

Selected immunological responses of the cow to  
Streptococcus agalactiae with special  
reference to the mammary gland

by

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## INTRODUCTION

Bovine mastitis is universally recognized as an economically important disease. Although many investigative studies have been done, it has been impossible up until the present to devise definite control measures including immunization for the disease. Some possible reasons could be that many different organisms can cause mastitis and that the susceptibility from cow to cow varies considerably depending upon age, lactation stage, environment, heredity, etc.

During the 1960's much work was directed toward the study of the defenses of the cow against the various antigens which may be introduced into the udder.

Most of the studies of the immunological reactions in the udder have been directed toward the demonstration of antibodies. Only limited attention has been given to cell-mediated aspects of the response of the udder to foreign antigens. It is suggested that the application of techniques which relate to cellular immunity might yield information that could provide a better understanding of the total immune response of the bovine mammary gland.

To study the cell-mediated responses which may occur in relation to mastitis, selected techniques were applied to the cow, her lymphocytes and her milk. These included skin hypersensitivity, local and generalized reactions to the introduction of antigens and inhibition of macrophage migration.

Humoral antibody responses in the udder were studied by the introduction of killed bacterial antigens and foreign serum proteins into either the mammary gland or by parenteral injection. Attempts to elucidate the mechanism of the Hotis reaction constitute an important part of the investigation.

## LITERATURE REVIEW

Nocard and Mollereau in 1887 incriminated the streptococci as the cause of mastitis. Since that time much research has been directed toward forms of mastitis caused by the streptococci but the specific types were unidentified until 1930 when Streptococcus agalactiae was shown to be responsible for about 80 to 90 percent of chronic mastitis (93).

Wall in 1918 (141) in his study of "Mastitis of the Cow" gave special importance to streptococcal mastitis.

Stableforth et al. (131) reported that Streptococcus agalactiae is widely spread on the outside of the udder and body of the cow and that it could survive in these places for long periods. He isolated it from one-third of the milkers' hands after normal washing and found that up to 35 percent of mastitis was due to Streptococcus agalactiae, 3 percent to Streptococcus dysgalactiae, 5 percent to Streptococcus uberis and 11 percent to Staphylococcus aureus. A few years later, in a six year survey of milk samples submitted by practicing veterinarians in Iowa, Packer (103) found only 7.8 percent incidence of mastitis due to Streptococcus agalactiae while as high as 70 percent of mastitis was caused by Staphylococcus aureus. Rendel and Sundberg (116) obtained similar results, in 1962, when they did a survey of Swedish cattle, i.e., less than 12 percent of mastitis

was caused by Streptococcus agalactiae and 65 percent was staphylococcal mastitis.

It has been recognized that bovine mastitis caused by Streptococcus agalactiae is somewhat unique when compared to other forms of the disease. The only source of Streptococcus agalactiae is an infected udder and the ability of the organism to exist outside of the host animal is apparently limited (68). Streptococcus agalactiae is an obligate parasite of the mammary gland. It spreads from cow to cow and therefore transmission can be interrupted by segregation of the infected cows (121). The other streptococci are not dependent upon the mammary gland or milk for survival and for this reason mammary gland infections produced by them are less frequent and are related to the management practices of the herds in which they occur. Numerous environmental conditions and dairy practices may serve to render the udder more susceptible to infection by various microbiological agents.

Lancaster and Stuart (60) found lack of sanitation is the cause of rapid spread of Streptococcus agalactiae infection. Murphy in 1959 (84) used three different stresses before exposure of the teat canal to Streptococcus agalactiae. He concluded that by removing the keratin lining of the teat canal three milkings prior to exposure to the organism, resistance was completely broken and infection always occurred but after treatment the teat was again resistant.

The clinical appearance of the disease varies from a severe acute mastitis in some cows of a herd to a mild or non-clinical disease in most of the others (82). The shifting between clinical stages is the result of a battle going on between the organism in the udder and the resistance mechanisms of the cow. Therefore, two things must be considered: the ability of the infecting bacteria to injure the udder and the udder's ability to combat it.

Age is an important influence in mastitis incidence (116). While 14.9 percent of first-calf heifers had the disease, as many as 35 percent of cows in their fourth or fifth lactation period contracted it. The high rise in the incidence of mastitis with increased age appears to be due to a combination of cumulative effects of previous mastitis infections and an increase in first mastitis cases with age. This conclusion is in complete agreement with the studies of Lancaster and Stuart (60). On the other hand, observations by Oliver et al. (97) attribute the rise in the total incidence of udder infections with age to reinfection and believe that first infection rates are not changed significantly by age.

Legates and Grinnells (67) proved that heredity is another factor that plays some role in the resistance to mastitis and, therefore, individual variation in susceptibility to mastitis is partly inherited..

### Somatic Cells in Milk

The method that has been most widely used for somatic cell counting is that developed by Prescott and Breed (113) in which a measured amount of milk (0.01 ml) is spread over a one square cm area on a slide and then stained with methylene blue. It is then examined by counting the cells in 100 microscopic fields using a calibrated microscope. The number of cells per ml is calculated by multiplying the average number of cells per field by the microscope factor.

New techniques have been devised in recent years with the same objective. Some workers have used the Millipore filter membrane (32) to collect the stained cells of milk on the filter. The membrane is cleared in immersion oil, examined microscopically and the cells counted.

Another technique used is the electronic counter (25). Although this technique counts higher levels of cells than those counted by the microscopic method, it appears that it is difficult to standardize the cell suspensions in such a way that only one size of cells remain in it. However, this technique could be very precise for milk with more than 300,000 cells/ml (115).

The somatic cell counting of milk is now recognized as the best method of detecting poor quality of milk due to mastitis.

In the opinion of some workers (81) the polymorpho-nuclear leucocytes of milk are the most important cells to



indicate a pathological process in the udder.

Epithelial cells are also present when the mammary gland tissue is injured. As a result of the injury, the dead tissues and the epithelial cells will disintegrate, liberating chemical substances that attract the polymorphonuclear leucocytes (120). The cells that have their origin in bone marrow come to the site of injury and enter into the milk in very high numbers. Any injury to mammary tissue or infection accounts for the presence of leucocytes forming an exudate. In addition to these two facts there are several other factors that have a very marked influence on the changes in numbers of somatic cells in milk and many workers have found data to support this conclusion. Based on the knowledge that the California Mastitis Test is an indicator of the presence of cells in the milk, Whittlestone et al. (142) concluded that the reaction in this test is due to the unravelling of the nuclear DNA. They also found that stress has a decisive influence on the changes in DNA levels. The sudden occurrence of a cold storm was accompanied by increases of up to 3 ug/ml in the DNA levels of the milk. Such things as the isolation of one cow from the rest of the herd for examination can produce sufficient stress to cause a marked rise in the number of leucocytes in milk.

Nelson et al. (91) concluded that extreme changes in temperature have a definite effect on the rise of cell counts in milk. On the contrary, those cows maintained at constant

temperatures of 32 C did not show changes in the count of circulating erythrocytes, leucocytes and somatic cells of milk (100). Cows milked by machine have higher leucocyte counts than those milked by hand (23).

There is a very wide range in the number of cells from normal and mastitic milk. In normal milk the numbers have been considered to be as low as 20,000 cells per ml (20), up to more than 700,000 cells per ml (128). In mastitic milk there is an extraordinarily high number of cells in acute infection, whereas mild infection may not show notable increases in the number of cells. It has been reported that streptococcal mastitis produces a higher leucocytic count than mastitis caused by staphylococcus or coliforms (122).

It was found that normal milk has a leucocyte formula similar to that of blood, i.e., a lymphocyte to polymorph ratio of about 2:1 but in mastitic milk nearly all leucocytes are polymorph. The increase in the number of cells is thought to be due to chemotactic stimuli which result from the presence of microorganisms in the udder (24).

A general conclusion is that any milk with more than 500,000 cells per ml is considered as an expression of an inflammatory defense mechanism in the udder (99, 101). There are differences in the number of lymphocytes present in the milk, ranging from 0.5 percent up to 36 percent (24). In addition to the above mentioned factors, there are many more (physiological, genetic, nutritional) that have a very

important influence on the changes in the number of cells in milk.

The viability of the cells in the milk has been studied by the absorbance of dyes into the dead cells. This is the basic concept for the study with Trypan blue technique reported by Jain and Jasper (51) and used in this study.

Leucocytes of milk have been studied very extensively in relation to immunological phenomena.

#### Immunology

Many research workers have directed their attention to various factors that may be involved in the resistance of the udder to infection (11, 133). There is evidence that circulating antibodies are of great importance in mastitis. In some cases antibodies are easily detectable and their presence can be related to the incidence of the clinical disease. In others antibodies can be detected only in minimal amounts or are apparently absent (50).

In the 1930's considerable work was directed toward the prevention of the disease by means of vaccination. Carpenter (17) found that those cows which received several subcutaneous inoculations of Streptococcus agalactiae experienced a shorter period of clinical disease and a less severe inflammatory reaction than unvaccinated cows. Plastridge and Hartsell (110) referred to one case of Streptococcus agalactiae mastitis in which only one quarter was infected. The in-

fection disappeared and two years later the same type of infection appeared again in the same quarter. The explanation for the reinfection was that any immunity which may have resulted from the first attack did not persist for two years. Similar findings were reported by other workers (60, 68).

Resistance to Streptococcus agalactiae has been shown experimentally in mice, guinea pigs and goats. Serum from goats immunized by intracutaneous or intraperitoneal inoculations of Streptococcus agalactiae was reported to confer to mice passive protection which was type specific (48). Some success with formalized Streptococcus agalactiae vaccine was demonstrated by Howell *et al.* (50). Two weeks after vaccination, more animals became free of Streptococcus agalactiae than those not vaccinated. It was also observed that the vaccinated cows had lower neutrophil counts in milk following intramammary challenge with  $10^5$ - $10^8$  Streptococcus agalactiae cells. When the vaccine was tested under field conditions there was a minimum level of statistical significance in favor of the vaccinated cows (9).

Failure of Streptococcus agalactiae to establish in quarters infected with other streptococcal species led to the hypothesis that cross-reacting streptococcal antibodies may be significant in suppressing the growth of Streptococcus agalactiae in the udder (124). Although Stark and Norcross (132) found cross-reactive antigens in Streptococcus agalactiae, Streptococcus dysgalactiae and Streptococcus uberis, he

showed a virtual lack of protective effects after either active or passive protection of mice immunized with a heterologous streptococcal species. It was concluded that no cross-protection occurs among these three different species.

Kevin and Norcross (58) suggest that antigenic similarity between *Streptococcus agalactiae* and components of bovine tissues could explain the failure of detectable serum antibodies or very low serum titer after stimulation with a variety of *Streptococcus agalactiae* preparations. Norcross (95) found a high level of antibodies against extracellular antigens (polysaccharides) of *Streptococcus agalactiae* after experimental inoculation. The level of antibodies was higher in colostrum than in milk. He concluded that antibodies were stimulated to the greatest degree against extracellular products of the streptococcus and that it would not be expected that those antibodies could be active as a defense against reinfection with the same organism or closely related one. Later he was able to detect antibodies to extracellular antigens of *Streptococcus agalactiae* after 457 days of continuous infection. In this experiment colostrum was very rich in anti-streptococcal antibodies (94).

Murphy and Stuart (88) found that following intramammary inoculation of small numbers of *Streptococcus agalactiae*, every normal gland became infected. They thought that the bactericidal or bacteriostatic properties of

normal uninfected glands would not be able to protect against the introduction of small numbers of Streptococcus agalactiae shortly after milking (88). This is contrary to Jones and Little's finding (54) that Streptococcus agalactiae grows more slowly in fresh milk because the fresh milk has a bacteriostatic or bactericidal property.

Murphy and Stuart (89) stress the importance of the teat canal as a barrier to infection but believe that this barrier is more effective in some animals than in others. In at least four out of six animals this difference was constant over a period of five months and did not appear to be affected by the exposure process itself in either the resistant or susceptible cows. They also tried different stresses in a group of resistant first-calf heifers. These stresses consisted of the insertion of a small cotton swab into the teat, repeated application of the milking machine ten minutes after milk had been removed from the gland by normal machine milking and removal of the soft keratin lining from the streak canal by means of polyvinyl tubing. The last of these stresses broke the resistance of the udder. It was concluded that the teat canal is the main barrier to infection in those glands said to be normal and uninfected (84).

#### Hypersensitivity

Jones and Little (54) reported that the udder should first be sensitized by frequent exposure to infection before

an organism can establish itself. Several inoculations of Streptococcus agalactiae were necessary before typical mastitis appeared.

Enhanced inflammatory reactions following successive subcutaneous inoculations are evidence that cows become specifically sensitized to Streptococcus agalactiae. In contrast to enhanced reactions of the skin, the response of the mammary gland was less following sensitization. The author (130) suggests that this is due to the greater dilutions of the antigens in the mammary gland than in skin.

Lancaster and Stuart (60) demonstrated that previously infected cows were more susceptible to infusion of small amounts of abnormal milk containing Streptococcus agalactiae than were cows which had resisted previous natural exposure. They concluded that cows differ in susceptibility to Streptococcus agalactiae and they associated this difference with the interior of the udder.

Spencer and Angevine (129) in 1950 reported that cows infected with Streptococcus agalactiae responded to intradermal injection of a streptococcal antigen with longer and more persistent swelling than did normal animals. They supported their evidence with similar experimentation in rabbits. Those rabbits repeatedly injected intravenously or intracutaneously with Streptococcus agalactiae showed an exaggerated response following intramammary injection of the antigen.

In 1966 Zarkower and Norcross (143) demonstrated classical delayed hypersensitivity response to Streptococcus agalactiae and soluble extracts of the same organisms on the dermis of sensitized rabbits. Previously infected cows failed to react to intradermal inoculation of Streptococcus agalactiae extracts but an acute response of the cow to intramammary infusion of the Streptococcus agalactiae extract was present.

Campbell and Norcross (16) found that colostrum of many first-calf heifers contain antibodies which will react with the soluble cellular antigen of Streptococcus agalactiae. It is possible that these antibodies might play a significant role in the production of hypersensitive response to mastitis later in life.

Schalm et al. (123) in their studies of experimental coliform mastitis indicated that the infrequency of coliform mastitis in the younger cows may be ascribed to an effective natural barrier to bacteria from a normally functional streak canal.

Thomas et al. (136) have suggested that the periodic flushing of the streak canal during milking constitutes a partial barrier to the entrance of bacteria into the udder. They observed that of eight lactating cows which were milked regularly and exposed to various bacteria, only two quarters became infected, while in eight lactating cows in which milking had suddenly ceased, 22 quarters became infected.

Other authors think that cells in milk are an important



factor not only as a sign of abnormality of the udder but also as a defense against pathogens. Some workers (43, 81) have stated that when many leucocytes are observed in milk this is reliable evidence that something is wrong and one must then search for the cause of the trouble. Delay in the cellular response to streptococci during the first 12 to 24 hours after several exposures, permitted survival and multiplication of the streptococcus leading to establishment of chronic infection. Leucocytosis in milk was associated with depressed numbers of streptococci (125).

Experimental infection with Staphylococcus aureus gave rise to mastitis of varying degrees of severity. In all the animals the cell content of the lymph before infection was composed almost entirely of medium and small lymphocytes. Elevation of polymorphonuclear and cells of the plasma cell series in the lymph was present during the course of acute mastitis (98).

Milk containing larger numbers of cells possessed an inhibiting power not seen in low cell count milk from uninfected glands. This conclusion was reached by Murphy and Stuart (88) when they failed to infect quarters naturally infected with staphylococci and corynebacteria.

Repeated infusions of sodium chloride into the mammary gland of the cow elicited leucocytosis in the quarter tested and no response in those quarters not treated. A pre-existing leucocytosis protected most of the quarters against

inoculation of Staphylococcus aureus. The streptococcus became established in all quarters but there was definite inhibition of the multiplication of streptococci in those quarters in which a leucocytosis had previously occurred (4a).

Kent and Newbould (57) found fewer efficient phagocytes among leucocytes collected from artificially irritated mammary glands than among the leucocytes from blood of the same animals. Leucocytes isolated from mastitic milk were washed and some disrupted and crude lysosomal preparations from such leucocytes were inoculated into the mammary gland. This produced signs of acute inflammation, rise in the concentration of whey serum albumins and diapedesis of neutrophil leucocytes from blood to milk for three to five days. They concluded that infiltration of leucocytes into the milk acts as a catalyst in the inflammatory reaction (52).

#### Antibodies

Immunoglobulins are large glycoproteins that include all molecules with antibody activity. They are uniquely heterogeneous among milk proteins because they are synthesized by large numbers of cells or cell clones that each produce a different amino acid sequence.

The major immunoglobulin of bovine milk is IgG<sub>1</sub> (69). Serum albumin, immunoglobulin,  $\alpha$  lactoalbumin and  $\beta$  lactoglobulin are proteins found in milk whey. The first two are proteins which pass into the milk from the blood and the latter

two are synthesized in the mammary gland. It has been suggested that increases in the blood protein fractions in whey, especially the blood serum albumin fraction, may be a sensitive indicator of increased capillary permeability which occurs in inflammation (7).

The bactericidal activity of cell-free mastitis milk generally coincided with increases in serum albumin and immune globulin fractions of the whey protein. Since the presence of plasma proteins depends upon capillary permeability which is increased during inflammation, the bactericidal effect of mastitis milk could be related to the presence and degree of udder inflammation (50).

Murphy et al. (82) when comparing the gammaglobulin components of bovine colostrum and dry secretion with serum, found that the proportional amount of each component varies according to the character of the secretion. It was also observed that both 7S slow and fast gammaglobulin accumulate in the secretion of the dry udder in relatively high levels. Colostrum and milk have fast 7S gammaglobulin, IgA and IgM.

In 1965 Pierce and Feinstein (109) demonstrated that the mammary gland has a high selective preference for the electrophoretically fastest serum immunoglobulin and the ability to concentrate it in colostrum. The slowest serum immunoglobulin was present in the colostrum as a comparatively minor component. In contrast to the mammary gland, the intestine of the new born calf showed no selectivity. The

selection of proteins from maternal plasma for admission to the calf's circulation occurs within the mammary gland during the formation of colostrum but not during absorption across the calf's intestinal mucosa (13).

