; Slavik and Switzer, 1972). Roberts and Little (1970) have reported the presence of CF antibodies reactive with porcine lung extract in 17 of 40 swine sera with CF antibody titers to M. hyopneumoniae. Sera with high titers against M. hyopneumoniae had CF titers against lung extract. Whittlestone (1972) speculated that lung cells may have been altered antigenically during mycoplasma infection by some mycoplasmal product or during antigen-antibody-complement reactions at the

cell surface. In M. mycoides infection of cattle, onset of symptoms (Pearson and Lloyd, 1972) and persistence of viable organisms in lymph nodes (Davies, 1969b) correlated with onset and persistence of CF antibodies. And in rats, Cole et al. (1969) were able to correlate the dose of M. arthritidis injected and severity of arthritis with the levels of CF antibodies. At this time, the possible role of the marked initial CF antibody response in the pathogenesis of arthritis in Hampshires may be speculated. The particular microenvironment in the synovial tissues of susceptible Hampshires, including synovial tissue damage due to environmental stress, poor joint conformation, etc., may allow the mycoplasmas to grow to higher titers in the joints, resulting in the induction of higher levels of CF antibodies and, possibly alteration of tissue antigens by some toxic factor. Complement proteins and immunocompetent cells have been detected in joints. It seems feasible that if there is early initiation of CF antibody production in the joints, antigen-antibody-complement complexes may play a role in the acute as well as chronic phases of M. hyosynoviae arthritis. Fixation of complement by antigen-antibody complexes may lead to many other physiological responses besides immune lysis. Certain fixed components of complement attract leukocytes, which release lysosomal enzymes that can cause local necrosis of the host tissues. When the phagocytized M. hyosynoviae-antibody-complement complexes are degraded, inflammation should subside unless tissue antigens have been altered in some way by the mycoplasmal infection. Antibodies produced against altered tissue antigens may fix complement and promote chronic inflammation.

The latex agglutination test involves adsorption of washed mycoplasmas onto carrier latex particles. It would be assumed that LA antibodies are directed against the cell membrane. Studies with indirect hemagglutination, which involves adsorption of mycoplasmas onto erythrocytes, have indicated that agglutinating antibodies are directed against the cell membrane antigens (Hollingdale and Lemcke, 1969). Antibodies produced against membrane fragments in the vaccinated pigs may not be able to effectively agglutinate intact organisms due to steric factors, whereas antibodies produced in infected pigs may be better able to bind to intact organisms. Adsorption of mycoplasmas onto the latex also masks certain antigenic determinants and may alter conformation of others. Therefore, the LA test does not measure the total amount of agglutinating antibodies in the serum, but it is more sensitive than agglutination because of the increased mass of the reactants.

Several difficulties were encountered with the LA test in the present study. Antigen preparations had to be grown in special PPLO serum fraction medium (D-SF) and then washed, homogenized and sonicated to ensure that the sensitized latex did not autoagglutinate. Positive controls were included with each run and sometimes agglutination with such controls was weak. When this occurred, sensitized latex was prepared again and used only if there was strong agglutination in the positive controls.

The protective value of LA antibodies detected in this study is not known. As mentioned previously, Goiš et al. (1974) found that whole serum and specific IgM as well as IgG fractions from a pig hyperimmunized with M. hyorhinis prevented dissemination of the organism in gnotobiotic

piglets. The IgM fraction had mainly LA and IHA antibody activity and the IgG fraction had mainly MI activity. However, whether LA antibodies were protective was not determined.

The requirement for a supplemental factor(s), as supplied by NRS, in addition to those provided by unheated horse serum and unheated guinea pig serum for optimal MI activity against mycoplasmas has not been reported previously. It is possible that the particular lots of horse and/or guinea pig sera used were deficient in certain factors required for metabolic inhibition by swine antibodies, but that these factors were in sufficient supply for rabbit or horse antibodies. Possibly swine antibodies require certain factors that cannot be provided at all by horse or guinea pig serum. The supplemental factors present in NRS were apparently different from those gained by use of GPS since use of unheated NRS without GPS resulted in loss of MI activity of swine and rabbit serum. Rather similar problems were encountered in development of complement fixation tests for detection of M. hyopneumoniae antibodies in swine serum. With traditional CF tests using only guinea pig complement, antibodies were detected in sera from hyperimmunized rabbits (Boulanger and L'Ecuyer, 1968), but detection of antibodies in swine serum required supplementation with fresh calf serum (Boulanger and L'Ecuyer, 1968) or swine serum (Slavik and Switzer, 1972). The nature of the supplemental factor(s) was not determined.

Since one of the proposed mechanisms for metabolic inhibition is complement-mediated lysis, a possible explanation for the changing requirements for supplemental factors with length of time after induction of the immune response may involve changes in antibody affinity. Six et al.

