# The Immune Response of Calves given Mycoplasma bovis Antigens

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## ABSTRACT

Seven calves seven to 30 days of age were given Mycoplasma bovis antigen by different routes. Immunization was in two phases. The first consisted of single or multiple SC, IV or oral doses of antigen for two to four weeks. The second phase consisted of multiple SC or ID injections given from the eighth to the 19th week. The experiment was terminated at 26 weeks. Antibody titers were followed by indirect hemagglutination, growth inhibition and tetrazolium reduction inhibition. Total serum protein, protein fractions and IgG and IgM concentrations were determined in serums of one calf and the distribution of indirect hemagglutination antibodies in IgG and IgM classes were determined in serums of two of the calves.

Indirect hemagglutination titers of 1280 and peak titers of >20,480 occurred after the first and second phases respectively. There was no relationship between total serum IgG or IgM concentrations and indirect hemagglutination titers. In one calf given M. bovis antigen in one dose SC and five weekly doses IV in phase I, indirect hemagglutination antibodies appeared in IgM within one week and IgG by four weeks, IgG antibody activity rose steadily until the 17th week but declined at the 26th week, whereas IgM activity after the initial rise dropped at the 13th week but rose even higher as a result of second phase ID injections. Another calf given six weekly IV doses of M. bovis antigen in phase I developed indirect hemagglutination antibodies in IgM peaking at four weeks then declining but with no IgG response. Activity in both IgM and IgG occurred after the second phase. Growth inhibition antibodies were found only on two occasions in one calf serum and tetrazolium reduction inhibition activity when tested never gave titres exceeding 1:32.


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## RÉSUMÉ

Cette expérience visait à injecter l'antigène Mycoplasma bovis, par diverses routes, à sept veaux âgés de sept à 30 jours. La planification de cette immunisation comportait deux phases. La première consistait à administrer par les voies sous-cutanée, intra-veineuse ou orale, une ou plusieurs injections d'antigène, échelonnées sur une période de deux à quatre semaines. La seconde consistait à administrer, par la voie sous-cutanée ou intra-dermique, plusieurs injections de l'antigène, de la huitième à la 19e semaine de l'expérience, laquelle dura 26 semaines. La vérification des titres d'anticorps s'effectua à l'aide des épreuves de l'hémagglutination indirecte, de l'inhibition de la croissance du mycoplasme et de l'inhibition de la réduction du tétrazolium. On détermina la teneur du sérum d'un veau en protéines totales, en fractions protéiniques, ainsi qu'en IgG et en IgM; on rechercha par ailleurs, dans le sérum de deux veaux, la quantité d'anticorps décelés par l'hémagglutination indirecte que contenaient les IgG et les IgM.

Les titres d'anticorps décelés par l'épreuve d'hémagglutination indirecte, à la fin de chacune des deux phases de l'immunisation, atteignirent respectivement 1:1280 et > 1:20,480. On ne décela pas de relation entre la teneur du sérum en IgG ou IgM et en anticorps décelés par l'hémagglutination indirecte. Chez le veau qui avait reçu une injection sous-cutanée et cinq injections intra-veineuses hebdomadaires de l'antigène M. bovis, au cours de la première phase de l'immunisation, les anticorps décelés par l'hémagglutination indirecte apparurent dans les IgM, en l'espace d'une semaine, et dans les IgG, durant la quatrième semaine. L'activité des anticorps IgG s'éleva progressivement jusqu'à la 17e semaine, pour ensuite diminuer, durant la 26e semaine. Par ailleurs, l'activité des IgM, après une élévation initiale, baissa au cours de la 13e semaine, pour s'élever davantage à la suite des injections intra-dermiques de la deuxième phase de l'immunisation. Un autre veau qui avait reçu six injections intra-veineuses hebdoma-

**daires de l'antigène M. bovis, au cours de la première phase de l'immunisation, développa dans les IgM des anticorps décelés par l'hémagglutination indirecte, lesquels atteignirent un sommet en quatre semaines et diminuèrent ultérieurement; ce veau ne développa cependant pas de IgG. Après la deuxième phase de l'immunisation, on décela une activité impliquant les IgM et les IgG. On ne décela des anticorps de l'inhibition de la croissance du mycoplasme, qu'à deux reprises, dans le sérum d'un veau. Les épreuves visant à rechercher l'activité de l'inhibition de la réduction du tétrazolium ne révélèrent jamais de titres supérieurs à 1:32.**