IgA is the most abundant immunoglobulin in exocrine glands except for the mammary gland and the reproductive tract. Antibody activity has been demonstrated in all classes and subclasses of bovine immunoglobulin. IgM and IgA are effective agglutinating antibodies.

Nilsson (92) examined milk samples from cows with staphylococcal or Streptococcus agalactiae mastitis and normal cows. No changes in the amount of total protein in mastitic milk compared to normal milk were detected but with ninhydrin staining he saw six fractions in normal milk and in milk from animals with chronic mastitis, seven fractions were present. He postulated that this additional fraction may represent antibodies formed locally in mammary tissue.

Schanbacher et al. (126) postulated that one of the whey proteins that is able to kill bacteria or arrest their growth, is lactoferrin. They stated that this protein is abundant in dry secretion and is a part of the 7S protein peak. It appears that the potential protective effect of lactoferrin is due to its ability to tightly bind two molecules of iron/molecule of protein. This protein stops growth of bacteria and may be important in enhancing resistance of the dry mammary gland to infection.

Partfield et al. (105) believe that the udder is a potent source of local antibodies but some other workers found very high levels of antibodies in blood and low levels of antibodies in whey after immunization with diphtheria toxoid and Salmonella pullorum. Therefore, they suggested that the antibody present in milk comes from circulation and it is not locally produced (41). Norcross et al. (96) reported that immunization of cows by inoculating inactivated bacteria into the supra-mammary lymph node will result in the appearance of protective antibodies in the milk of the quarters on the same side as the node which has been inoculated. Some time later antibodies can be demonstrated in the milk from the quarters on the opposite side.

Mackenzie et al. (71) followed for 40 hours the specific activity of  $\gamma$  globulin inoculated with  $I^{131}$  in the efferent lymphatic duct of one side. Following the introduction of staphylococci into the teat cistern of the same side, they observed a greater capillary permeability during infection as indicated by three- to four-fold increase in the lymph flow and a two-fold increase in the concentration of radioactivity. The increases in lymph flow and radioactivity were greater and persisted longer in the cases of severe mastitis.

The question of transport or local synthesis of the immunoglobulins into the lacteal secretion is not yet fully answered. Most of the cells found in mammary tissue which

are involved in local synthesis of immunoglobulins are concerned with the production of IgG and only a small number produce other immunoglobulins. The development of bovine lymphoid tissue and immunoglobulin-producing cells follows the same sequence of appearance as has been reported for man. The first lymphoid tissue is seen in the thymus at 42 days and the first IgM-containing cells appear at 59 days. Immunoglobulin G-containing cells appear at 145 days, however, IgA-containing cells are not observed even in older fetuses (15). IgM from cattle is a macroglobulin with properties similar to IgM of other animal species. It has a sedimentation coefficient of 19S (59). The primary immune response of the cow is found almost all in the IgM fraction (118). Butler and Maxwell (14) found a discontinuous distribution of IgM in bovine serum by using a radial diffusion assay technique and they also concluded that IgM exists in small amounts in normal serum. It was eluted by gel filtration just before IgG and its molecular weight was estimated to be 900,000.

The carbohydrate content of bovine IgM was found to be very similar to that reported from human IgM, i.e., hexose 5.5 percent, glucosamine 2.08 percent, galactosamine 0.72 percent (80). IgA is another gammaglobulin found in the mammary secretion of the cow and it is derived from serum. It was not demonstrated in the newborn calf which had not received colostrum but in adult cows its level in sera

ranges from 51 to 115 mg/100 ml (111).

In the bovine species the particular function of the mammary gland to transport selectively IgG from serum to colostrum does not affect other secretory organs. In those organs which secrete IgA as a major immunoglobulin, the transport from serum seems improbable. There is a definite difference in bovine species between secretory organs such as salivary glands which synthesize IgA locally and the mammary gland in which transport of the immunoglobulin from blood to milk occurs (70). The most abundant and most extensively studied immunoglobulin in the cow belongs to the IgG class. The homology of bovine IgG to the IgG of other species is supported by the finding that human gamma chains share antigenic determinants with bovine, caprine and ovine gamma chains.

The more basic IgG molecules are IgG<sub>2</sub> but they occur in low concentrations in milk, colostrum and saliva. IgG<sub>1</sub> consists of the less basic IgG immunoglobulins which often appear more heterogeneous than IgG<sub>2</sub> (12).

In anaplasmosis a progression of agglutinating antibody from IgM to IgG occurs and the latter can be detected after 18 months post-infection (83). In serum and whey giving positive titers for Brucella, the agglutinins usually occur in a class characterized as 7S, but serum and whey from selected animals yielded agglutinins of higher molecular weights and were found to have sedimentation constants of

19S and 12S (53).

Human sera from patients with infections due to Streptococcus Group A have higher levels of gammaglobulin rich in antibodies to extracellular products of the organisms and relatively low levels of antibodies to cellular components (45).

### Lymphocytes

Lymphocytes are relatively numerous in the blood of most species of domestic animals. In the cow they comprise about 60 to 65 percent of the total leucocyte count (134). The small lymphocyte has a cell diameter of less than 8  $\mu$ , a round deeply-staining nucleus and a narrow pale-staining cytoplasm. By electron microscopy it is observed that they have an irregular nucleus with abundant electron-dense chromatin at the periphery and some of this substance in the center. Occasional cells possess a nucleolus and the cytoplasm is scarce with abundant ribosomes and mitochondria. After stimulation with PPD some changes were seen including round nucleus with an area of dense chromatin in the periphery. The cytoplasm has abundant ribosomes, polyribosomes and Golgi apparatus (36). Stem cells are the primary origin of lymphocytes. These cells can be differentiated in specific cell lineage and pluripotential stem cells; the latter form the marrow stem cells.

Marrow pluripotential cells enter the thymus where they



become differentiated (34).

It has been suggested that in the adult as well as in the embryonic state, myeloid tissue is a major source of stem cells for the thymus. Embryonic liver was not immunologically competent to produce graft vs host reaction but competence was acquired when the liver explants were cultured for several days in combination with thymic tissue (140).

Small lymphocytes arise from the division of large and medium lymphocytes which are located throughout the lymphoid system. Small lymphocytes recirculate from blood into the lymph and then back to blood. These cells are long-lived small lymphocytes that are derived from thymus and in turn these cells are derived from bone marrow (74). These thymus-derived cells will reach the periphery, enter at the germinal center of lymphoid follicle and proliferate as antigen reactive cells. Long-lived small lymphocytes can transform to a large blast cell by a sequence of changes (34). The region where lymphocytes most likely make contact with the antigen is in the zone surrounding the germinal centers and it has primarily long-lived recirculating cells. A high percentage of lymphocytes in Peyer's patches and white pulp of the spleen belong to the long-lived recirculating cells (46). Approximately one-third of small lymphocytes in blood are the short-life variety. In contrast, in thoracic duct lymph about 90 percent of the small lymphocytes are long-lived and 10 percent short-lived. (34).

The majority of large lymphocytes are precursors to themselves and the number formed from cells of different morphology is small compared to the number of large lymphocytes present. Large lymphocytes have the same properties as small lymphocytes. The only difference is that large lymphocytes synthesize DNA and divide (40). Few of them give rise to small lymphocytes but the great majority of those in thoracic duct lymph migrate into the submucosa of the intestine where they develop into plasma cells.

Hall and Morris (45) reported that the large lymphocytes in the efferent lymph draining the sheep popliteal node were produced within the node. This report is in agreement with Everett and Tyler (34) who suggested that large lymphocytes are not recirculating but are added in route as the lymph passes through the lymph nodes.

Other types of lymphocytes which are very important in immunity are the so-called B cells. B cells in birds are derived from the bursa of Fabricius. It has been suggested that its equivalent in mammals is the gut-associated lymphoid tissue (22).

Claman and co-workers (21) demonstrated the interaction of T and B cells by inoculating lethally irradiated mice I.V. with normal mouse lymphoid cells and sheep red blood cells. One week later slices of recipient's spleen were incubated at 37 C in agar to which complement was added. As a result, hemolytic foci were present in the agar after incubation.

These foci occurred when normal mouse spleen or lymph node cells were in the original inoculum but did not appear when thymus cells or bone marrow cells alone were inoculated. They concluded that bone marrow population contained the effector cells capable of producing antibody but only in the presence of auxiliary cells present in the thymus (21).

Dennert and Lennox (30) came to similar conclusions while studying the cell interaction in humoral and cell-mediated immunity. They expressed the view that T-derived lymphocytes trigger B lymphocytes to produce target-cell specific antibody. Thus, the role of the T cell in cell-mediated and humoral immunity is to cooperate with B cells.

#### Mediators produced by sensitized lymphocytes

Sensitized lymphocytes are thought to play a key role in certain biological reactions including delayed hypersensitivity, resistance to certain microorganisms, rejection of transplanted tissue and possibly autoimmune disease. Two different types of cells are involved in hypersensitivity reactions, lymphocytes and macrophages (26). Macrophages employ their phagocytic activity to attack or destroy foreign material. After contact with antigen, lymphocytes become sensitized to that antigen and elaborate cell-free factors after stimulation by the specific antigen.

The cell-free factors produced by sensitized lymphocytes

were enumerated by David (27) and called "lymphokines".

1. Factors affecting macrophages
  - a. Migration-inhibitory factor
  - b. Macrophage-activating factor
  - c. Chemotactic factor for macrophages
  - d. Macrophage-aggregation factor
2. Chemotactic factors
  - a. Chemotactic factor for neutrophils
  - b. Chemotactic factor for eosinophils
  - c. Chemotactic factor for lymphocytes
3. Cytotoxic factor
4. Growth-inhibitory factors
  - a. Clonal-inhibitory factor
  - b. Proliferation-inhibitory factor
5. Skin-reactive factor
6. Blastogenic or mitogenic factor
7. Interferon
8. Immunoglobulin
9. Transfer factor

David defined lymphokines as follows: "Biological activities mediated by cell-free soluble factors which are generated during interaction of sensitized lymphocytes with specific antigen but which are expressed without reference to immunological specificity."

Gell (39) observed numerous variations in the quality of the delayed hypersensitivity skin reaction depending upon the chemical nature of the agent used as antigen, i.e., gammaglobulin produced a large, well-defined indurated reaction while ovalbumin reactions were widespread and only

slightly erythematous and indurated. His explanation for this phenomena is that the antigens have different capacities to remain at the injection site.

In 1940 Freund et al. (37) found that tubercle bacilli incorporated in paraffin oil resisted destruction and maintained a persistent reaction. By repeated subcutaneous injection, this material induced in rabbits an intense sensitivity and abundant antibody formation without suppuration or necrosis.

Bennet and Bloom (3) developed conditions for cultivating guinea pig lymph node lymphocytes in serum-free medium. When the supernatant from those cultures was concentrated and inoculated into the skin of normal guinea pigs, only the supernatant from cultures of antigen stimulated lymphocytes produced reactions. The reactions were characterized by palpable induration with little or no erythema. At four hours, the cells of these lesions were predominantly mononuclear with relatively few neutrophils and by 16 hours about equal numbers of neutrophils and mononuclear cells were present.

Chase (19) studied the cellular transfer of cutaneous hypersensitivity to tuberculin and observed maximal reactivity of the recipient at 72 and 96 hours after injection of the cells from guinea pigs hypersensitive to tuberculin. He did the same experiment with serum of the donors of active cells but failed to transfer the hypersensitivity.

Several workers have tried passive induction of tuberculin sensitivity by means of peritoneal exudate cells from sensitized donors (63). Metaxas and Metaxas-Buehler (77) found that cutaneous reactivity in guinea pigs which have received cells intraperitoneally is not established until about 20 hours after cell transfer.

Lawrence (64) passively transferred generalized cutaneous streptococcal reactivity of the delayed tuberculin type to human recipients by means of viable leucocytes. Delayed cutaneous reaction did not develop when leucocytes were obtained from negative donors.

The first observation of migration-inhibitory factor was reported by Rich and Lewis (117). They inhibited the migration of cells obtained from spleen and lymph nodes from tuberculous rabbits by adding tuberculin to the cell culture medium.

Bennet and Bloom (3) obtained inhibition of migration in peritoneal exudate cells from guinea pigs sensitized with BCG but at the same time they did not detect circulating antibodies in those animals. They did not obtain inhibition of migration in guinea pigs' peritoneal exudate cells if the animals had high titers of antibody.

The experiments of David et al. (29) were directed toward the finding of the cell responsible for the production of migration-inhibitory factor. They reported macrophages and lymphocytes of the peritoneal exudate of hypersensitive

guinea pigs and found that only one percent of purified peritoneal lymphocytes from these guinea pigs was able to inhibit the migration of a population of normal macrophages.

Thor and Dary (137) in 1968 described the inhibition of migration of guinea pig macrophages produced by stimulated human lymphocytes.

Aalund et al. (1) suggested the possible application of the migration test in the diagnosis of Johne's disease. They found a lower migration index of the bovine cells with the homologous antigen.

#### Hotis Test

The Hotis test was developed during the years 1933 to 1935 by R. P. Hotis. He observed that when some samples were incubated in glass tubes for 24 hours, small round flakes appeared on the walls of the tube. He found that the addition of brom cresol purple to the milk sample would permit easier recognition of the flakes after incubation. He subjected 753 samples of milk to this procedure and to blood agar plate culture. It was found that 74.4 percent of the samples showed typical changes of the colors and flakes on the sides of the tube and from these same samples Streptococcus agalactiae was isolated on the blood agar plate cultures (49).

Miller and Heishman (79) during a survey of 1500 milk samples found that the Hotis test is about 85 to 90 percent

as accurate as cultures on blood agar plates for the detection of Streptococcus agalactiae in milk.

Miller (78) suggested that the test be applied for routine examination. He established the significance of the reaction of the test and suggested that the tendency of Streptococcus agalactiae to grow in chains and to ferment lactose is probably responsible for the changes in color and the formation of the flakes that permit its recognition.

The changes have been described by Murphy (87) who stated that upon incubation the milk with brom cresol purple as indicator, changes color from purple to a blue gray, distinct olive, through a yellowish-green and greenish-yellow to a yellow color. The test could also indicate the presence of Staphylococcus aureus but in that case the colonies are rust color.

Merchant and Packer (76) in 1952 suggested that the appearance of canary yellow colonies of organisms along the sides of the tube or in the bottom indicates infection due to Streptococcus agalactiae. They believe that the Hotis test is quite specific for Streptococcus agalactiae.

McCulloch and Fuller (73) inoculated raw milk from a Hotis negative cow with a pure culture of Streptococcus agalactiae and found the Hotis test positive. The addition of 10 to 15 percent bovine or equine blood serum to the milk followed by inoculation with a positive Hotis strain of streptococcus produced typical flakes and color change.



They stated that several workers earlier recognized the tendency of streptococci to grow in clumps when the medium contained agglutinins specific for them. The same surface factor which causes bacteria to clump would make them migrate toward any interface (in the Hotis test the wall of the tube) and adhere. Milk from one herd was negative when inoculated with Streptococcus agalactiae but when human serum was also added, the Hotis test was positive. The milk from the animals tested was probably free from agglutinins to this organism. The opinion of the workers is that the Hotis test appears not to be specific for Streptococcus agalactiae.

The Hotis test is sometimes positive but no colonies of Streptococcus agalactiae are detected on blood agar plates. This situation is present with unusually occurring strains of Streptococcus agalactiae that require an atmosphere of 10 percent carbon dioxide in air for colony development on the surface of the agar (122).

Schalm (119) also found the Hotis test useful for the detection of Staphylococcus aureus but the color of the colonies is different from those of Streptococcus agalactiae.

## MATERIAL AND METHODS

## Experimental Cows

A group of ten cows from the Obstetrics Laboratory of the College of Veterinary Medicine was utilized in this study. Three of these cows were used in the preliminary work and seven were in the second part of the experiment. The cows were housed in a stanchion barn. The age, breed and number of lactations of these animals are shown in Table 1. The cows were fed on dry hay which was supplemented at milking time with a grain mixture. They were milked by hand for the duration of the experiment.

Table 1. Cow number, age, breed and lactation number

Cow	Age	Breed	Lactation number
1	4	Jersey	2
2	3	Holstein	1
3	3	Guernsey	1
10	6	Guernsey	4
5	4	Guernsey	2
6	5	Guernsey	3
4	4	Holstein-angus	2
9	4	Guernsey	2
8	3	Guernsey	1
7	4	Guernsey	2

## Source of Organisms

The organisms used in the study were Streptococcus agalactiae strain 6-4 and Salmonella pullorum strain 275. They were obtained from the stock cultures maintained in

the Department of Veterinary Microbiology at Iowa State University.

The strains have been maintained on tryptose agar slants through many transfers over several years. Before use they were streaked on blood agar plates made with 5 percent bovine blood in tryptose blood agar base. From the blood agar plates a colony was inoculated into tryptose broth and utilized after 24 hours incubation. Growth characteristics of Streptococcus agalactiae were determined in 10 percent serum broth and 5 percent bovine blood agar plates. Biochemical characterization of the two organisms were made using standard media and reagents. The characteristics and biochemical reactions corresponded to those reported for Streptococcus agalactiae and Salmonella pullorum and are seen in Table 2.

#### Bacterial Antigens

After characterization of the bacteria, three different antigens were prepared.

#### Sonicated cells

The streptococcal cells were grown in three liters of Todd-Hewitt<sup>1</sup> broth during 24 hours at 37 C. Following incubation, the cells were sedimented and washed three times with saline solution.

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<sup>1</sup>Difco Laboratories, Detroit, Michigan.

Table 2. Characteristics of Streptococcus agalactiae 6-4 and Salmonella pullorum 275

	<u>Streptococcus agalactiae</u>	<u>Salmonella pullorum</u>
Lactose	+	
Sucrose	+	
Salicin	+	
Trehalose	+	
Litmus milk	acid, coagulation	
Sodium Hippurate	+	
Hemolysis	narrow beta	
CAMP test	positive	
Gram stain	positive	negative
Growth in broth	flocculent in bottom	diffuse
Dextrose		⊕
Fructose		+
Glactose		+
Xylose		+
Arabinose		+
Mannitol		+
H <sub>2</sub> S		+
Nitrate reduction		+
Indol		-
Microscopically	rather long chains of gram positive coccus	small gram negative rods

+ = positive

⊕ = positive with gas

- = negative

A heavy suspension of cells was sonicated for a total of 30 minutes in a Bronwill Biosonik II<sup>1</sup> using the standard probe at 20,000 Hz. In order to more completely disrupt the cells, the heavy suspension of the organism was mixed 1:1 with fine beads<sup>2</sup> before the sonication treatment.