(1973), in investigations on the role of antibody affinity in complement-dependent damage of biological membranes, found that low affinity IgM antibodies, as found early in the immune response, were more efficient in causing membrane damage than low affinity IgG. High affinity IgG antibodies, as might be found 40 to 60 days after induction of the immune response, were more efficient in causing membrane damage than low affinity IgG. Certain early sera from M. hyosynoviae infected pigs had slight MI activity when the original MI procedure was used. In those sera, IgM antibodies may have been present in high enough titers to cause some immune lysis without large amounts of supplemental factors. With increasing time after inoculation, amounts of high affinity IgG increase and its efficiency in immune lysis may require smaller amounts of supplemental factors. The addition of unheated NRS to the test system may have provided necessary factors for immune lysis by the predominantly lower affinity antibodies in the early immune response and augmented the total measured MI response in later sera.

Others have found that MI activity of early sera required a heat-labile factor(s) provided by guinea pig serum and activity in late sera was enhanced by, but did not require, a heat-labile factor(s) (Coleman and Lynn, 1972a,b; Davies, 1969a). In further work, Coleman et al. (1974) detected two MI antibody populations in the late sera of rabbits immunized with M. pneumoniae. One group required no complement components whereas the other group required certain heat-stable complement components.

The difference in the MI responses of the pigs infected and vaccinated with M. hyosynoviae is probably due to the method of preparation of the vaccine. Metabolic inhibiting antibodies are directed against anti-

genic components of the mycoplasmal membrane (Soběslavský et al., 1966; Williams and Taylor-Robinson, 1967; Hollingdale and Lemcke, 1969) and antibodies produced against fragments of that membrane may not have a good affinity for the antigenic determinants in the whole organism. In the MI test, where one mechanism of inhibition is complement-mediated lysis, antibodies inefficient at fixing the entire complement sequence would not mediate lysis and would result in low MI titers as noted in the vaccinated pigs. However, with the CF test, no difference was detected in the magnitude of the responses of vaccinated and infected pigs because the test does not distinguish between incomplete complement fixation and fixation of the entire complement sequence.

The protective properties of this MI activity are not completely known. In localized infections, such as mycoplasmal pneumonias, serum MI antibodies do not appear to correlate with protection against infection (Smith et al., 1967; Davies and Hudson, 1968; Fernald, 1969). However, as indicated earlier, serum containing MI activity against M. hyorhinis as well as IHA and LA activity did protect pigs against systemic infection (Goiš et al., 1974). It has also been indicated that there are often higher levels of MI as well as CF antibodies in synovial fluid than in serum of M. hyorhinis infected pigs, suggesting possible local production of antibodies (Barden and Decker, 1971; Ross et al., 1973). The protective role of MI antibodies in synovial fluid was questioned by Barden and Decker because they isolated M. hyorhinis from many of the joints with high antibody levels.

In certain mycoplasmal diseases, a positive correlation has been detected between the ability of the organisms to produce disease and lack of

MI antibody production by the susceptible host. In their work on M. arthritidis disease in rats, Cahill et al. (1971) found a heterogenetic antigen(s) common to M. arthritidis and rat tissues. They suggested that organisms possessing heterogenetic antigens would be less immunogenic in the host and more resistant to primary defense mechanisms. This phenomenon of biological mimicry may also play an important role in other mycoplasmal diseases. No or low MI antibody responses have been reported for chickens and turkeys with natural M. gallisepticum infection (Jordan and Kulasegaram, 1968; Taylor-Robinson and Berry, 1969). An immunologic relationship between the galactan of M. mycoides and pneumogalactan of bovine lung has also been reported (Shifrine and Gourlay, 1965; Gourlay and Shifrine, 1966), thus, cattle might develop a partial immune tolerance to that organism.

Detection of high levels of MI antibodies with the modified MI technique, low levels of MC antibodies utilizing actively growing mycoplasmas and no early MC activity using resting mycoplasmas suggests that either MI and MC tests measure different antibodies or that the MC system, as with the original MI procedure, lacks certain required factors for optimal activity. Reports have varied as to the correlations between MI and MC antibodies. Differences were noted in the immunoglobulin classes reactive in the MI and MC tests using early and late sera from rabbits immunized with M. pneumoniae (Coleman and Lynn, 1972a,b). In contrast to these differences between MI and MC antibodies, several workers have found direct correlations between the two. Purcell et al. (1967) examined the relationship between MI antibodies and MC antibodies against M. hominis, M. pneumoniae, M. fermentans and M. pulmonis and demonstrated close

correlations between the dilutions of antisera inhibiting color changes in the MI test and those inhibiting growth in liquid medium. Good correlations were found between MI and MC titers with M. gallisepticum (Taylor-Robinson and Berry, 1969; Woode and McMartin, 1973).

Several observations seem to indicate that the MC test was not working optimally in the present study. Antibody titers detected with the procedure were similar to those detected with the original MI procedure and were not detectable until 6 weeks PI or later. The modified MI procedure, however, detected earlier and much higher levels of MI antibody. In addition, sera from two of the pigs infected with M. hyosynoviae (H9-3B and H9-4B) were also tested for MI and MC antibodies by another laboratory. In that work, levels of antibodies were similar to those detected with the original MI test and the MC procedure in this study, but minimal titers were detected as early as 12 days PI.[1] In the present MC test, it was also found that different lots of fetal calf serum varied in their effectiveness in the MC procedure. All of these results seem to indicate that if a certain, as yet, unknown serum factor(s) is provided in the required amounts, detection of earlier and higher MC titers might be possible. Preliminary results with 1% unheated NRS indicated that this supplemental serum does not supply the needed factor(s).