## INTRODUCTION

Considerable work of practical and theoretical interest has been done with *Mycoplasma agalactiae*. Various strains, vaccines and adjuvants have been used in efforts to develop protection against mastitis in sheep and goats (14, 15). Complement fixing (CF) and growth inhibiting (GI) antibodies developed in all animals inoculated with live vaccines. Although CF antibody titers were low, resistance to challenge was demonstrated in vaccinated animals. On the other hand, only limited work has been done with *M. bovis* (2). Immunological tests have been used in identification (3, 10, 17, 21, 22) and it has been shown that agglutinating titers (latex and plate test) developed in serums of cows given *M. bovis* antigens intravenously or in the udder (19, 20). Constant antigenic stimulus was necessary to maintain antibody levels. Indirect hemagglutinating (IHA) and GI antibodies to *M. bovis* measured in dairy cows in Canada revealed that IHA titers greater than 1:80 were present in cows with a history of udder infection with the organism (8).

In continuing studies on the immunological aspects of *M. bovis* mastitis, rabbits were used initially (7). IHA antibodies appeared to give a satisfactory measure of antibody development. Titers reaching $10^5$ in four to six weeks were found but despite continued antigen administration titers fell to low levels by nine to ten weeks. In the present study, trials were conducted in calves given *M. bovis* antigen with the intent to gain information on the effects of route of administration of antigen, on the time of appearance and magnitude of the antibody response and the relative distribution of antibodies in the different antibody classes with time.

## MATERIALS AND METHODS

### CALVES

The seven calves used were obtained from a dairy which had been under close observation for several years. Frequent culturing of milks from the cows during this time revealed no evidence of mycoplasma mastitis. Calves 1 through 6 were purebred Holstein-Friesian, calf 7 was a crossbreed. Calves 1 and 2 were fraternal twins. Each calf received pooled colostrum the first day and colostrum from the dam the second day postpartum. Thereafter, they were fed a standard milk replacer diet and were housed in groups of two calves/pen. Calves 1 through 3 were one month of age, calf 4 was three weeks and calves 5 and 6 were a week old at the start of antigen administration. Calf 7 was kept as an untreated control separate from the other calves until the tenth week after the start of *M. bovis* treatment at which time it was placed in the pen with calves 5 and 6.

### SAMPLING

The calves were bled by jugular venipuncture before and after receiving colostrum the first day, several times the first week and at regular intervals thereafter. Blood and colostrum samples were also collected from the dams. Serums were removed from blood after clotting and centrifugation and were stored at –40°C in small aliquots until testing.

### MYCOPLASMA STRAIN

*M. bovis* strain 201 was used as antigen. It has been referred to previously with regard to its relationship to other strains (7).

## ANTIGENS

*M. bovis* 201 was grown in 100 ml of biphasic PPLO medium (7) for at least 48 hours. The broth was asceptically poured through sterile gauze into 500 ml of PPLO broth (7) in a one liter flask. After 48 hours incubation, which consistently gave viable counts of $10^8$ to $10^9$ mycoplasma/ml, the mycoplasma were sedimented by centrifugation and washed three times in phosphate buffered saline (PBS) solution, pH 7.2. Antigens were reconstituted to 10% of the original volume and were stored at –40°C in two ml aliquots. Experience had shown that *M. bovis* survived long periods of storage at this temperature. Antigen given calves 1 and 2 in the first SC dose was mixed with Freud's complete adjuvant[1] in a ratio of 1:1 (v:v) after thawing and was homogenized by application of light bursts of sonic energy.

## IMMUNIZATION SCHEDULE

The immunizations were conducted in two phases. The first was to determine the initial immune response to a series of antigenic exposures by various routes. The second phase was to determine the effects of subcutaneous and intradermal injections of large numbers of organisms.