<sup>1</sup>Bronwill Scientific Co., Rochester, New York.

<sup>2</sup>Glass beads 45-90 u. Sigma Chemical Co., St. Louis, Missouri.

Excessive heating of the sample was avoided during the process by the aid of a crushed ice bath. The sonication was performed in the following manner: sonication at full power for 30 seconds, then interrupted for about two minutes to allow the suspension to cool. The disrupted cell suspension was centrifugated at 2,000 g in a refrigerated centrifuge for two hours. The supernatant was then sterilized by passing it through a 0.45 u Millipore filter<sup>1</sup>.

#### Preparation of protein antigen

Protein extract was produced using the method of Lancefield and Perlmann (62) for the production of type-specific M antigen from group A streptococci. The streptococci were grown in 12 liters of Todd-Hewitt broth for 24 hours. The suspension was collected in 250 ml centrifuge bottles and the cells were spun down at 1600 RPM<sup>2</sup> for two hours. The cells were resuspended in 150 ml of saline solution. The pH was adjusted to 2.5 by adding 1N HCl. The preparation was placed in a water bath at 95 C for ten minutes and the pH adjusted to 7.5 with N NaOH. Following centrifugation for 30 minutes at 1,000 RPM<sup>2</sup>, the supernatant fluid was removed and the bacterial sediment washed once with saline solution at pH 7.5. The extraction of cells was repeated once more and then the

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<sup>1</sup>Millipore Filter Corporation, Bedford, Massachusetts.

<sup>2</sup>International centrifuge model UV. International Equipment Co., Needham, Massachusetts.

supernatants were pooled and filtered through a 0.45 u filter<sup>1</sup>.

Protein and ribonucleic acid were precipitated by acidifying the filtrate to pH 2.0 with 6N HCl followed by solution in 20 ml of 0.01 M phosphate buffer at pH 8.0. Twenty ug of ribonuclease<sup>2</sup> were added and the solution was dialyzed in cellophane tubing<sup>3</sup> during digestion at 37 C against 0.01 M phosphate buffer at pH 8.0. After digestion for four hours, the suspension was dialyzed for two days at 3 C against 0.01 M phosphate buffer changed every 12 hours to eliminate digestion products. The protein was precipitated between 30 and 60 percent saturation of  $(\text{NH}_4)_2 \text{SO}_4$  at pH 6.0 and 8.0. The product was dialyzed against saline solution at 3 C for two days and then it was kept frozen.

#### Extraction of polysaccharide

The Formamide method described by Fuller (38) was followed. Four tubes, each containing 25 ml of an 18 hour Todd-Hewitt broth culture were centrifuged at 1500 RPM for 45 minutes and the supernatant removed. Five ml of formamide was added to each one of the four tubes. After shaking the tubes several times, they were placed in an oil bath at 150 C for 15 minutes. The tubes were allowed to cool and

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<sup>1</sup>Falcon disposable filter. Benton Dickinson and Co., Oxnard, California.

<sup>2</sup>Calbiochem, Los Angeles, California.

<sup>3</sup>Fischer Scientific Corp., Pittsburgh, Pennsylvania.

12.5 ml of acid-alcohol<sup>1</sup> was added. The supernatant from the four tubes was drawn off by pipette and distributed in ten test tubes and 7 ml of acetone was added to each tube. The resulting precipitate was collected by centrifugation and the supernatant discarded. The precipitates were pooled and then dissolved in four ml of saline solution containing one drop of phenol red indicator. The solution was neutralized with a trace of sodium carbonate and then frozen.

#### Antistreptococcal Serum

Two rabbits were inoculated intravenously with a suspension of formalin-killed cells of Streptococcus agalactiae that were resuspended in a solution composed of one part 1:10,000 merthiolate and nine parts of 0.85 percent sodium chloride. Turbidity was adjusted to give a final concentration equivalent to the McFarland nephelometer tube number 10 or approximately  $3 \times 10^9$  cells per ml. The schedule of inoculation was the one described by McCarthy and Lancefield (72) with the slight difference of one week instead of four days of rest between two series of inoculations. The rabbits received 0.5 ml of the suspension on three consecutive days. After one week of rest, the rabbits were given four injections, one each day, of 1 ml of the suspension; one week later blood was taken from the heart. Rabbit antisera were used to test the protein and polysaccharide

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<sup>1</sup>95 parts of anhydrous alcohol with 5 parts of 2N HCl (1 part concentrated acid with 4 parts water).

antigens.

For the protein antigen the capillary precipitin test described by Swift et al. (135) was followed. The titer of the protein antigen was 1:640. The polysaccharide was tested by the precipitin test described by Lancefield (61). Two-fold dilutions of the polysaccharide solution were made from 1:10 to 1:160 in a total volume of 0.4 ml with saline solution. The concentration of the original polysaccharide ranged from 10 to 1.25 percent. An amount of 0.4 ml of rabbit antiserum was added and the tubes were incubated for two hours at 37 C and then they were refrigerated overnight. The best precipitation was 1:40 or 2.5 percent concentration. This dilution was utilized for the MIF test.

#### Determination of Protein

Quantitative determination of protein for antigen 1a and 2 was made following the Lowry procedure as described by Kabat and Mayer (55). The reagents used were reagent A made up of 2 percent  $\text{Na}_2\text{CO}_3$  in 0.1 M NaOH; reagent B made up of one percent  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and 2 percent sodium tartrate mixed 1:1. Reagent C consisted of one ml reagent B and 50 ml reagent A. Reagent D was 1N Folin's solution<sup>1</sup>. The diluent used was 0.1 M Tris-buffer pH 8.0. Two and one-half ml of reagent C was added to 0.5 ml of the sample. The mixture stood for ten minutes and 0.25 ml of cold reagent D

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<sup>1</sup>Fischer Scientific Corp., Pittsburgh, Pennsylvania.



was added and allowed to stand an additional 45 minutes.

For the standardization of the curve for protein determination, Labtrol<sup>1</sup> containing 7.1 mg/ml of protein was diluted 1:100 and from this, two-fold dilutions up to 1:64 were made. They were read in a Coleman Spectrophotometer at a wavelength of 660 nm. These readings were used to make the standard curve. The protein content of the three bacterial antigens and the two globulin fractions separated from whey and blood serum are summarized in Table 3a.

Table 3a. Concentration of protein in bacterial antigens and immunoglobulin fractions

Antigen or Immunoglobulin	Spectrophotometer Reading	Protein $\mu\text{g/ml}$
Filtered, sonicated streptococcal cells	0.035	400 $\mu\text{g/ml}$
Protein extract from <i>Streptococcus agalactiae</i>	0.088	800 $\mu\text{g/ml}$
Polysaccharide extract from streptococcus	0.021	200 $\mu\text{g/ml}$
IgM from serum cow 2	0.035	350 $\mu\text{g/ml}$
IgM from whey cow 2	0.040	450 $\mu\text{g/ml}$
IgG from serum cow 6	0.049	500 $\mu\text{g/ml}$
IgG from whey cow 6	0.045	480 $\mu\text{g/ml}$

<sup>1</sup>Dade Labtrol. Scientific Products, Division of American Hospital Supply Corp., Miami, Florida.

### Cleaning of Glassware

All glassware items used for collection, isolation and culture of bovine lymphocytes from blood and milk were thoroughly washed in Haemo-Sol cleanser<sup>1</sup>, rinsed ten times in tap water, ten times in distilled water and two times in deionized water. This was followed by sterilization by autoclaving and drying.

The 500 ml flasks used for the collection of blood were prepared prior to autoclaving with 13 wooden applicator sticks held together at one end with a rubber band and the other ends spread apart forming a cone. This cone was arranged in the flask so that the stopper of the flask pressed against it and prevented its rotation during defibrination of the blood.

Tubes utilized for lymphocyte cultures were screw-cap tubes 16 x 125 mm and for the centrifugation of the blood 35 ml screw-cap tubes 16 x 150 mm were used.

For the collection of cells all the glassware was washed and rinsed ten times in deionized water, dried and sterilized by dry-heat. The slides and rings for the chamber were washed by hand in distilled water followed by three rinses in deionized water. Each slide was wiped with gauze and then polished with lens paper. They were then wrapped in gauze and aluminum foil and sterilized in the autoclave.

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<sup>1</sup>Scientific Products, Evanston, Illinois.

## Media

Cell culture media used were purchased from a commercial source<sup>1</sup>. The various components were stored at 4 C before use except the antibiotics and fetal calf serum which were kept frozen at -20 C.

For the in vitro culture of macrophages, the medium used was minimum essential medium<sup>1</sup> (MEM) with Hanks' salts and L-glutamine<sup>1</sup>. Hanks' balanced salt solution<sup>1</sup>, calcium and magnesium free, was used to wash the cells.

For the in vitro culture of blood lymphocytes, Medium 199 (10X)<sup>1</sup> with L-glutamine and without sodium bicarbonate was used.

## Sensitization of the Cows

The cows were sensitized by subcutaneous inoculation of complete Freund's adjuvant<sup>2</sup> (containing 1 mg/ml Myco-bacterium tuberculosis H37Ra) and Streptococcus agalactiae. The inoculations were made once a week for three weeks and each dose consisted of 1.5 ml Freund's complete adjuvant

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<sup>1</sup>Grand Island Biological Co., New York, New York.

<sup>2</sup>Bacto Adjuvant Complete. Difco Laboratories, Detroit, Michigan.

and 2 ml Streptococcus agalactiae  $3 \times 10^9$  cells/ml (Table 3b). The material was inoculated into the lateral aspects of the neck or both sides of the brisket.

Table 3b. Schedule of sensitizing injections

	Inoculum	Route of Injection	Dates
Cow 1	Freund's complete adjuvant, 2.5 ml	Subcutaneous	7-20-72 7-27-72
Cow 2	Freund's complete adjuvant, 1.5 ml + <u>Streptococcus agalactiae</u> , 2 ml	Subcutaneous	8-17-72 8-24-72 8-30-72
Cow 3	Freund's complete adjuvant, 1.5 ml + <u>Streptococcus agalactiae</u> , 2 ml	Subcutaneous	9-11-72 9-18-72 9-25-72
Cow 4	Freund's adjuvant, 2.5 ml	Subcutaneous	12-01-72 12-08-72
Cows 5, 6	Freund's complete adjuvant, 1.5 ml + <u>Streptococcus agalactiae</u> . 2ml	Subcutaneous	12-08-72 12-15-72 1-04-73

Before the first inoculation the cows were milked and such tests as the Wisconsin mastitis, catalase, Hotis tests, somatic counts and aerobic and anerobic cultures on blood agar plates were performed to verify that the cows were free of mastitis.

#### Harvest of Peritoneal Cells

Guinea pigs weighing 400-500 grams were the donors of the peritoneal macrophages. A total of 16 guinea pigs were

utilized. Cells from three were used as normal cells and the others were sensitized with complete Freund's adjuvant and Streptococcus agalactiae  $9 \times 10^8$  cells/ml. The number of streptococci was standardized against the tube number 3 of the McFarland nephelometer. To obtain the peritoneal exudate cells, a modification of the technique presented by J. David and R. David (28) was followed. Twenty ml of light mineral oil was injected intraperitoneally four days before the harvest of the cells. The animal was anesthetized with ether<sup>1</sup> and its abdomen shaved and wiped thoroughly with 70 percent alcohol. A mid-line incision was made through the skin from the lower abdomen to the neck and the skin was reflected to each side. The abdominal wall was lifted with a hemostat and a small incision made. Using a 50 ml syringe and a 12 gauge needle with a rounded end, 30 ml of Hank's balanced salt solution (HBSS) with 20 units/ml of heparin were introduced<sup>3</sup> through the incision. Without removing the needle the abdomen was very gently massaged for four or five minutes and then the fluid was withdrawn very carefully into the syringe. The fluid was delivered into siliconed test tubes and then centrifuged at 1200 RPM<sup>2</sup> for ten minutes. During this period of centrifugation the oil phase came to the top and it could be removed with the HBSS by aspiration with a pipette. The cells were resuspended in 5 ml fresh HBSS and

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<sup>1</sup>Fischer Scientific Corp., Pittsburgh, Pennsylvania.

<sup>2</sup>International Equipment Co., Boston, Massachusetts.

transferred to clean tubes and then centrifuged at 750 RPM for seven minutes. The cells were washed two more times and centrifuged each time for seven minutes at 750 RPM. After the last washing, the supernatant was discarded and the cells suspended one to ten in Eagle's minimal essential medium<sup>1</sup> containing 100 units/ml penicillin and 100 µg/ml streptomycin and sufficient fetal calf serum<sup>1</sup> to give a concentration of 15 percent.

#### Collection of Lymphocytes from Blood

The technique developed by Kay and Kaeberle (56) was followed for the lymphocyte separation. Two hundred and fifty ml of blood was aseptically collected from the jugular vein of the cow into sterile 500 ml Erlenmeyer flasks. The flask was swirled during the time necessary for the fibrin to adhere to the cone of applicator sticks, usually about ten minutes.

The defibrinated blood was aseptically poured into 35 ml screw-cap tubes and then centrifuged for 45 minutes at 750 g in a swinging-bucket-type centrifuge<sup>2</sup>. At the end of centrifugation, the buffy coat was aspirated with a pipette and delivered into a 35 ml plastic syringe<sup>3</sup> that had been

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<sup>1</sup>Grand Island Biological Co., New York, New York.

<sup>2</sup>International centrifuge Model UV. International Equipment Co., Boston, Massachusetts.

<sup>3</sup>Monogest Sherwood Medical Industries, Inc., Deland, Florida.

prepared previously in the following manner. A one inch square of gauze that had been shredded was pressed into the 35 ml plastic syringe to form a small gauze column. At the needle end of the syringe, a rubber tube 1.5 inches in length was attached for easy delivery of the cells from the syringe. The syringe previously prepared was washed with HBSS, wrapped in foil and sterilized at 120 C for 15 minutes.

After sterilization, the needle end of the syringe was placed into a sterile plastic tube so that it fit tightly. The buffy coat was aspirated with a propipette and about 7 ml was poured into the column. It was covered with aluminum foil to avoid drying of the column and incubated at 37 C for 45 minutes.

After incubation, the syringe was filled with HBSS and the cells were flushed into the plastic tube using a sterile plunger. The suspension of cells was placed in 25 ml screw-cap tubes, 7 ml per each tube.

It was necessary to hemolyze the red cells present in the suspension by mixing one volume of cell suspension with two volumes of Tris-buffered isotonic ammonium chloride pH 7.2

The ammonium chloride was prepared following Boyle's method (8). Tris-buffer was prepared dissolving 20.6 gm Tris-base<sup>1</sup> in one liter of distilled water and adjusting

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<sup>1</sup>Tris-Base 2-amino-2 (hydroxymethyl) -1, 3-propanediol. Eastman Kodak Co., Rochester, New York.

the pH to 7.65 with HCl. It was mixed with nine volumes of 0.83 percent ammonium chloride<sup>1</sup> and the final solution adjusted to pH 7.2 with HCl. The cell suspension plus the ammonium chloride was left standing for three to five minutes until hemolysis was nearly complete. The tubes then were centrifuged at 225 g for ten minutes. The cells were rinsed again in ammonium chloride solution, centrifuged and resuspended in culture medium.

#### Enumeration of Leucocytes

The cells were mixed in serial dilution 1:10, 1:100, 1:1000 with one percent acetic acid. A hemocytometer chamber<sup>2</sup> was filled with the highest dilution and the cells were counted. Adjustment was made in the original suspension to obtain a final concentration of  $2 \times 10^6$  cells/ml.

The percentage of lymphocytes was determined by using smears of the cell suspension and staining with Wright's stain. The result was given in the number of lymphocytes per 100 cells.

#### Viability of Blood Cells

The method described by Merchant et al. (75) was followed to determine cell viability. Trypan blue solution,

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<sup>1</sup>J. T. Baker Chemical Company, Phillipsburg, New Jersey.

<sup>2</sup>C. A. Hauser and Son, Philadelphia, Pennsylvania.



0.1 percent, in saline solution was mixed 1:1 with the cell suspension and allowed to stand 5 to 15 minutes. A hemocytometer chamber<sup>1</sup> was filled with the suspension and the stained and unstained cells were counted. Those cells which absorbed the stain were considered dead while those which were unstained cells were considered to be alive. The viability varied from 84 to 95 percent with the exception of one week in which it was 75 percent. The percentage of lymphocytes in the total count of leucocytes varied between 89 and 96 percent and the number of cells in blood were concentrated to  $2 \times 10^6$  cells/ml.

#### Culture of Lymphoid Cells

The cells were distributed in tubes with 2 ml of medium TC199 and cultured as follows:

1. Normal cells: 2 ml of cell suspension containing  $2 \times 10^6$  cells/ml in TC199.
2. Cells plus antigen: 2 ml of cell suspension containing  $2 \times 10^6$  cells/ml plus the respective antigen: 15 ug/ml PPD or streptococcal antigen 25 ug/ml or 0.5 ml 1:40 polysaccharide dilution.
3. Controls that were made up of TC199 only.

The caps of the tubes were loosened and the tubes placed in a humidified incubator at 37 C with an atmosphere of 5 percent CO<sub>2</sub> and 95 percent air for three days. Each day the cell-free supernatant was carefully removed with a

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<sup>1</sup>C. A. Hauser and Son, Philadelphia, Pennsylvania.

pipette and replaced with fresh medium with antigen. The supernatant fluid collected each day from each set of samples was pooled and stored at -20 C.

If the supernatants were to be kept longer than four weeks before they were tested for migration inhibitory factor, they were dialyzed in the cold against 100 volumes of 0.15 M NaCl for 18 hours. They were then redialyzed against distilled water for 36 hours and the samples lyophilized and stored in the refrigerator. On the day the supernatants were tested they were diluted 1:5 with TC199. Before testing they were passed through a 0.45 u Millipore filter<sup>1</sup>.

#### Collection of Milk Lymphocytes

The procedure for collection of milk lymphocytes followed for the most part the technique developed by Kay and Kaeberle (56) for collection of lymphocytes from peripheral blood. Some modifications were necessary to get the lymphocytes from milk. Eighteen hours before milk was drawn, 25 ml of sterile saline solution was aseptically introduced into each quarter of the bovine mammary gland.