The value of MC antibodies in protection against infection is not completely known. High MC titers in preinoculation sera of volunteers infected with M. pneumoniae correlated well with failure to develop

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[1]Dr. B. C. Cole, University of Utah Medical Center, Salt Lake City, Utah. Personal communication. 1973.

illness (Brunner et al., 1973b) but Davies and Hudson (1968) found no correlation between immunity to M. mycoides infection in cattle and circulating MC antibodies. As mentioned earlier, the MC antibodies against M. arthritidis detected by Cole and Ward (1973a) in rats were not effective against actively multiplying organisms. It was speculated that since the antibodies could not kill active organisms, the combination of antibody with M. arthritidis could play some role in pathogenesis of the disease.

Smith et al. (1966) have demonstrated that composition of growth medium can affect the antigenic structure of mycoplasmas. Such alterations may be detected serologically and are generally attributed to adsorption of serum components of the medium by the growing mycoplasmas (Bradbury and Jordan, 1971). Whether such medium components may have had any influence on the serological results in this study was not determined. None of the control swine had been vaccinated with turkey serum-containing medium (D-TS) alone. However, all of the test antigens were specific for M. hyosynoviae and did not react with sera from swine infected with M. hyorhinis and M. hyopneumoniae, indicating that the responses were probably not directed against medium components. Cultures prepared in D-TS and horse serum-containing medium (D-HS) were compared in the modified MI test and the MC test and essentially no differences were noted in the titers detected. However, neither D-HS antigen nor D-TS antigen could be used in the LA test because of the tendency to autoagglutinate and a D-HS antigen prepared for comparative use in the CF test was anticomplementary.

As has been mentioned, the pathogenesis of a disease may affect the protective role of humoral antibodies. Local immunity, both secretory (Brunner et al., 1973a) and cellular (Fernald et al., 1972; Fernald, 1973), may have a more important role than humoral immunity in prevention of mycoplasmal pneumonias. However, humoral antibodies may have a protective role in mycoplasmal infections that have a septicemic phase, such as M. hyosynoviae arthritis of swine. The protective value of serum has been documented for rat (Cole et al., 1969; Cole and Ward, 1973b) and swine (Goiš et al., 1974) arthritides. The possible roles of the humoral antibodies detected in the M. hyosynoviae vaccinated and infected pigs may be speculated. Agglutinating antibodies may promote phagocytosis of the mycoplasmas by increasing their mass. Metabolic inhibiting and mycoplasmacidal antibodies may promote complement-mediated lysis of the organisms or may prevent multiplication of the mycoplasmas. Complement fixing antibodies may promote phagocytosis or lysis of the organisms, but also may play a role in pathogensis of the arthritis.

SUMMARY

Swine vaccinated or infected with Mycoplasma hyosynoviae developed antibody responses detectable by complement fixation, latex agglutination, metabolic inhibition and mycoplasmacidal techniques. Vaccinated swine were inoculated intramuscularly with one or two doses of a sonicated, heated M. hyosynoviae vaccine with incomplete Freund's adjuvant. Infected swine were inoculated intranasally with a young culture of M. hyosynoviae. Complement fixing antibodies were detected in the vaccinated and infected pigs by 9 days postinoculation (PI), with high peak titers occurring in Hampshire swine from 12 days PI through 4 weeks PI and in Yorkshire swine from 8 weeks PI through 24 weeks PI. Vaccinated and infected swine had detectable latex agglutinating antibodies by 6 days PI, with titers peaking at about 2 weeks PI and gradually declining thereafter. The magnitude of the responses differed, with the infected pigs having much higher levels of latex agglutinating antibodies than the vaccinated pigs. Using a modified metabolic inhibition (MI) test with 1% unheated normal rabbit serum as a medium supplement, transient, low to moderate titers were detected in the vaccinated pigs. Onset of the MI response in the vaccinated pigs was usually by 9 days PI, with the early response ending by 4 weeks PI and the late response, when present, ending by 10 weeks PI. The infected pigs had detectable MI antibodies by 9 to 12 days PI with titers peaking at about 2 weeks PI and declining thereafter. Using a mycoplasmacidal test with resting mycoplasmas, none of the vaccinated pigs had detectable antibodies, but the infected pigs had detectable antibodies by 6 to 8 weeks PI which increased in titer through the end of the study.

Selected sera from vaccinated and infected pigs were fractionated by Sephadex G200 gel filtration and the resultant three peaks were characterized serologically. Complement fixing antibodies in sera collected 9 days, 6 weeks and 24 or 26 weeks PI were found mainly in the IgG fraction in both vaccinated and infected pigs. Latex agglutinating antibodies were detected in the IgM fraction for both groups of pigs. Early (9 days PI) sera had metabolic inhibiting activity in the IgM peak, while later sera had activity primarily in the IgG fraction. Mycoplasmacidal antibodies from the infected pigs were detected only in the IgG fraction.

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