The calves were treated as follows:

*Phase I* — Calves 1 and 2 received 5.0 ml of antigen distributed in two sites subcutaneously in the prescapular region on days 1 and 4. Three ml of antigen without adjuvant was given intravenously at four weekly intervals thereafter.

Calves 3 and 4 received 4.0 ml of antigen intravenously on day 1 and 3.0 ml of antigen intravenously at five weekly intervals thereafter.

Calves 5 and 6 received 2.0 ml of antigen added to the milk daily for a total of two weeks.

*Phase II* — Calves 1 through 4 were given 2 x $10^{10}$ viable mycoplasma in 5.0 ml of PBS subcutaneously in the eighth and again at the ninth week. All calves were given an intradermal dose of about 2 x $10^{10}$ viable mycoplasma in 0.1 ml of PBS in the 13th week in a prepared skin site on the middorsal region. Similarly, calves 1, 3, 5 and 7 were skin tested in the 17th week as were calves 2, 4, 6 and 7 in the 19th week. The latter injections were given to evaluate the *in vivo* skin reactivity to live mycoplasma as a part of a separate study.

## ANTIBODY TITRATION

Serums were titered for IHA antibodies as described (7) in microtiter plates using *M. bovis* strain 201. All serums were titered at the same time using the same batch of antigen coated cells. GI antibodies were tested according to the method of Clyde (9). Growth precipitation (GP) was done according to the method of Kronsgaard-Jensen (22) and the tetrazolium reduction inhibition test (TRI) was done essentially as described by Ernø *et al* (11). For this test 0.025 ml of serum dilutions or of column fractions, 0.05 ml of a $10^{-8}$ dilution of a 24 hour culture of *M. bovis* and 0.15 ml of PPLO broth containing unheated horse serum and 2,3,5-triphenyl tetrazolium chloride (0.05%) were incubated from two to five days in microtiter plates.

## SERUM FRACTIONATION

Serums of calf 1 were applied in 3.0 ml amounts to a 1.5 x 90 cm column of G-200 Sephadex[2] and eluted with 0.1 M Tris HCl-0.2 M NaCl pH 8.0, 75-drop fractions were collected. This effectively resolved macroglobulin antibodies (IgM) in the first peak and IgG antibodies in the second as confirmed by immunoelectrophoresis but the amount of protein applied and column characteristics did not allow for the resolution of serum into three peaks as is normal for serum on G-200. Calf 3 serums in 1.0 amounts were added to 2.5 x 45 cm columns of G-200 and eluted with 0.1 M Tris-1.0 M NaCl, pH 8.0, 60 drop fractions were collected. The normal three peak distribution of protein was produced by this method. Protein content of each fraction was determined at 280 nm and the contents of the tubes were pooled in groups of two and IHA, TRI and GI titers of each pool were determined without further treatment. The identity of the frac-

[1]Difco Laboratories, Detroit, Michigan.

[2]Pharmacia Fine Chemicals, Piscataway, New Jersey.

TABLE I. Titers (Reciprocal of Dilution) in Colostrum and Serum of the Dams and Serums of the Calves Before Administration of *Mycoplasma bovis* Strain 201 Antigen as Determined by Indirect Hemagglutination

| Calf | Colostrum | | Dam's Serum | Calf Serum Titers — Preimmunization | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | First Feeding[a] | Second Feeding[b] | | Precolostrum | Postcolostrum Day 1 | Week: 1 | 2 | 3 | 4 |
| 1 | 256 | 256 | 160 | 10 | 20 | 20 | 40 | 20 | 20 |
| 2 | 256 | 256 | 160 | 80 | 80 | 80 | 60 | 20 | 20 |
| 3 | 128 | 128 | 320 | 0 | 80 | 80 | | 40 | 20 |
| 4 | 256 | 64 | 20 | 20 | 0 | 0 | 0 | 0 | 10 |
| 5 | 32 | 16 | 160 | 0 | 0 | | | 0 | 0 |
| 6 | 32 | 16 | 80 | 0 | | | | | 0 |