In order to obtain a sample of milk as free of contamination as possible, the udder was washed with tap water at approximately 100 F and then dried with disposable towels. The teats were wiped with a cotton soaked in 75 percent

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<sup>1</sup>Millipore Filter Corp., Bedford, Massachusetts.

alcohol and a sheet of plastic with an opening cut for each teat was placed over the udder. The teats were cleaned for a second time and the milk sample taken. The hands of the milker were washed previously and wiped with 75 percent alcohol.

Samples were collected in bottles of 250 ml capacity. Four ml of an antibiotic mixture which contained sufficient amounts of penicillin and streptomycin to make a final concentration 160 units/ml of penicillin and 160 µg/ml of streptomycin were added. As soon as the milk reached the laboratory, it was passed through four folds of sterile gauze into a 250 ml centrifuge bottle and centrifuged at 700 RPM for 75 minutes. The fat layer and a small portion of milk were removed by vacuum aspiration.

In order to obtain as many cells as possible, the supernatant was saved and approximately 5 ml of milk was left in the centrifuge bottle to resuspend the sediment which was transferred to a 15 x 125 mm screw-cap tube. The supernatant was passed through a 5 µ Millipore filter. The filter pad was placed in a sterile petri dish and then washed with 3 ml of Hanks' balanced salt solution. The product of this washing was pooled with the suspension of cells from the sediment and was centrifugated for 20 minutes at 700 RPM. The objective of the last centrifugation was to remove the remaining fat.

After removal of the fat, the cells were resuspended

in HBSS and placed in a small column of shredded gauze that was prepared previously. This column was set up in the same manner as it was described in the collection of blood lymphocytes except that it was washed in Hanks' solution just before use and not before sterilization. This change in procedure was necessary because differences in the pH of the milk samples required the preparation of Hanks' solution of different pH.

After 30 minutes of incubation, the cells were flushed into a plastic centrifuge tube. The suspension was poured into sterile screw-cap tubes and centrifuged 15 minutes at 600 RPM. The cells were washed twice with the Hank solution centrifuging each time 10 to 15 minutes at 600 RPM. Finally, the cells were suspended in medium TC199 which contained fetal calf serum 15 percent and antibiotics, 100 units/ml of penicillin and 100  $\mu$ g/ml streptomycin.

#### Viability of Milk Lymphocytes

Viability of the cells in milk was determined by use of Trypan blue in a concentration of 1:1600, adding 1 ml of one percent stock solution to 15 ml of saline solution. The cell suspension was mixed 1:1 with Trypan blue and allowed to stand for five minutes. A hemocytometer chamber was filled with the mixture and 100 cells were counted. The number of cells which were unstained was taken as the percentage of viability.

The viability of the cells in milk varied from 87 to 92 percent and the percentage of lymphocytes was within the limits of 75 to 82 percent (Figure 1). It is necessary to note that many cells from milk smears were not classified because we did not have enough confidence to give them any classification although they did have similarity to lymphocytes.

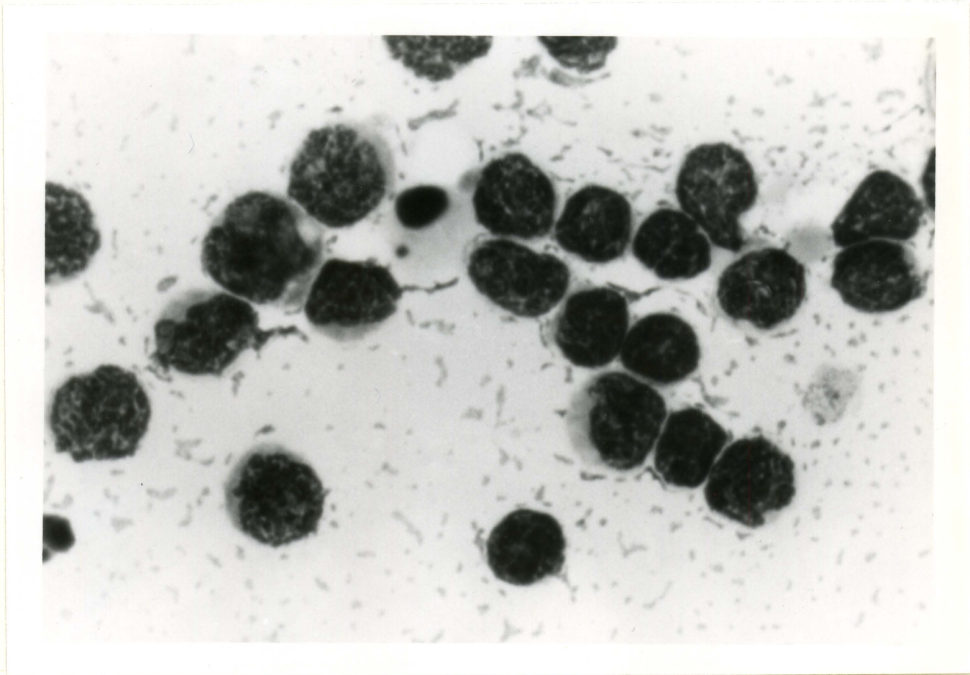
#### Enumeration and Culture of Lymphocytes from Milk

The cells were counted as described for blood lymphocytes with the exception that in milk the suspension was adjusted to  $1.5 \times 10^6$  instead of  $2 \times 10^6$  as was used for blood. The procedure for culture of the cells was the same as used for blood lymphocytes and the migration test was carried out in the same manner.

#### Viability of Cells After Culture

The viability of the cells was checked after three days of culture using Trypan blue exclusion. Most of the cultures showed several clumps of cells but from those cells it was possible to count, the viability was between 25 and 52 percent. The viability of lymphocytes from milk was always lower. After three days of culture, it varied from 18 to 39 percent.

Figure 1. Lymphocytes isolated from milk of a sensitized cow. Wright's stain (x 1600)



## Technique of MIF Test

The capillary tubes used were 1.6-1.8 x 90 mm with one closed end<sup>1</sup>. The tubes were filled with the suspension of peritoneal cells by using a 2 ml syringe with 20 gauge needle four inches long. To fill the capillary tubes it was necessary to hold the tube with sterile forceps. After the capillary was filled, it was placed in a test tube and centrifuged for three minutes at 600 RPM at 4 C. After centrifugation, one by one each capillary tube was placed in a sterile petri dish and cut with a diamond pencil at the interface of the sedimented cells and the liquid. The capillary was held with sterile forceps while a cut was made with the diamond pencil. The capillary was rotated 180° and another cut made continuing until it came apart.

The chamber used was a variation of the one described by David and David (28). It was made of two glass slides and a rubber "O" ring<sup>2</sup> which provided a 1.5 mm working distance inside the chamber. The rubber ring was placed over one side and the piece of capillary tube containing the cells was placed on the slide within the ring. To have the tube adhere to the slide, it was necessary to place a very small amount of silicone stopcock grease<sup>3</sup> on the slide.

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<sup>1</sup>KIMAX. Fischer Scientific Corp., Pittsburgh, Pennsylvania.

<sup>2</sup>Bellco Silicone Rubber #1025-0. Bellco, Vineland, New Jersey.

<sup>3</sup>Fischer Scientific Corp., Pittsburgh, Pennsylvania.



It was important that there was no silicone grease near the open end of the capillary which would interfere with cell migration. In placing the capillary tube in the silicone spot, it was necessary to press down with the forceps to be sure it was lying flat on the top of the slide. The second slide was placed over the ring and both slides held together with a piece of plastic tape around each end. Enough pressure had to be applied while the slides were tightened so as to prevent air from entering the chamber.

To fill the chamber, a one ml syringe with a 26 gauge needle was filled with the supernatant from the lymphocyte culture. Care was taken in filling the syringe and the chamber without leaving air bubbles. A 26 gauge needle was placed in the other side of the ring to allow the air to escape as the chamber was filled. The needle delivering the fluid should not be pointed to the open end of the capillary tube because it could wash out some cells and as a consequence interfere with migration. The filled chambers were placed in a tray leaving free spaces between chambers and incubated for 24 hours at 37 C in 5 percent CO<sub>2</sub> humidified atmosphere.

#### Calculating area of migration

After incubation, the slides were taken very carefully from the incubator and then the area of migration was measured using a Zeiss Automatic Photomicroscope<sup>1</sup> with a

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<sup>1</sup>Carl Zeiss, Oberkachen Wuertt, West Germany.

projection head. The image of the capillary tube was projected into the head and the area traced on standard memo bond paper. The area so outlined was measured with a planimeter and the area of migration determined by using the following formula:

$$\% \text{ migration} = \frac{A}{B} \times 100$$

A = area of migration of the macrophages in the supernatant from lymphocytes cultured in the presence of antigen

B = area of migration of the macrophages in the supernatant from lymphocytes cultured without the presence of antigen

The enlargement of the area of migration on the microscope screen was 40. The measurement of the drawing from the screen with the planimeter<sup>1</sup> is given in square centimeters.

#### Delayed Hypersensitivity Reactions

In order to determine the skin sensitivity of cows which had previously been injected with Freund's adjuvant and intact cells of Streptococcus agalactiae, 0.1 ml of P.P.D. or protein extract of Streptococcus agalactiae was injected intracutaneously into the caudal fold. The injected sites were observed at 20 hour intervals for four days. The resulting reactions were measured according to the method of Spencer and Angevine (129) using the following formula

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<sup>1</sup>Kauffel and Esser Co.

to determine the volume of a cone:

$$V = 1.047 r^2 h$$

V = volume of cone

r = radius

h = height of cone (thickness)

Two dimensions, the thickness and diameter of the swelling, were utilized. The swelling was measured in two perpendicular directions and the average considered as the diameter. The thickness of the swelling was calculated as one-half the difference between the thickness of an inoculated skin fold and a fold of adjacent normal skin.

#### Transfer of Sensitivity

Attempts to transfer hypersensitivity from a sensitized cow by lymphocyte transfer employed two different methods. One procedure used intact lymphocytes recovered from peripheral blood and the second employed lysed or disrupted cells. Two hundred milliliters of blood from donor (sensitized cow) was collected in a sterile flask containing 10 ml of a 20 percent sodium citrate solution. The citrated blood was mixed with 6 percent dextran<sup>1</sup> solution at a 1:10 ratio and distributed into sterile tubes which were incubated as long as necessary to allow the erythrocytes to settle down.

The plasma with the leucocytes in suspension was withdrawn with a pipette and delivered into sterile tubes which

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<sup>1</sup>Dextran Grade B "Clinical" MW 100,000-200,000. Schwarzmann, Orangeburg, New York.

were centrifuged at 750 RPM for 30 minutes. The supernatant was discarded and the red cells, if any, were lysed with Tris-buffer-ammonium chloride solution, 0.83 percent, mixed 1:1 v/v with HBSS. The centrifugation was repeated and the cells were washed two times more. After the supernatant from the last washing was discarded, the cells were suspended in medium TC199 in a proportion of 1:10. The suspension was inoculated subcutaneously in the vulvar fold at the junction of the skin and the mucous membrane. Five days later the cow was challenged intracutaneously with a dose of 0.1 ml of the same antigen as injected before.

The same method used for transfer of sensitivity employed peripheral blood leucocytes from the donor (sensitized cow) which were lysed by consecutive freezing and thawing in liquid nitrogen. The suspension of disrupted cells was divided into two portions; one portion was inoculated subcutaneously and the other portion was dialyzed against physiological saline and the dialysate was concentrated and inoculated subcutaneously. After four days, the animal was challenged with 0.1 ml of antigen inoculated intracutaneously in the caudal fold.

#### Hypersensitivity Reactions Following Intramammary Inoculation

Six weeks after the last injection for sensitization, the systemic and local reactions of the cow to intramammary inoculation of 2 ml of formalinized whole Streptococcus

agalactiae ( $3 \times 10^9$  cells/ml) suspended in 3 ml of distilled water, were observed.

The reactions recorded included temperature, cell counts of milk and local inflammatory reaction of the inoculated quarters.

#### Hotis Reaction

One of the results of Streptococcus agalactiae infection of the bovine mammary gland is the secretion of milk which causes the organism to grow in colonies which adhere to the walls of the tube when the milk is incubated for a period of 24 hours at 37 C. The mechanism of this reaction has not been explained. Samples for the study of the reaction were carefully collected in the following manner. The udder was thoroughly washed with tap water, dried with disposable towels and the teats wiped with 75 percent ethyl alcohol. The milk was drawn into sterile glass bottles then taken to the laboratory and pipetted into sterile screw-cap tubes in amounts of 9.5 ml of milk and 0.5 ml of 0.5 percent brom cresol purple (BCP). The tubes were incubated 24 hours at 37 C.

The milk utilized as negative samples was taken from a cow in the Iowa State University dairy herd. Streptococcus agalactiae infection had not been diagnosed in the herd for the past 29 years. Milk from one cow in the Obstetrics Laboratory herd which apparently had not had contact with the

microorganism was also used.

The test was performed using several different combinations.

1. Milk from each experimental cow + BCP only
2. Milk from each experimental cow + Streptococcus agalactiae
3. Serum from each experimental cow + negative milk
4. Serum from each experimental cow + negative milk + Streptococcus agalactiae
5. Whey from each experimental cow + negative milk
6. Whey from each experimental cow + negative milk + Streptococcus agalactiae
7. Negative milk + immunoglobulin IgG
8. Negative milk + immunoglobulin IgM
9. Negative milk + immunoglobulin IgG + Streptococcus agalactiae
10. Negative milk + immunoglobulin IgM + Streptococcus agalactiae
11. Whey + immunoglobulin IgG + Streptococcus agalactiae in negative milk

#### Separation of Immunoglobulins

Assuming that immunoglobulin might play a significant role in the Hotis reaction, fractionation of blood serum and whey from milk by gel filtration and ion exchange chromatography was performed. In order to obtain serum, two cows positive to the Hotis reaction were bled. The blood was distributed into sterile tubes and left to stand overnight at room temperature. Once the clot was contracted, the serum

was withdrawn and centrifuged for 30 minutes at 1200 RPM. The serum thus clarified was transferred to sterile tubes and kept frozen at -20 C.

To obtain the whey from milk, the first step was to remove the fat by centrifugation at 1600 RPM for two hours. The fat was then aspirated and 0.1 ml of rennet extract, 100 percent strength<sup>1</sup>, was added to 200 ml of milk and placed in a water bath at 37 C for one hour. The whey was drawn off, centrifuged and filtered through a 0.45  $\mu$  filter<sup>2</sup> and stored at freezing temperature.

#### Gel filtration procedure

Gel filtration using Sephadex G-200<sup>3</sup> was done following the method described by Flodin and Killander (35). Serum or whey from sensitized cows was passed through a Sephadex G-200 column which was prepared in Tris-buffer according to the following formula: Tris<sup>4</sup> solution, 0.5 M made of Tris base 60.57 gm/liter and  $\text{NaN}_3$  2 gm/liter.

To prepare the Tris-buffer, 66 ml of 0.5 M HCl was added to 133.3 ml of 0.5 M Tris stock at room temperature and made up to one liter with 0.1 M NaCl. The pH of the

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<sup>1</sup>Miles Laboratories, Elkhart, Indiana.

<sup>2</sup>Falcon, Oxnard, California.

<sup>3</sup>Pharmacia, Upsala, Sweden.

<sup>4</sup>2-amino-2(hydroxymethyl) 1,3, propanodiol. J. T. Baker, Phillipsburg, New Jersey.

buffer was 8.0. The size of the column used was 100 x 2.5 cm and the flow rate 18 ml/hour. The aliquots of serum were concentrated with the Tris-buffer in an ultrafiltration cell with a XM 100 A membrane<sup>1</sup>.

After concentration, the aliquot of serum was placed in a 5 ml syringe and passed through the column in a reverse flow process. After the serum entered the bed of the Sephadex, Tris-buffer was allowed to continuously flow through the column. The effluent was monitored by an ultra-violet light set at a wavelength of 282 nm. The portion of effluent that corresponded to the peaks of UV absorbance was collected in 3 ml amounts and identified later by immunoelectrophoresis.

#### Ion exchange chromatography

Isolation of IgG and IgA from blood serum was tried by ion exchange chromatography using the stepwise gradient technique of Mach and Pahud (70). Buffers of different molarities were prepared.

1. 0.01 M phosphate buffer pH 7.4

$\text{KH}_2\text{PO}_4$  130 ml

$\text{K}_2\text{HPO}_4$  370 ml

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<sup>1</sup>Amicon Corporation, Lexington, Massachusetts.



## 2. 0.02 M phosphate buffer pH 7.2

 $\text{KH}_2\text{PO}_4$  175 ml $\text{K}_2\text{HPO}_4$  325 ml

## 3. 0.06 M phosphate buffer pH 7.0

 $\text{KH}_2\text{PO}_4$  225 ml $\text{K}_2\text{HPO}_4$  275 ml

## 4. 0.01 M phosphate buffer pH 6.0

 $\text{KH}_2\text{PO}_4$  425 ml $\text{K}_2\text{HPO}_4$  75 ml

To prepare the column, DEAE Sephadex<sup>1</sup> was suspended in 0.01 M phosphate buffer pH 7.4 and left to stand in the buffer for 24 hours; during this period the buffer was changed three times. The Sephadex was poured and distributed as uniformly as possible into the column so it would not form any sediment. The sample of serum was dialyzed against buffer number 1 for 24 hours; the buffer was changed four times during this period.

After dialysis, 30 ml of the sample were applied at the top of the column. As soon as the serum descended into the gel, it was followed by a gradient of buffers of increasing molarity. Three hundred milliliters of buffer number 1 was placed into a reservoir beaker with a magnetic stirrer. The

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<sup>1</sup>Pharmacia Fine Chemicals Inc., Piscataway, New Jersey.

rate of flow from the beaker was regulated so as to keep the level in the column constant. As the beaker emptied, the gradient was continued utilizing, in order, buffers number 2, 3 and 4.

The effluent from the column was collected in portions of about 10 ml and the components were identified by immunoelectrophoresis. Protein content of the eluent was monitored by ultraviolet light absorbance at a wavelength of 282 nm.

#### Whey fractionation

Whey was dialyzed in the cold for 36 hours against 0.01 M phosphate buffer. During this time buffer was changed every 12 hours. Forty milliliters of whey was placed in the column. The phosphate buffer continuous-gradient technique with some modifications of the one described by Aalund et al. (2) was used for the chromatography of whey.

A gradient mixing apparatus was formed by connecting two beakers in series at the top of the column with pieces of rubber tubing. Phosphate buffer number 1 containing no NaCl was placed in the beaker nearest the column and buffer number 2 (8.16 gm NaCl/300 ml) in the second. When the second beaker was nearly empty it was filled with buffer number 3 (13 gm NaCl/300 ml).

Mixing was accomplished by a magnetic stirrer. As the gradient progressed, a higher amount of sodium chloride was added to the buffer. The chamber was placed about 20 cm

above the top of the DEAE Sephadex column and in this way hydrostatic pressure was applied to the column. The fractions were collected in portions of 10 ml and identified by immunoelectrophoresis.

#### Immuno-electrophoresis

The method used was described by Scheideger (127). Clean glass slides<sup>1</sup> were rinsed in deionized water, then dried and coated with 0.1 percent agarose solution<sup>2</sup> which had been autoclaved five minutes at 15 pounds pressure.

Agar for the slides was made up of 0.1 gm of agarose and 0.5 gm of agar noble<sup>3</sup> dissolved in 100 ml of 0.05 M Barbitol buffer, pH 8.6<sup>4</sup>. The agar was dissolved by boiling and then autoclaved for ten minutes at 250 C. Twenty-five ml. of melted agar was placed on the slide and allowed to harden and then stored in a refrigerator at 4 C to be used the next day.

Two pairs of wells with a center trough between each pair were cut in the agar. The agar was removed from the wells and one well was filled with normal bovine serum while the other three wells were filled with the sample to be

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<sup>1</sup>3½ x 4 inches. Kodak, Rochester, New York.

<sup>2</sup>Gallard Schlesinger Chemical Corp., New York, New York.

<sup>3</sup>Difco Laboratories, Detroit, Michigan.

<sup>4</sup>Kallestad Laboratories Inc., Minneapolis, Minnesota.

identified.

Three slides were placed in a Gelman immunoelectrophoresis apparatus<sup>1</sup> filled with barbital buffer. The samples were electrophoresed for 60 minutes at a current of 100 milliamps.

The agar of the center trough was removed and filled with rabbit antiovine serum. The slides were placed in a moist chamber at room temperature and the immunoelectrophoretic patterns were allowed to develop and examined after 48 hours.

Those samples from serum or whey that appeared to be pure IgG or IgM were pooled and used in experiments with the Hotis test. Each fraction was mixed in a proportion of 1:10 with the milk and the test was set up using 5 ml of milk instead of 10 ml and 0.25 ml of brom cresol purple solution instead of 0.5 ml.

#### Microscopic Slide Agglutination

Serum from each cow was diluted using two-fold serial dilutions and one drop of Streptococcus agalactiae suspension ( $3 \times 10^9$  cells/ml) was mixed with one drop of the respective dilutions. The slide was rotated several times and the reaction was observed after five minutes.

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<sup>1</sup>Gelman Instrument Co., Ann Arbor, Michigan.

## Intrammary Injections of Antigens

In preliminary work three antigens were inoculated in the udder of three lactating cows. The antigens inoculated were Salmonella pullorum, horse serum and fresh and inactivated rabbit serum.

Salmonella pullorum was cultured in tryptose broth<sup>1</sup> for 18 hours at which time the organisms were sedimented by centrifugation and washed three times with sterile saline. The bacteria were standardized to a concentration equal to tube number 2 of a McFarland nephelometer ( $6 \times 10^8$  cells/ml). Two milliliters of the suspension of heat-killed (two hours at boiling temperature) Salmonella pullorum were inoculated into the right front and the right rear quarter of cow number 1.

Blood was aseptically drawn from the jugular vein of a horse and allowed to clot. After refrigeration overnight, the serum was recovered by centrifugation for 45 minutes at 1000 RPM and stored in the freezer. Two milliliters of the serum were inoculated into the left front and the right front quarter of cow number 2. Twenty-one days later the inoculation was repeated using the same amount in the same quarters and a third injection was administered 13 days later. The cow was challenged with one intravenous injection of fresh

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<sup>1</sup>Difco Laboratories, Detroit, Michigan.

horse serum on the 21st day following the first injection.

The third antigen utilized was rabbit serum. Two milliliters of inactivated (56 C for 30 minutes) rabbit serum was inoculated into the right rear quarter and two milliliters of fresh rabbit serum at the left rear quarter of cow number 3. This trial was repeated in two other cows.

To assess the reactions of the cows to the intramammary injection of the antigens, the following tests were conducted after inoculation of the respective antigens.

#### Catalase test

The enzyme catalase arises from the various cellular elements of the mammary gland, aerobic bacteria and in free solution. To detect catalase, 10 ml of milk was delivered in 16 x 125 mm sterile screw-cap tubes, 1 ml of 3 percent hydrogen peroxide was added. The tube was then filled with sterile distilled water, capped loosely and placed in an inverted position in a rack and left to stand for three hours at room temperature. The result was read as the percent of oxygen collecting in the closed end of the tube (18).

#### Somatic cell counts in milk

The test used was described by Brazis (10) in which 0.01 ml of milk was placed on a glass slide and spread over an area of 1 cm. After it was dry, the slide was stained with Newman's stain and allowed to dry. The slide was

examined microscopically and the cells from a number of fields were counted. The following formula was used to calculate the number of cells per ml. The microscopic factor was 392,000.

$$\text{Number of cells/ml} = \frac{\text{Leucocytes in fields} \times \text{microscopic factor}}{\text{Number of fields examined}}$$

#### Wisconsin mastitis test

The procedure described by Postle (112) was followed. Two ml of milk were pipetted into a tube and then the WMT reagent was added below the surface of the milk in each tube to provide good mixing of the sample and reagent. The tubes were capped and the mixture was mixed by gently rocking the rack back and forth ten times in about ten seconds. The tubes were allowed to stand for 30 seconds and the rack was then inverted and held in a vertical position to permit outflow for 15 seconds. At the end of this period, the rack was returned to an upright position and set aside for three minutes. The remaining fluid column in each tube was recorded in millimeters.

#### Agglutination test and double-diffusion test

To test for the presence of antibodies to Salmonella pullorum, the tube agglutination procedure was used. The agar double-diffusion test for antibodies to horse and rabbit serum was performed. The agglutination test was performed by using serial two-fold dilutions of the antiserum mixed with

an equal volume of Salmonella pullorum antigen. Tests were incubated in a water bath at 52 C for four hours and read after standing at room temperature overnight.

The agar diffusion test of Ouchterlony as described by Kabat and Mayer (55) was employed to detect precipitins to horse and rabbit serum proteins. A glass slide was covered with 0.01 percent agarose which was allowed to dry. A solution of one percent Noble agar in saline containing 0.01 percent merthiolate was prepared and sterilized by autoclaving and then 4 ml was poured onto each glass slide. The agar was allowed to harden and wells were cut in it arranged with one in the center and four around it at equal distances apart. Agar from the wells was drawn off with a pipette attached to a vacuum line. Serum from the cow which had been given intramammary injections of horse or rabbit serum was placed in the central well and normal horse or rabbit serum was placed in the outside wells and vice versa. The slides were kept at room temperature in petri dishes containing moistened filter paper and observed for four days.

#### Preliminary Tests for Pathogenic Microorganisms

Before the experiments were started, the cows were tested for the presence of pathogenic microorganisms by the following tests.



### Blood agar plate culture, aerobic and anaerobic

From the milk of each quarter two blood agar plates were streaked. One was incubated at 37 C for 24 hours. The plates were read and then incubated for 24 hours more. The other blood agar plate was incubated under anaerobic conditions for 48 hours in Gas Pak jars<sup>1</sup>.

The first group of cows tested by blood agar plates aerobically showed only organisms considered to be contaminants, i.e., micrococci and bacilli. In the second group of cows, one yielded Staphylococcus aureus from the left rear and left front quarters. Blood agar cultures incubated anaerobically were negative for pathogenic microorganisms.

### Catalase test

The results of this test varied from 1 to 2 ml of gas, i.e., from 10 to 20 percent except the two quarters infected with Staphylococcus aureus which gave a result of 40 percent catalase in the left front quarter and 55 percent in the left rear. This cow was treated before being used as an experimental animal.

### Somatic cell counts

Cell counts averaged from 60,000 to 120,000 cells/ml in the normal quarters. In the two infected quarters, the cell counts were from 800,000 to 1,000,000/ml.

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<sup>1</sup>Bioquest Laboratories, Baltimore, Maryland.

## RESULTS

## Migration Inhibitory Factor Test

The results of various migration inhibitory factor (MIF) tests are presented in Figures 2 through 7. In Figure 2 the responses of three cows given Freund's complete adjuvant are shown. Cows numbers 1 and 2 responded minimally but cow number 3 had a definite response. Figure 3 represents another group of cows treated in the same manner. Each of these cows (numbers 4, 5 and 6) had a constant response to PPD antigen.

Figure 4 shows the results of the MIF test of cows sensitized with Streptococcus agalactiae. The cells were cultured in the presence of Streptococcus agalactiae protein. In cows 2 and 3 the response was definitely positive. Cow 5 was also positive but the response was not as high as in cows 2 and 3. Cow 6 had completely negative results.

Figure 5 is the representation of the results when the test was performed utilizing the supernatant from cells cultured in the presence of streptococcal polysaccharide. Cow 2 showed a minimum positive reaction but cows 3, 5 and 6 were essentially negative. There was a very close similarity in the response of cows 5 and 6 in that they showed some inhibition of migration at first followed later by almost a complete lack of inhibition.

Figure 2. Results of MIF test using lymphocytes from cows 1, 2 and 3 cultured in the presence of PPD. Each bar represents the average from three samples. Cow 1 was sensitized with Freund's complete adjuvant. Cows 2 and 3 were sensitized with adjuvant and Streptococcus agalactiae cells.

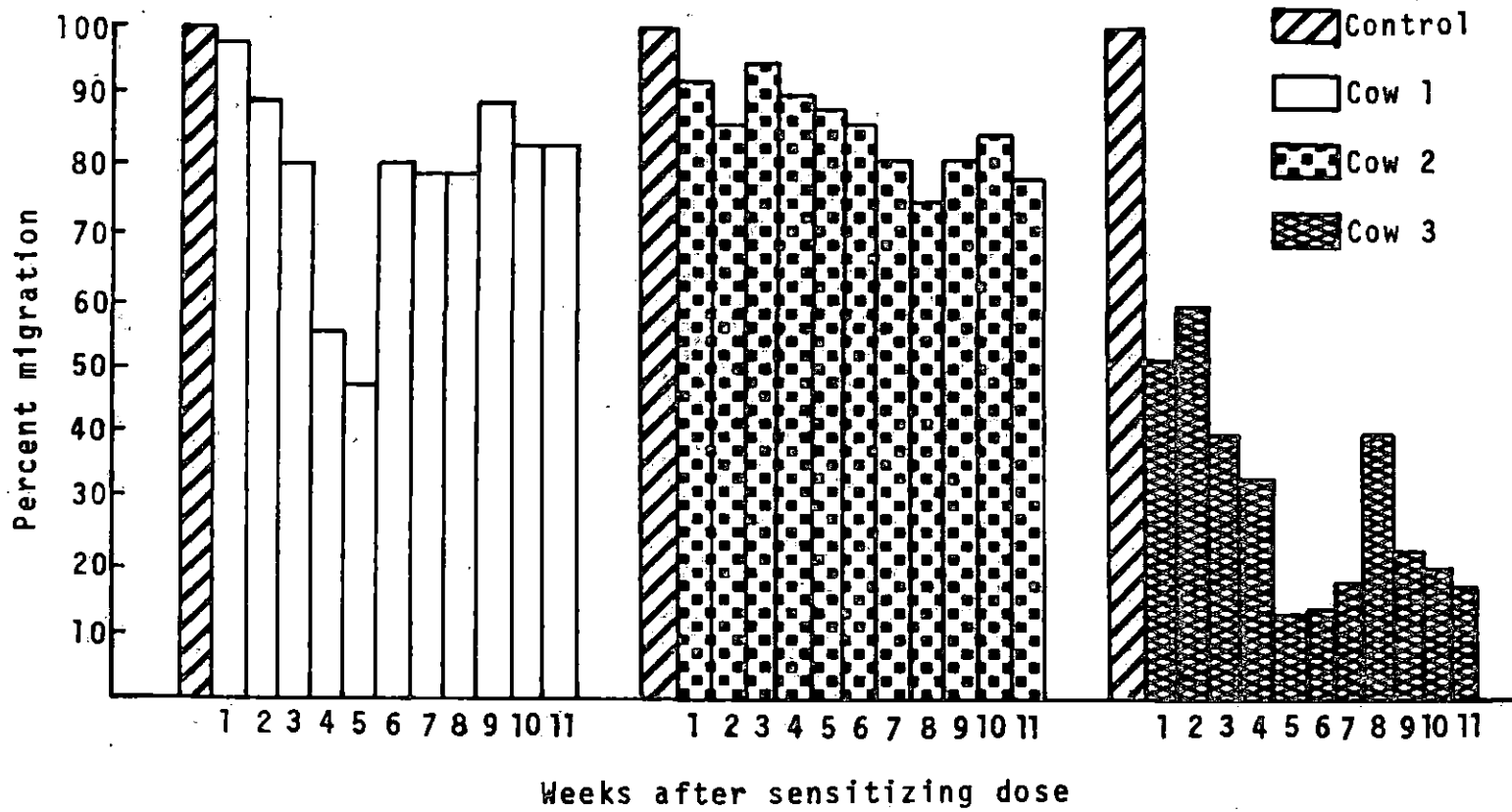


Figure 3. Results of MIF test using lymphocytes from cows 4, 5 and 6 cultured in the presence of PPD  
Each bar represents the average from three samples.  
Cow 4 was sensitized with complete Freund's adjuvant.  
Cows 5 and 6 were sensitized with complete Freund's adjuvant and Streptococcus agalactiae cells.

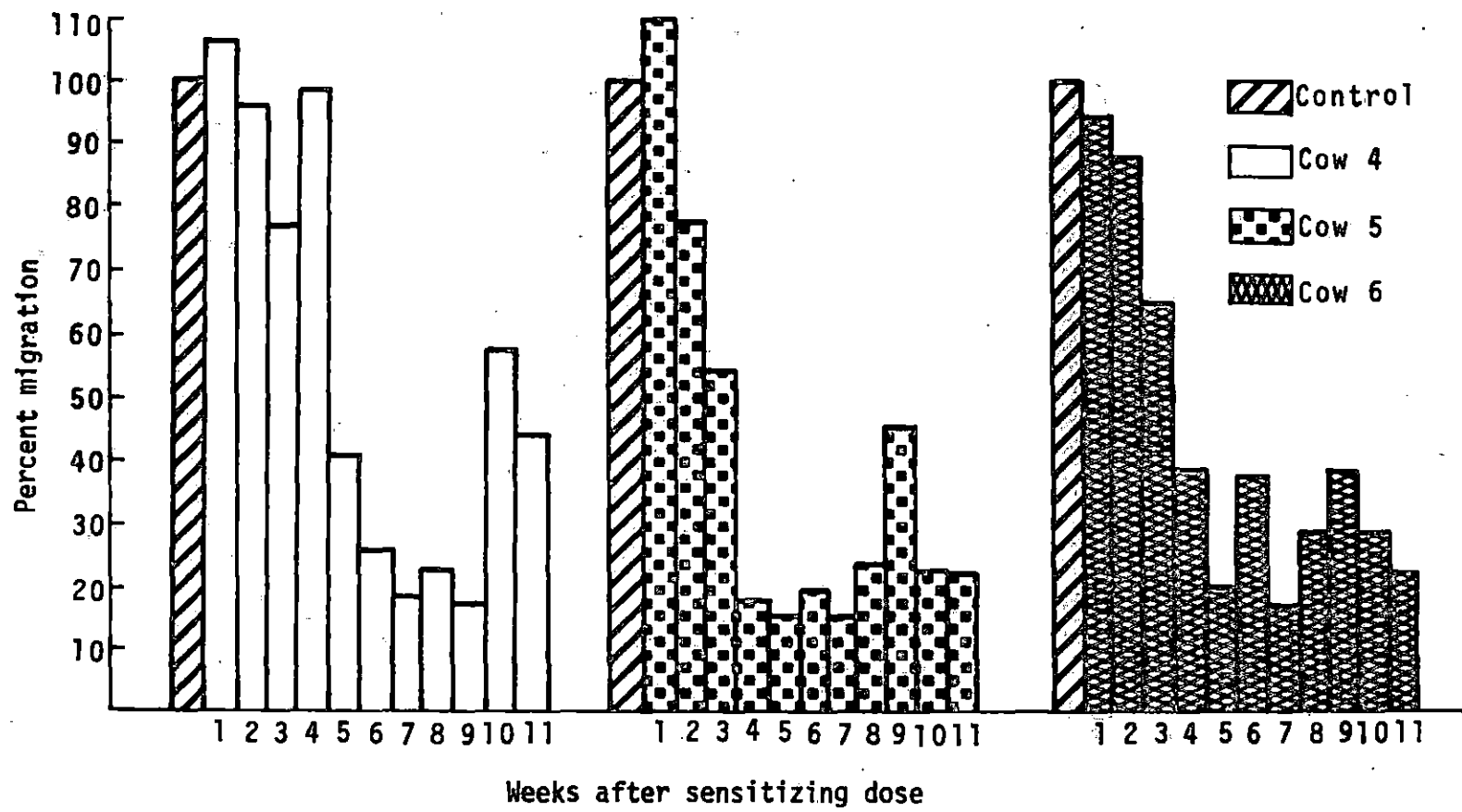


Figure 4. Results of MIF test using lymphocytes from cows 2, 3, 5 and 6 cultures in the presence of Streptococcus agalactiae protein. Each bar represents the average from three samples. These cows were sensitized with complete Freund's adjuvant and Streptococcus agalactiae cells.