[a]Pooled colostrum
[b]Colostrum from the dam, given on the second day

tions as to immunoglobulin class was first presumed by their position in the elution profile and confirmed by immunoelectrophoresis on microscope slides using antisera to whole bovine serum or to antibovine IgM and/or IgG. Immunoglobulin concentrations in serum were determined by radial diffusion in gels as described (6). Total protein was determined by refractometer[3] and serum protein fractions were separated and quantitated by electrophoresis on cellulose acetate membranes.[4]

## RESULTS

### IHA Titers

Results of the IHA titrations of the colostrum fed, serums of the dams and serums of the seven calves before giving *M. bovis* antigen are given in Table I. Titers of 80 or less are not considered significant. The colostrum had varying levels of IHA antibodies to the mycoplasma (titers of 16-256) as did the serums of the dams (titers of 20-320). Both calves 2 and 3 had titers of 80 within the first week postparturition but these declined when immunization was started.

After immunization (Fig. 1) an IHA titer of 640 was found in calf 1 serum by the third week and by the fourth week calves 1 through 4 had titers of 320-1280. Calves 5 and 6 given the antigen in milk did not develop significant titers despite persistent nasal colonization by *M. bovis*. After these peaks as a result of phase I immunization, titers declined until a response was obtained to the subcutaneous boosters given at the eighth and ninth week. This stimulus was more effective in calves 1 and 2 than in calves 3 and 4. The large intradermal doses of live antigen given the 13th week elicited the highest titers obtained (>20,480). Calves 5 and 6 did respond to the skin injections but not to the degree seen in calves 1 through 3. It appeared that IHA titers obtained at the last bleeding were declining in four out of the seven calves.

### Growth Precipitation, Growth Inhibition and Tetrazolium Reduction Inhibition

The GP test was not used extensively but serum at the 14th week from calf 1 developed two lines, serums from calves 2, 3, and 4 developed one line whereas serums from the remaining calves were inactive. Serums of the 16th week from calves 5 and 6, but not calf 7, then developed one line.

All serums were tested for GI antibodies and in fact, high titered sera by IHA were retested several times. This was done because clear cut inhibitory zones were never obtained when compared with tests of high titered adult cow serums run concurrently. Zones of partial clearing were observed in serums of calves 1 through 4 obtained at the 16th and 21st week. This partial inhibition was the only evidence of GI antibodies obtained. The zones were not greater than 2.0 mm. On the other hand, the active adult cow serum control

[3]TS meter, American Optical Co., Buffalo, New York.

[4]Microzone system, Beckman Instruments, Palo Alto, California.

routinely produced 3.0 mm zones of complete clearing.

Not all serums were tested for TRI antibodies. Preliminary results showed that the TRI test was much less sensitive than the IHA and the results did not give any significant information. For example, the cow serum used as a positive control always gave titers >20,000 by IHA whereas TRI titers were always between 64 and 128. TRI titers for calf 1 serums are given in Table II and show very low values compared with those given by IHA. However, it does not appear that TRI activity is a direct correlate of the IHA activity as shown by several observations (Table II).

## Serum Fractionation

Serums from calves 1 and 3 were studied intensively. Total protein, $\gamma$-globulin, the