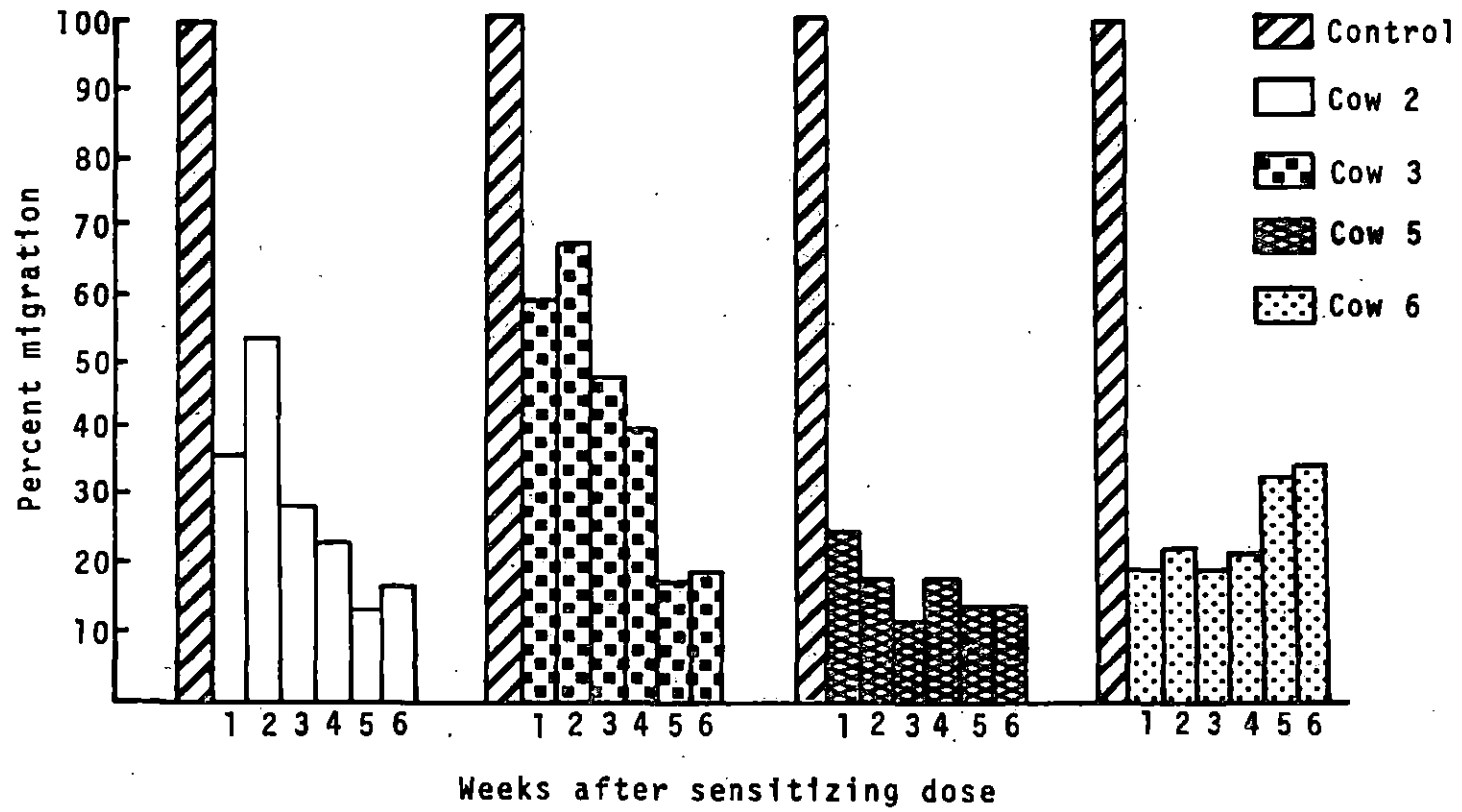




Figure 5. Results of MIF test using lymphocytes from cows 2, 3, 5 and 6 cultured in the presence of streptococcus polysaccharide. Each bar represents the average from three samples. These cows were sensitized with complete Freund's adjuvant and Streptococcus agalactiae cells.

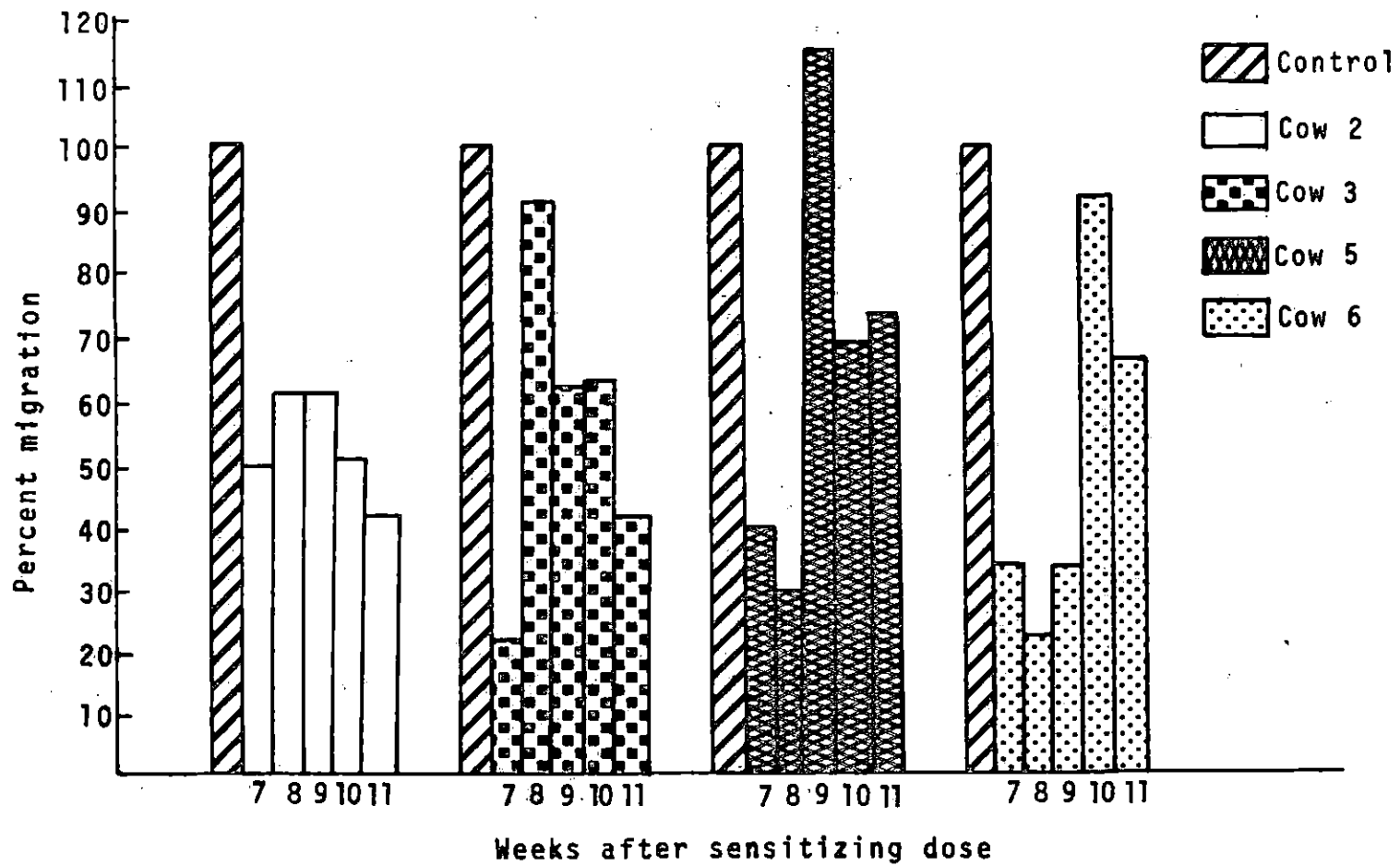


Figure 6. Results of MIF test using lymphoid cells from the milk of cows 4 and 6 cultured in the presence of PPD and cows 5 and 6 cultured in the presence of protein extracted from Streptococcus agalactiae. Cow 4 was sensitized with complete Freund's adjuvant. Cows 5 and 6 were sensitized with complete Freund's adjuvant and Streptococcus agalactiae cells.

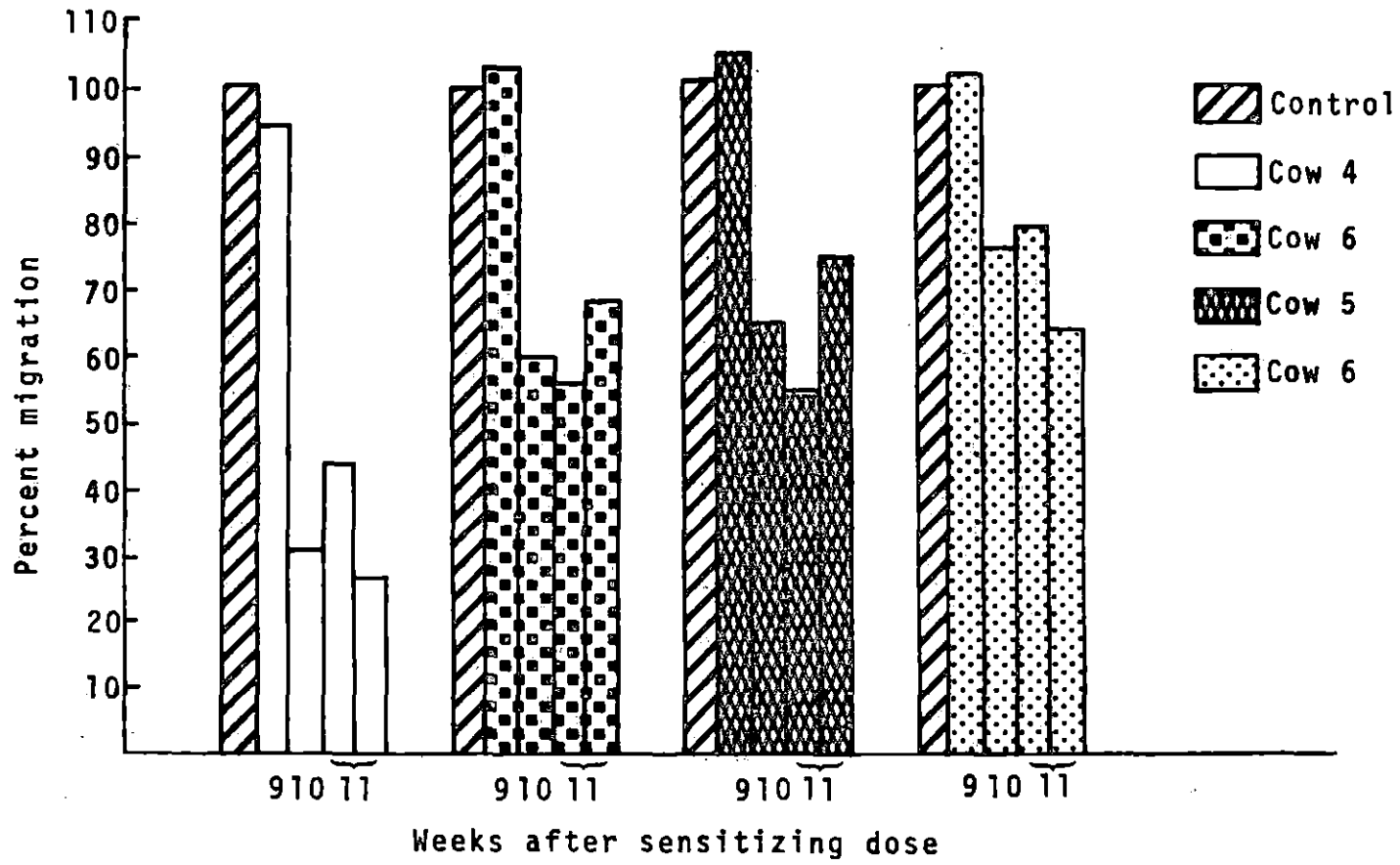
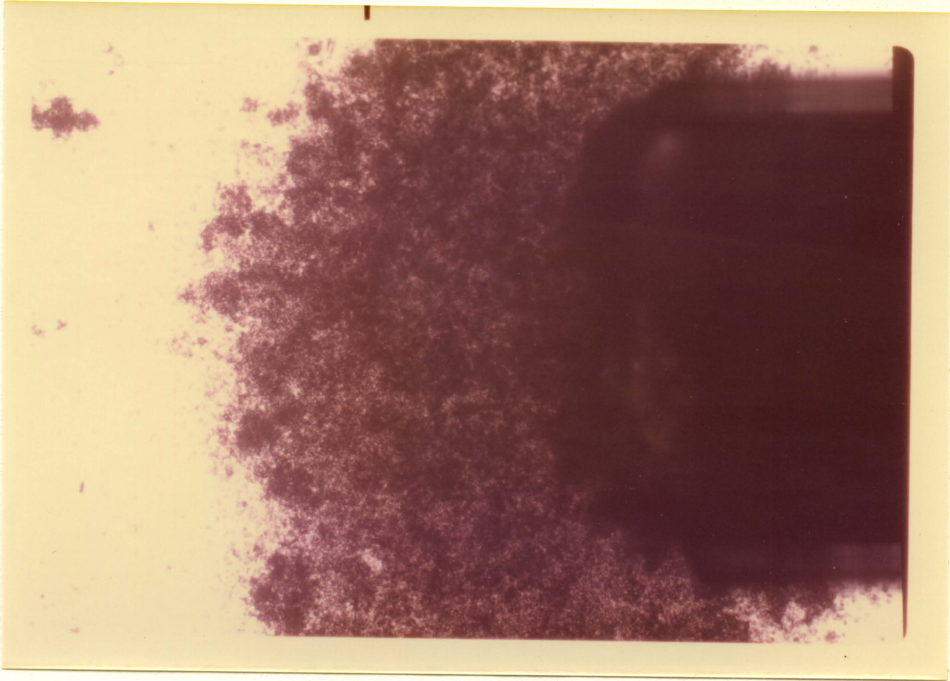
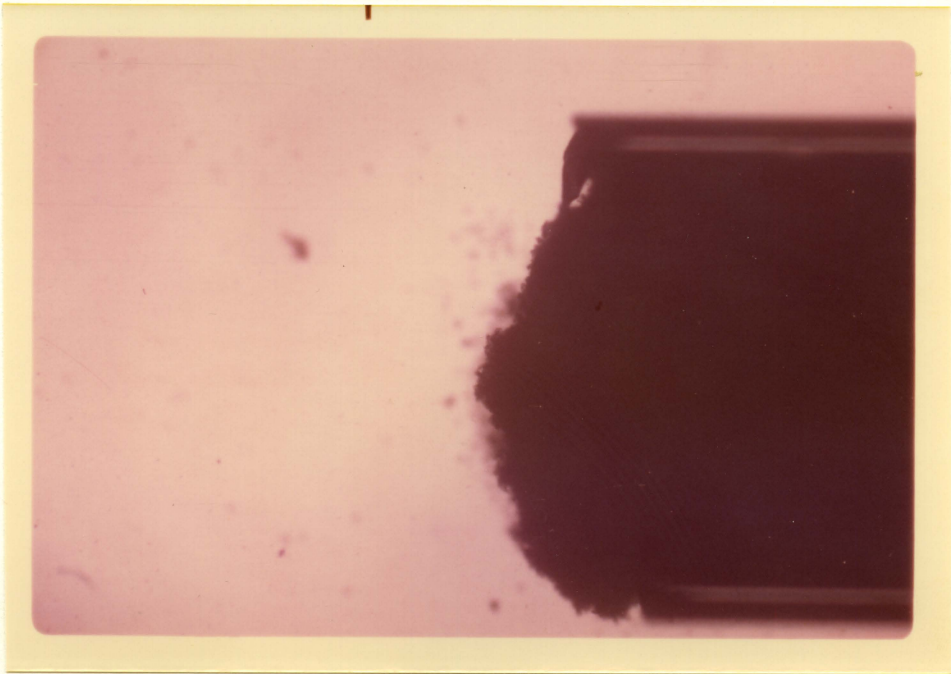


Figure 7. MIF test with supernatant from milk lymphocytes  
A. Supernatant with Streptococcus agalactiae protein  
B. Control



B



A

Figure 6 shows the results obtained from milk lymphocytes. Cows 4 and 6 show the results when cells were cultured in the presence of PPD. Cows 5 and 6 show the results when cells were cultured in the presence of Streptococcus agalactiae protein. All cows showed definite positive results to the MIF test. See Figure 7.

#### Skin Sensitivity

The response to intradermal injection of antigen (streptococcal protein or PPD) into previously sensitized cows was characterized by a delineated area of edematous swelling that appeared about 20 hours after inoculation of antigen. At 24 hours the swelling was more consistent and by 40 hours it was converted into a well defined nodule which disappeared completely after four days. The above reaction was present in cow 3 but this reaction became visible only after the cow was inoculated for the second time with the antigen. See Table 4.

One negative cow was observed as a control for the skin sensitivity. The reaction was measured in millimeters following the method of Spencer and Angevine (129). Measurements were taken after 12 hours and followed every 20 hours for a total period of 80 hours.

Table 4. Skin sensitivity reaction presented by cows previously sensitized with Freund's adjuvant and Streptococcus agalactiae and challenged with S. agalactiae protein

Time after Challenge	Skin Reaction Measurement	
	Cow 3	Cow 5
12 hours	redness	slight edema
20 hours	214 cmm	484 cmm
40 hours	415 cmm	285 cmm
60 hours	302 cmm	165 cmm
80 hours	160 cmm	negative

#### Sensitivity Transfer

Recipient animals were selected on the basis of a negative skin reaction when inoculated with 0.1 ml streptococcal protein intradermally in the caudal fold. The cow chosen as donor of leukocytes was the one which gave the greater skin reactivity when inoculated with the antigen after being sensitized with complete Freund's adjuvant plus Streptococcus agalactiae.

In the trial in which complete cells were inoculated, the viability was 89 to 91 percent. In the trial using lysed cells, viability of cells before being lysed was 91 percent.

Two cows were inoculated twice with whole peripheral leucocytes from the sensitized donor and challenged four to five days later with the streptococcal protein antigen without any results. Two other recipients developed varying degrees of reaction, one considered to be typical and the other



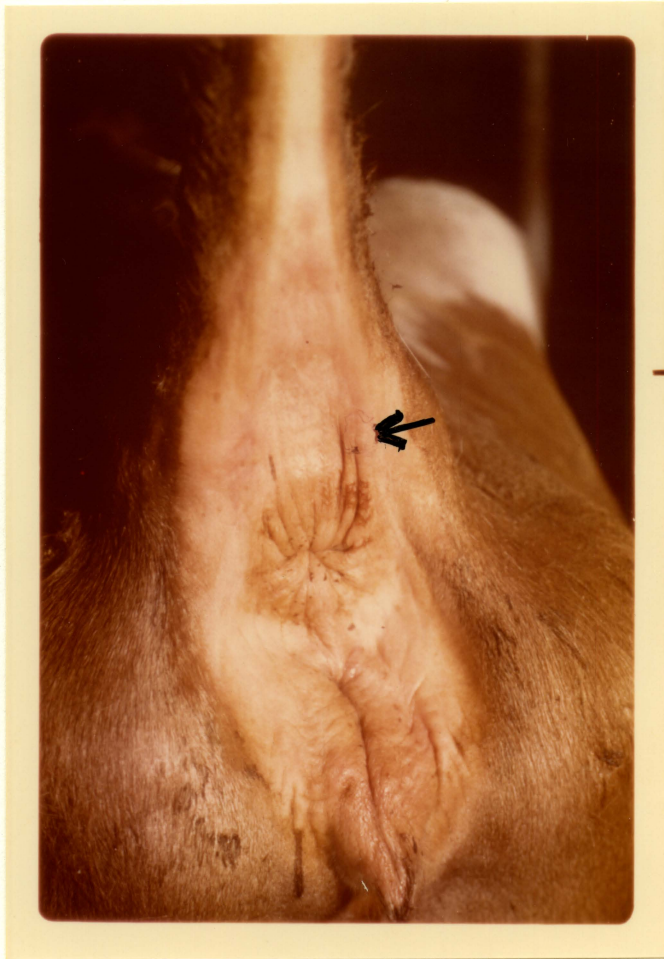
doubtful. One week after challenge with streptococcus antigen, a suspension of lysed cells was injected subcutaneously into one of them at the vulval fold at the junction of the skin and mucous membrane. Four days later the cow was challenged with streptococcus antigen and inoculated intracutaneously in the caudal fold. The reaction consisted of nodule formation swelling and an extensive area of redness along the inferior aspect of the tail (Figure 8). Results of the reaction appear in Table 5.

Table 5. Skin sensitivity reaction presented by two cows inoculated with lysed peripheral leucocytes (cow A) and concentrated dialysate of peripheral leucocytes (cow B) and challenged with S. agalactiae protein.

Time after Challenge	Skin Reaction Measurement	
	Cow A	Cow B
12 hours	214 cmm	redness
20 hours	506 cmm	226 cmm
40 hours	418 cmm	460 cmm
60 hours	201 cmm	376 cmm
80 hours	120 cmm	214 cmm
96 hours	slight reaction	slight reaction

A second portion of the lysed cell suspension was dialyzed at 4 C against physiological saline. The dialysate was concentrated and inoculated subcutaneously. After five days the animal was challenged and the reaction was observed daily for four days. In this cow the result was similar to the reaction presented by previously sensitized cows. See Table 5.

Figure 8: Reaction of negative recipient after inoculation of lysed lymphocytes from a sensitized cow and challenge with Streptococcus agalactiae protein.



### Reactions Following Intramammary Inoculation of Antigens

When cows were inoculated intramammary with a killed suspension of Streptococcus agalactiae, the reaction of the animal, local and general, was visible and parameters such as temperature, cell count in milk and catalase test were measured. Also swelling and pain in those inoculated quarters were present for about two or three days in the different quarters.

Before the suspension of Streptococcus agalactiae was inoculated into the udder, the body temperature was taken and varied from 100.9 to 101.4 F. in the different cows. Temperature readings after challenge are summarized in Table 6. The variation in temperature in cow 1 was considered only as physiological changes.

Cow 5 had a rise in temperature up to 3 F 20 hours after inoculation. All other cows presented normal temperature 1.5 F higher than the initial temperature.

#### Cell Count in Milk

Cell counts in milk were very high after intramammary infusion of killed Streptococcus agalactiae cells. Table 7 summarizes the cell count in each of the inoculated quarters.

Table 6. Body temperatures after intramammary inoculation of 2 ml of formalin-killed Streptococcus agalactiae mixed with 2 ml of sterile distilled water ( $1 \times 10^9$  cells/ml.)

Hours after Inoculation	Cow 1 <sup>a</sup>	Cow 2 <sup>b</sup>	Cow 3 <sup>b</sup>	Cow 4 <sup>a</sup>	Cow 5 <sup>b</sup>	Cow 6 <sup>b</sup>
0	101.3	100.9	101.6	101.7	100.9	101.4
6	101.7	102.4	102.3	102.4	103.7	102.3
20	101.5	102.9	104.6	103.8	103.9	103.8
28	101.4	102.3	103.6	102.9	103.4	103.2
42	101.2	101.8	102.9	102.3	102.9	102.2
48	101.4	102.0	102.2	101.8	103.1	102.5
60	101.2	101.4	101.4	101.3	102.4	101.9

<sup>a</sup>Cows sensitized with complete Freund's adjuvant only.

<sup>b</sup>Cows sensitized with Streptococcus agalactiae and Freund's adjuvant.

Table 7. Number of somatic cells in milk after intramammary inoculation of Streptococcus agalactiae in two quarters of each cow

Days after Inoculation	Cow 1		Cow 2		Cow 3	
	<u>RR</u>	<u>LR</u>	<u>RR</u>	<u>LR</u>	<u>RR</u>	<u>LR</u>
0	69 <sup>a</sup>	71	60	75	84	54
1	91	120	1321	1343	1234	894
2	225	142	1951	2842	1856	1125
3	261	178	2121	4140	2731	3731
4	256	175	1115	2200	2685	4327
5	101	180	974	112	1841	2118
6	93	105	854	725	1343	1110
8	92	93	195	241	934	832
15	89	90	91	110	92	71

	Cow 4		Cow 5		Cow 6	
	<u>RR</u>	<u>RF</u>	<u>RR</u>	<u>RF</u>	<u>RR</u>	<u>RF</u>
0	76	94	110	55	65	96
1	450	325	2617	132	1120	140
2	1795	854	2965	184	1584	952
3	1215	1110	1723	2125	2341	2875
4	1323	925	1180	3100	1985	1454
5	427	631	384	657	1756	1321
8	561	422	532	421	581	950
15	130	129	65	91	432	230
22	65	83	66	92	54	85

<sup>a</sup>Number of cells x 10<sup>3</sup>

### Hotis Reaction

A typical Hotis reaction has been reported to occur only in the milk of those cows in which Streptococcus agalactiae is an inhabitant of the mammary gland. It has been assumed that the reaction is immunologically related.

By repeated cultural examination of the milk of the cows used in this experiment, it was determined that they were free of Streptococcus agalactiae infection. Furthermore, none gave positive Hotis tests prior to the injection of sensitizing antigens.

As a result of the injection of Streptococcus agalactiae cells mixed with Freund's complete adjuvant, three out of four cows converted from negative to positive when live Streptococcus agalactiae cells were added to milk samples from them (Figure 9). Cow 3 was the exception.

It was also observed that when either the blood serum or whey from the positive cows and live Streptococcus agalactiae were added to Hotis negative milk, positive reactions occurred. Cow 4 which received injections of adjuvant only was also converted to positive Hotis test results as shown in Table 8.

Separation of IgG and IgM fractions from the serum of cows 2 and 6 were mixed with Hotis negative milk from cow 7. Inoculation of these samples with Streptococcus agalactiae followed by incubation, gave positive tests in the case of IgG but not IgM. See Table 9.

Figure 9. Hotis reaction from a cow sensitized with Freund's complete adjuvant plus Streptococcus agalactiae. From left to right, increasing dilutions of Streptococcus agalactiae culture.





Table 8. Hotis reaction in milk, serum and whey from cows sensitized with Freund's complete adjuvant only or adjuvant plus Streptococcus agalactiae

Cow	Sensitizing antigen	Milk	Milk + Strep. agal.	Serum + neg. milk	Serum + neg. milk + Strep. agal.	Whey + neg. milk	Whey + neg. milk + Strep. agal.	Milk + neg. milk	Milk + neg. milk + Strep. agal.
1	adjuvant	-	-	-	-	-	-	-	-
2	adjuvant + Strep. agal.		+	-	+		+		+
3	adjuvant + Strep. agal.	-	-	-	-	-	-	-	-
4	adjuvant		+	-	+		+		
5	adjuvant + Strep. agal.		+	-	+		+		
6	adjuvant + Strep. agal.		+	-	+		+		

Table 9. Hotis reactions given by milk of negative cow mixed with immunoglobulin from positive cow and Streptococcus agalactiae culture

Cow	Neg. milk + IgM	Milk + IgG	Neg. milk + IgM + Strep. agal.	Neg. milk + IgG + Strep. agal.
7	-	-	-	+

When whey from cows 1, 2 and 3 was mixed with negative milk and Streptococcus agalactiae and each one of the fractions, the reaction was positive in those tubes with IgG fractions.

Agglutinins for Streptococcus agalactiae in Serum

Agglutinins were detected in serial dilutions of serum as shown in Table 10.

Table 10. Agglutinins for Streptococcus agalactiae in serum

Cow	Dilutions						
	1/5	1/10	1/20	1/40	1/80	1/160	1/320
1	-	-	-	-	-	-	-
2	+	+	+	+	+	-	-
3	-	-	-	-	-	-	-
4	+	+	+	+	+	+	-
5	+	+	+	+	+	+	+
6	+	+	+	+	+	+	-

## Precipitation Test

This test was performed for the detection of antibody activity in the immunoglobulin. See Table 11.

Table 11. Antibody levels in serum and whey of sensitized COWS

	Streptococcal Protein Dilution								
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
whey IgM	+	+	+	+	+	-	-	-	-
whey IgG	+	+	+	+	+	+	+	+	-
blood IgM	+	+	+	+	+	+	-	-	-
blood IgG	+	+	+	+	+	+	+	+	-

Table 12 shows a summary of the results of delayed hypersensitivity tests, Hotis reaction and Streptococcus agalactiae agglutinins in the group of cows studied. Cow 1 had positive results in the MIF and skin sensitivity but was negative for the Hotis reaction and streptococcus agglutinins. The same results with respect to skin test and MIF were presented by cow 4 but she became positive for Streptococcus agalactiae for unknown reasons.

Cows 3 and 6 showed some interesting points. Cow 3 was positive for delayed hypersensitivity tests but was negative for Hotis reaction and streptococcus agglutinins. Cow 6 was

completely the opposite. She was negative for most of the delayed hypersensitivity reactions but was positive for streptococcus agglutinins and Hotis reaction. Cows 2 and 5 responded in a very similar way.

Cow number 1 that was inoculated with 2 ml of killed suspension of Salmonella pullorum 18 hours later showed swelling in the inoculated quarters and pain to palpation. The reaction lasted four days. The inoculated as well as the non-inoculated quarters showed higher readings in the catalase and Wisconsin mastitis tests. Figure number 10 shows the results for these tests in one inoculated and one non-inoculated quarter.

The presence of agglutinins to Salmonella pullorum was detected 48 hours after inoculation. Results of agglutination tests on whey are shown in Figure 11.

Left front and right front quarters of cow 2 were inoculated with 2 ml of fresh horse serum. Pain on palpation was evident for three days. The catalase and leucocyte values were higher in the right front quarter and showed up earlier and lasted a shorter time than those results detected in the left front quarter.

Table 12. Summary of results of delayed hypersensitivity tests, Hotis reaction and Streptococcus agalactiae agglutinins in cows studied

Cow	Sensitizing antigen	MIF to:			Skin Sensitivity to:		Transfer factor to Strep. agal. protein	Hotis reaction	Titer of agglutinin to Strep. agal.
		PPD	Strep. agal. protein	Strep. agal. polysaccharide	PPD	Strep. agal. protein			
1	adjuvant	+	0	0	+	0	0	-	-
2	adjuvant + Strep. agal.	+	+	-	0	-	-	+	40
3	adjuvant + Strep. agal.	+	+	+	+	+	+	-	-
4	adjuvant	+	0	0	+	0	0	+	40
5	adjuvant + Strep. agal.	+	+	-	0	+	-	+	160
6	adjuvant + Strep. agal.	+	-	-	-	-	-	+	80

Figure 10. Results of catalase and Wisconsin mastitis tests conducted on milk from the right front quarter after inoculation of 2 ml ( $6 \times 10^8$  cells/ml) Salmonella pullorum and catalase and Wisconsin mastitis tests in the left front quarter not inoculated

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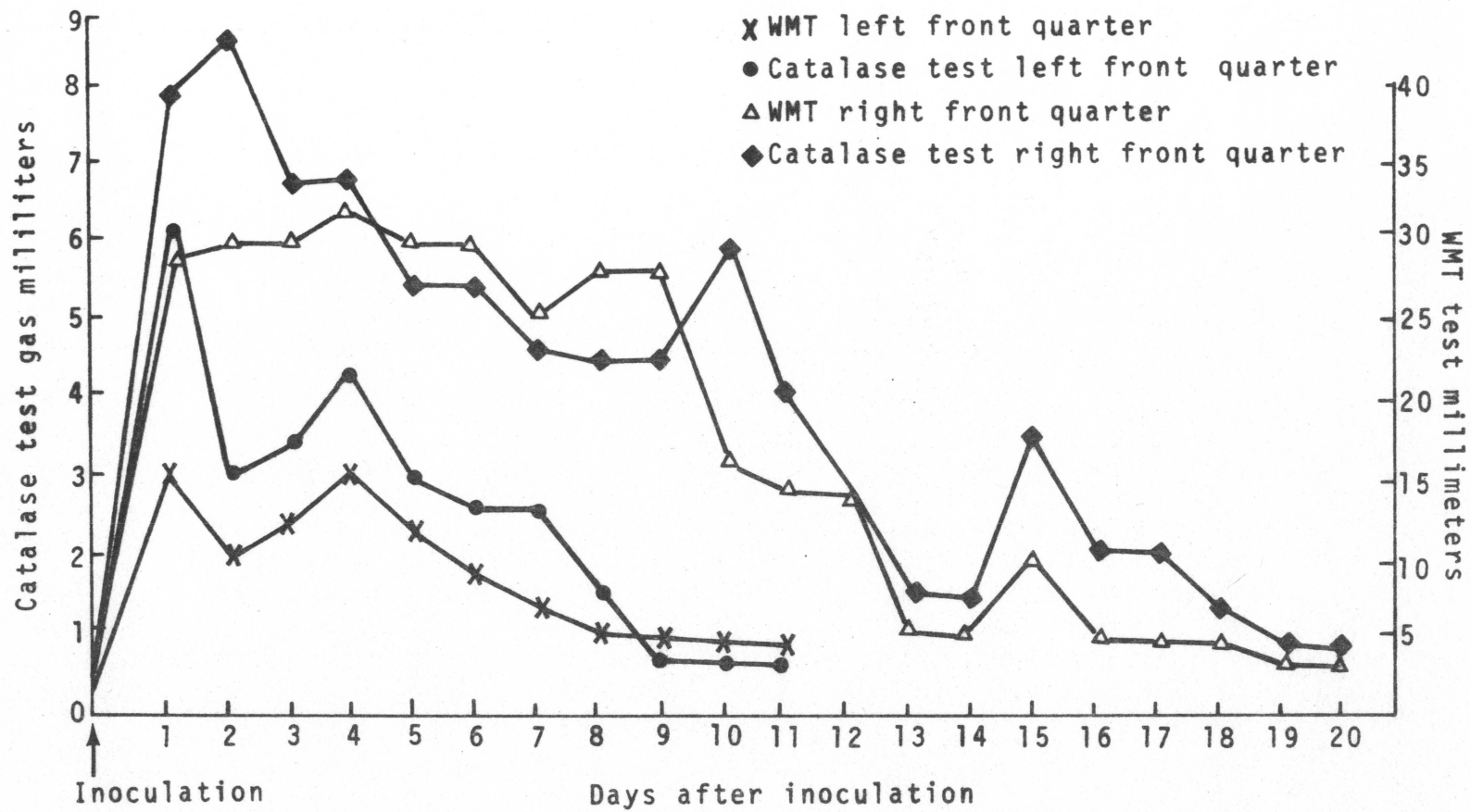
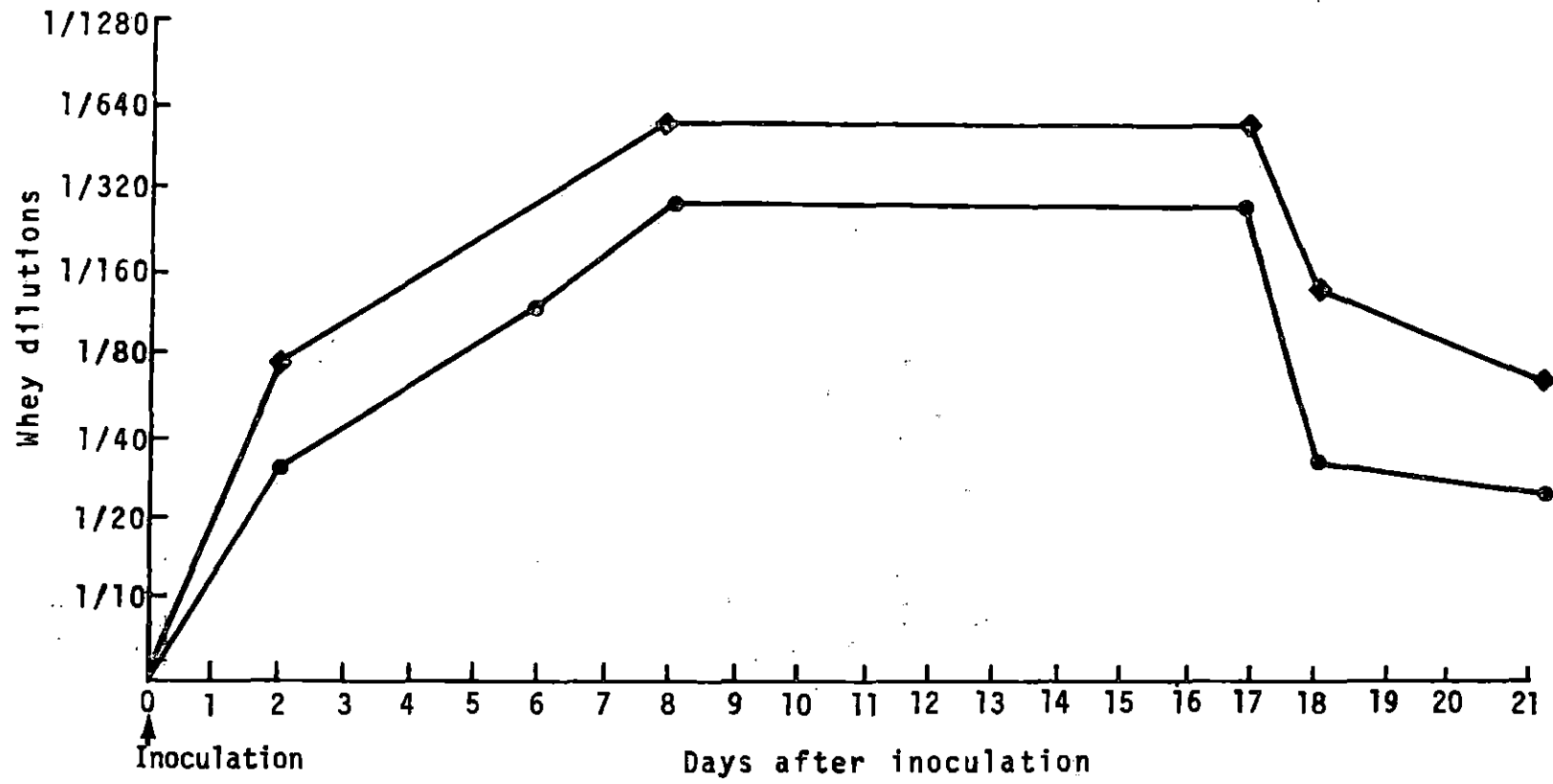




Figure 11. Whey-agglutinin titer to Salmonella pullorum following intramammary injection to the right front and right rear quarter



Twenty days after the first dose of horse serum, another dose was introduced into the same quarters. A third dose was given 13 days after the second in the right front quarter and one intravenous injection of 3 ml was administered to the animal. Although the left quarter was not given a third dose, the results of the tests were high in both quarters. No visible allergic reaction was observed in the animal after the intravenous inoculation of the horse serum. Results of the catalase test are shown in Figures 12 and 13. Results for leucocytic counts are shown in Figure 14.