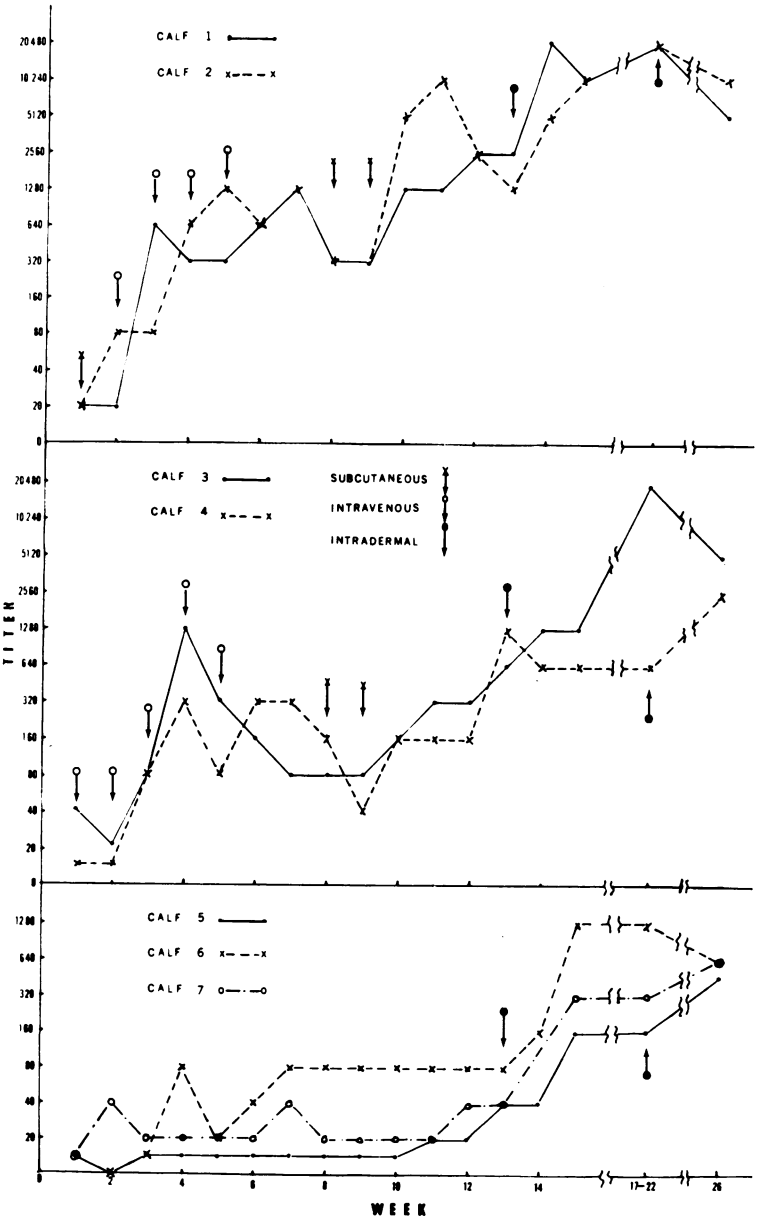


**Fig. 1. Indirect hemagglutination titers of serum of calves given Mycoplasma bovis by various routes. Calves 5 and 6 were given live mycoplasma in the milk during weeks 1 and 2 and calf 7 did not receive mycoplasma until week 13.**

A/G ratio, IgM and IgG concentrations, the IgG/IgM ratio and serum IHA and TRI titers are given for calf 1 in Table II. The preimmunization values reflect the ingestion and absorption of colostrum. Total protein peaked at 7.0 g/dl after the phase I period and at 10.2 g/dl after the skin treatment. IgG and IgM concentrations were somewhat variable but tended to fall after the initial postcolostrum peak despite the immunization procedure and IgM levels appeared to be rising between the 18 and 26th week. There appeared to be no correlation between total serum $\gamma$-globulin, IgG or IgM concentrations and IHA titers.

A determination of IHA antibodies in the pooled Sephadex column fractions indicated a good resolution of IgM and IgG antibodies. A plot of the sum of IHA titers for each pool representing IgM and IgG for each serum is shown along with the IHA titer of whole serum in Fig. 2 for calf 1 and Fig. 3 for calf 3. It can be seen in Fig. 2 that IHA activity was present in IgM after the initiation of antigen administration and remained relatively constant until the 8th week, declined somewhat then rose again after the 13th week with levels after the intradermal injections remaining above those seen during the phase I period. Activity in the IgG class first appeared in the sixth week rising more

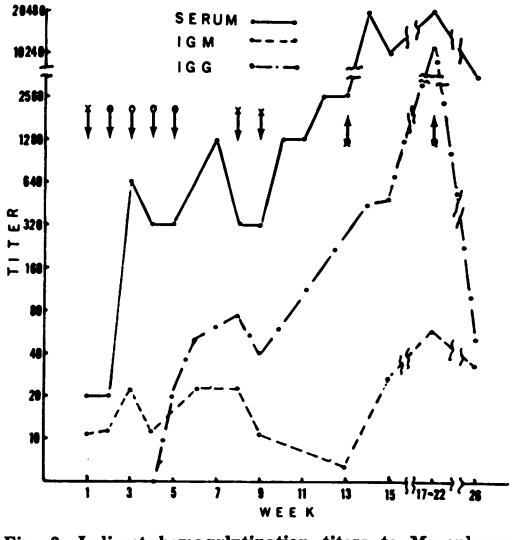


**Fig. 2. Indirect hemagglutination titers to Mycoplasma bovis in serum of calf 1 and the sum of the IHA titers present in IgG and IgM in pooled G-200 Sephadex column fractions at the various times. See Fig. 1 for additional details.**

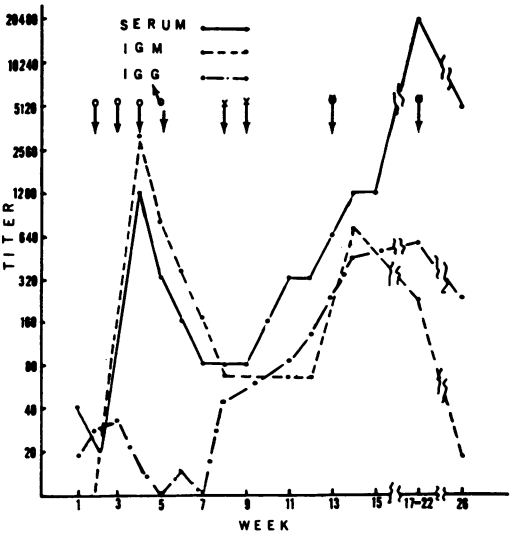


**Fig. 3. Indirect hemagglutination titers to Mycoplasma bovis in serum of calf 3 and the sum of the IHA titers present in IgG and IgM in pooled G-200 Sephadex column fractions at the various times. See Fig. 1 for additional details.**

or less continuously until the peak at the 17th week and declining thereafter. Values for preinoculation serums are not shown. However, titers from the first day of birth for calf 3 indicated that colostrum absorption was reflected in the IHA serum titers to *M. bovis*, since on the third day IHA antibodies were detected in IgM and a peak in IHA activity in IgG was found at three weeks after birth. The significance of these findings is not understood. Antigen injections (IV) began on the 30th day after birth and produced a rise in serum titers peaking at week 4 in phase I at which time titers in IgM also peaked (Fig. 3). Titers fell until the subcutaneous boosters produced a sharp rise in both total serum titers and in the IgM antibodies. An IgG response, not obtained from the series of intravenous injections in phase I, was also obtained and persisted until termination of the experiment.

## DISCUSSION

These results indicate that of the serological tests used, the IHA appears to be the most sensitive and are in agreement with those of Cho *et al* (8) who found the test to be sensitive, reproducible and spe-

TABLE II. Protein and Immunoglobulin Concentrations in Calf No. 1 Serum Before and After Immunization with *Mycoplasma bovis*

| Time | Serum Protein Concentration (g/dl) | | | A/G | Immunoglobulin Concentration (mg/ml) | | IgG-1/ IgM | IHA | TRI |
|---|---|---|---|---|---|---|---|---|---|
| | Total | Albumin | γ-globulin | Ratio | IgG-1 | IgM | Ratio | Titer | Titer |
| Preimmunization | | | | | | | | | |
| Day 1 | | | | | | | | | |
| Precolostrum | 4.6 | 2.16 | 1.08 | 0.85 | < 5.0 | <0.8 | | 10 | — |
| Postcolostrum | 9.0 | 4.34 | 0.93 | 1.73 | 32.3 | 7.0 | 4.60 | 20 | 0 |
| Day 3 | 7.2 | 3.37 | 0.88 | 1.50 | 34.5 | 7.0 | 4.92 | 20 | — |
| Day 31 | 6.2 | 3.38 | 1.20 | 1.20 | 18.0 | 2.1 | 8.57 | 10 | — |
| Postimmunization | | | | | | | | | |
| Week 1 | 5.3 | 2.75 | 1.02 | 1.08 | 16.2 | 1.8 | 8.97 | 20 | — |
| 2 | 5.5 | 2.19 | 1.61 | 0.66 | 18.0 | 3.3 | 5.45 | 160 | 0 |
| 3 | 5.7 | 2.10 | 1.82 | 0.58 | 15.5 | 4.5 | 3.44 | 640 | 2 |
| 6 | 7.0 | 3.76 | 1.25 | 1.16 | 15.5 | 4.0 | 3.87 | 1280 | 0 |
| 9 | 6.0 | 3.03 | 1.22 | 1.02 | 16.5 | 3.6 | 4.64 | 320 | 2 |
| 11 | 8.4 | 3.82 | 1.95 | 0.83 | 15.2 | 1.9 | 8.20 | 1280 | 16 |
| 12 | 6.7 | 3.19 | 1.44 | 0.91 | 11.0 | 3.5 | 3.13 | 2560 | 8 |
| 15 | 10.2 | 4.88 | 2.57 | 0.92 | 11.5 | 3.6 | 3.29 | 20,480 | 16 |
| 18 | 8.4 | 4.10 | 2.20 | 0.95 | 14.3 | 5.0 | 2.88 | >20,480 | 8 |
| 26 | 7.0 | 3.33 | 1.88 | 0.91 | 11.0 | 10.0 | 1.09 | 5,120 | 32 |

cific. The present results do not give too much information on the relative efficiency of the various routes of administration of antigen in producing an antibody response. It would appear that viable organisms given in the milk do not engender significant levels of antibodies. The data further suggest that continued antigenic exposure may be necessary to maintain circulating antibody titers and unless antigen is present, titers may fall abruptly. This pattern was also noted by Jain *et al* (19). In naturally infected cows, *M. bovis* can persist in the animals for long periods of time (18). The pattern of spread appears to be from an infected udder quarter to all four quarters and from the udder systemic spread can occur.

The response following phase II immunization suggests that large numbers of viable organisms given either SC, ID or perhaps IM and possibly with adjuvant may be the most satisfactory way of stimulating high antibody levels. Large numbers of viable organisms were cultured repeatedly from lesions observed at inoculated skin sites. The lesions progressed from small abscesses 1.5 cm in diameter to areas of fibrosis at the conclusion of the experiment.

The fractionation studies revealed that the first antibodies to be produced after immunization were IgM. In calf 1, this was followed by appearance of IgG antibodies as the result of phase I immunization. In calf 3, IgM antibodies were not succeeded by appearance of IgG. The difference at this time may have been the fact that calf 1 had had an initial SC injection of *M. bovis* antigen in Freund's complete adjuvant that calf 3 did not have. However, after phase II immunizations both calves developed an IgM and an IgG response.

In human infections with *M. pneumoniae*, it was shown that IHA antibodies were predominantly IgM with an increase in IgG relative to IgM after infection in only one-half of the cases. The remaining half produced IgM exclusively (5). Experiments in rabbits indicated that as IgM antibodies declined, the IgG component became prominent (13). In hamsters infected with the same organism, antibody reactivity was found only in IgG (12).

Cattle experimentally infected with *M. mycoides* var. *mycoides* produced IgM antibodies within a week but after two weeks IgG antibodies were found (4). Antibody activity was in both classes until 22 weeks at which time only IgG antibodies could be detected. The response of swine to *M. hypopneumoniae* showed that IHA antibodies were both IgG and high molecular weight, IgA (16).

Experiments are in progress to evaluate the relationship between circulating antibody titers and protection or resistance to *M. bovis* mastitis.

## REFERENCES

1. AL-AUBAIDI, J. M. and J. FABRICANT. Characterization and classification of bovine mycoplasma. Cornell Vet. 61: 490-518. 1971.

2. **ASKA, G. and H. ERNØ.** Elevation of **Mycoplasma agalactiae** subsp. **bovis** to species rank: **Mycoplasma bovis** (Hale et al) comb. nov. Int. J. syst. Bact. **26**: 323-325. 1976.
3. **BAAS, E. J. and D. E. JASPER.** Agar block technique for identification of mycoplasmas by use of fluorescent antibody. Appl. Microbiol. **23**: 1097-1100. 1972.
4. **BARBER, T. L., S. S. STONE and P. D. DeLAY.** Antibody in cattle experimentally infected with contagious bovine pleuropneumonia. Infection & Immunity **2**: 617-622. 1970.
5. **BIBERFELD, G.** Distribution of antibodies within 19S and 7S immunoglobulins following infection with **Mycoplasma pneumoniae**. J. Immun. **100**: 338-347. 1968.
6. **CARROLL, E. J. and G. L. CRENSHAW.** Bactericidal activity of neonatal calf serums for selected coliform bacteria in relation to total protein and IGG-1 and IG-M concentrations. Am. J. vet. Res. **37**: 389-394. 1976.
7. **CARROLL, E. J., M. ROLLINS and D. E. JASPER.** The immune response of rabbits to 3 strains of **Mycoplasma agalactiae** var. **bovis** isolated from mastitic bovine udders. Cornell Vet. **66**: 143-151. 1976.
8. **CHO, H. J., H. L. RUHNKE and E. V. LANGFORD.** The indirect hemagglutination test for the detection of antibodies in cattle naturally infected with mycoplasmas. Can. J. comp. Med. **40**: 20-29. 1976.
9. **CLYDE, W. A.** Mycoplasma species identification based upon growth inhibition by specific antisera. J. Immun. **92**: 958-965. 1964.
10. **ERNØ, H. and K. JURMANOVA.** Bovine mycoplasmas: Serological studies by double immunodiffusion, growth precipitation and growth inhibition. Acta vet. scand. **14**: 524-537. 1973.
11. **ERNØ, H., K. JURMANOVA and R. H. LEACH.** Bovine mycoplasmas: A serological study by the metabolic inhibition test. Acta vet. scand. **14**: 511-523. 1973.
12. **FERNALD, G. W.** Immunologic aspects of experimental **Mycoplasma pneumoniae** infection. J. infect. Dis. **119**: 255-266. 1969.
13. **FERNALD, G. W., W. A. CLYDE, JR. and F. W. DENNY.** Nature of the immune response to **Mycoplasma pneumoniae**. J. Immun. **98**: 1028-1033. 1967.
14. **FOGGIE, A., J. R. ETHERIDGE, O. ERDAG and F. ARISOY.** Contagious agalactia of sheep and goats. Preliminary studies on vaccines. J. comp. Path. **80**: 345-358. 1970.
15. **FOGGIE, A., J. R. ETHERIDGE, O. ERDAG and F. ARISOY.** Contagious agalactia of sheep and goats. Studies on live and dead vaccines in lactating sheep. J. comp. Path. **81**: 165-172. 1971.
16. **HOLMGREN, N.** On the immune response in porcine serum and tracheobronchial secretions following experimental infection with **Mycoplasma hypopneumoniae**. Zentbl. VetMed. **B21**: 188-201. 1974.
17. **JASPER, D. E., J. M. AL-AUBAIDI and J. FABRICANT.** Epidemiologic observations on mycoplasma mastitis. Cornell Vet. **64**: 407-415. 1974.
18. **JASPER, D. E., N. C. JAIN and L. H. BRAZIL.** Clinical and laboratory observations on bovine mastitis due to mycoplasma. J. Am. vet. med. Ass. **148**: 1017-1029. 1974.
19. **JAIN, N. C., D. E. JASPER and J D. DELLINGER.** Cultural characters and serological relationships of some mycoplasmas isolated from bovine sources. J. gen. Microbiol. **49**: 401-410. 1967.
20. **JAIN, N. C., D. E. JASPER and J. D. DELLINGER.** Serologic response of cows to mycoplasma under experimental and field conditions. Am. J. vet. Res. **30**: 733-742. 1969.
21. **KEHOE, J. M., N. L. NORCROSS and L. E. CARMICHAEL.** Bovine mycoplasma mastitis. Ann. N. Y. Acad. Sci. **143**: 337-344. 1967.
22. **KROGSGAARD-JENSEN, A.** Mycoplasma: Growth precipitation as a serodiagnostic method. Appl. Microbiol. **23**: 553-558. 1972.
23. **LEACH, R. H.** Further studies on classification of bovine strains of mycoplasmatales, with proposals for new species, **Acholeplasma modicum** and **Mycoplasma alkalescens**. J. gen. Microbiol. **75**: 135-153. 1973.