Cow 3 was inoculated with 2 ml of inactivated rabbit serum (56 C for 30 minutes) into the right rear quarter and 2 ml of fresh rabbit serum was inoculated into the left rear quarter.

Reactions to the catalase and Wisconsin mastitis tests were higher and appeared sooner in the quarter given the inactivated rabbit serum than those shown by the quarter inoculated with fresh rabbit serum. See Figure 15.

Swelling and acute pain in the inoculated quarters were observed for four days postinoculation. The udder was warm and congested. In looking for the possibility of different response to the inoculation of fresh and inactivated rabbit serum, the same dose used before was administered to two other cows but on this occasion the results were similar. The udder became painful to palpation for a period of five days and the catalase and Wisconsin mastitis test results were

gradually higher with a peak on the fourth day. See Figure - 16.

Agar double-diffusion tests were performed with both horse and rabbit serum against their respective antiserum produced in the cows. These tests were negative. The tests were repeated with similar results.

Figure 12. Catalase test results from milk obtained from the right front quarter and left front quarter after inoculation of fresh horse serum

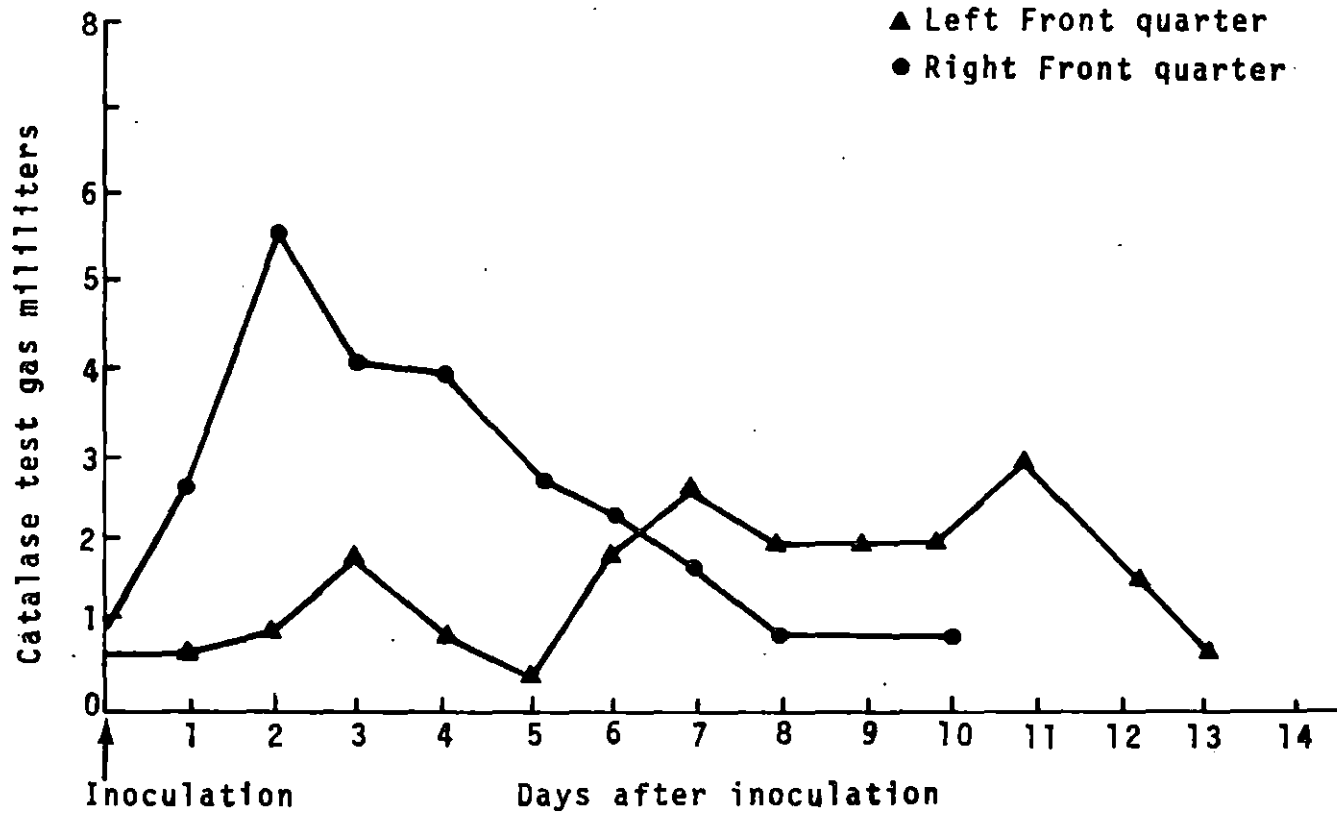


Figure 13. Catalase test results from milk obtained from the right front quarter and left front quarter after inoculation of fresh horse serum

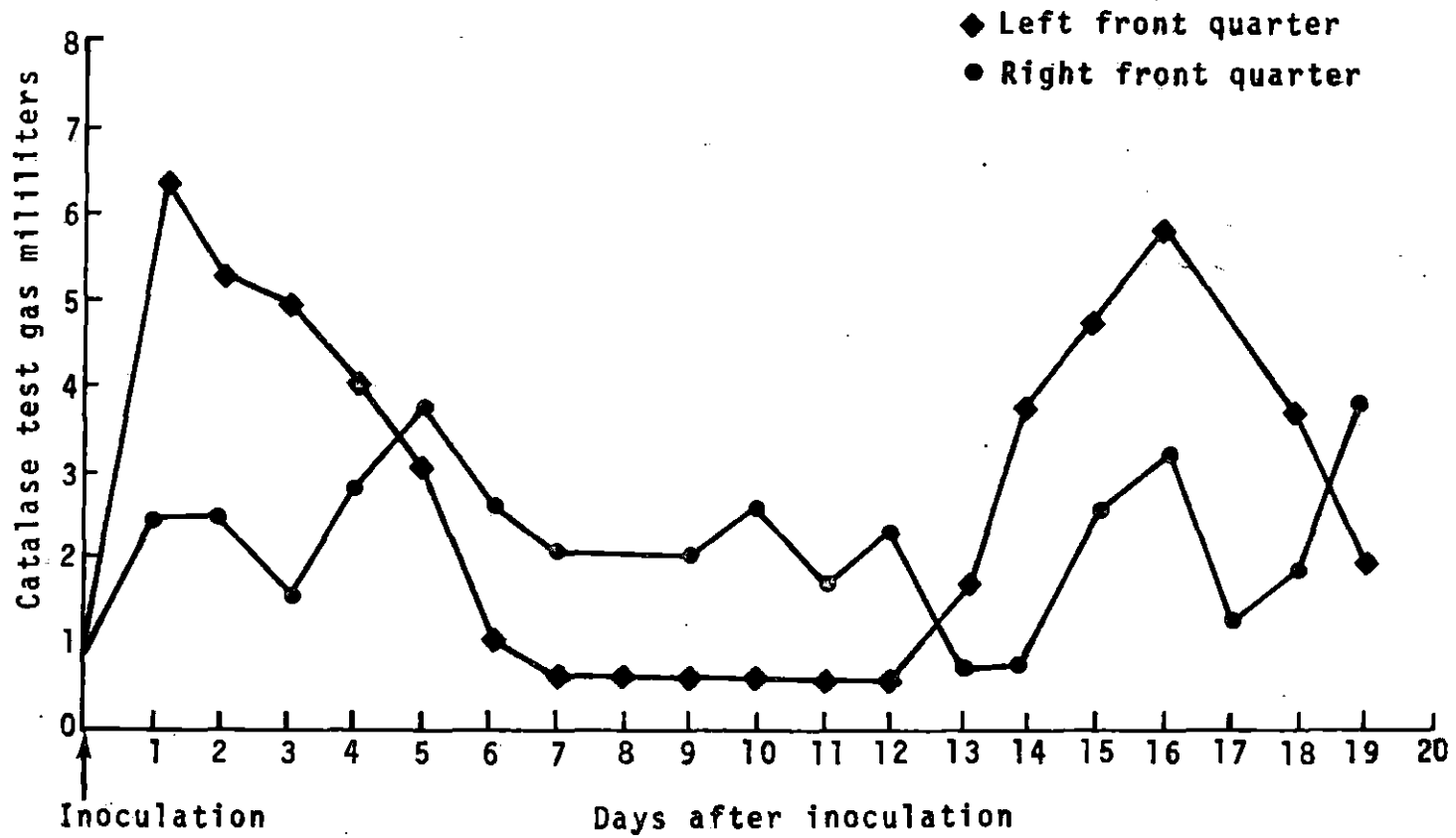




Figure 14. Leucocytic counts after inoculation of fresh horse serum in the right front and left front quarters

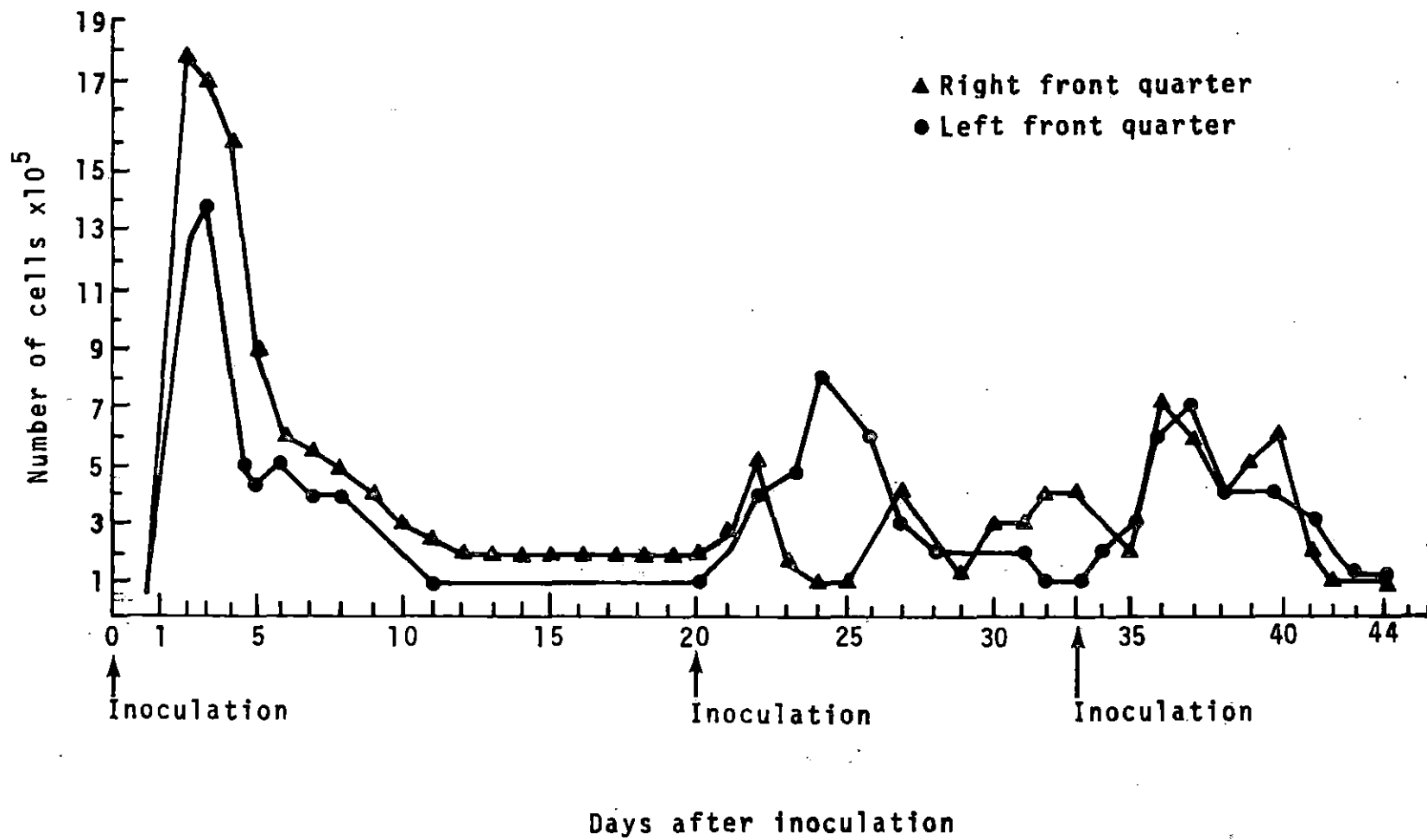


Figure 15. Catalase and Wisconsin mastitis test results from milk collected from the right rear quarter after inoculation of inactivated rabbit serum and the left rear quarter after inoculation of fresh rabbit serum

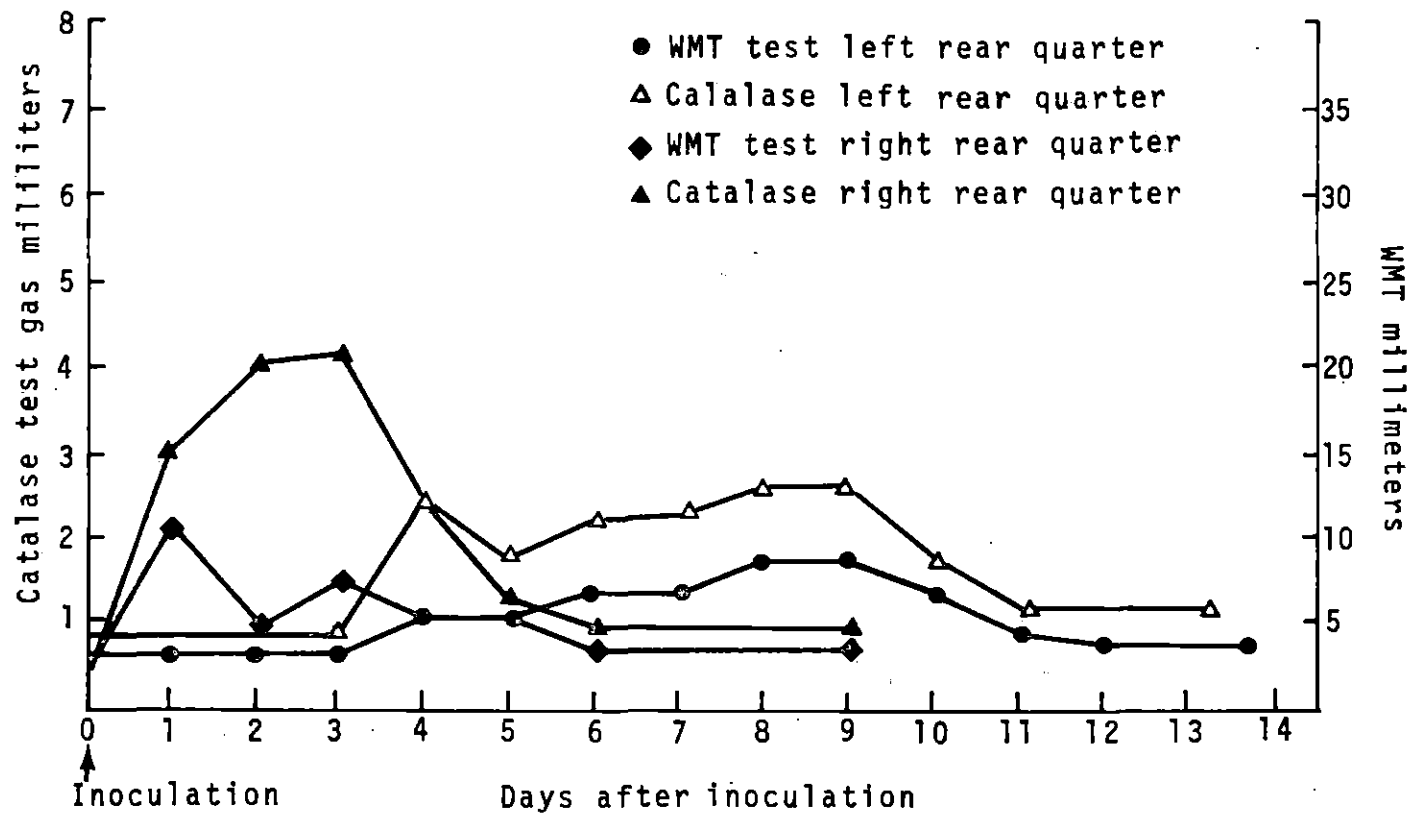
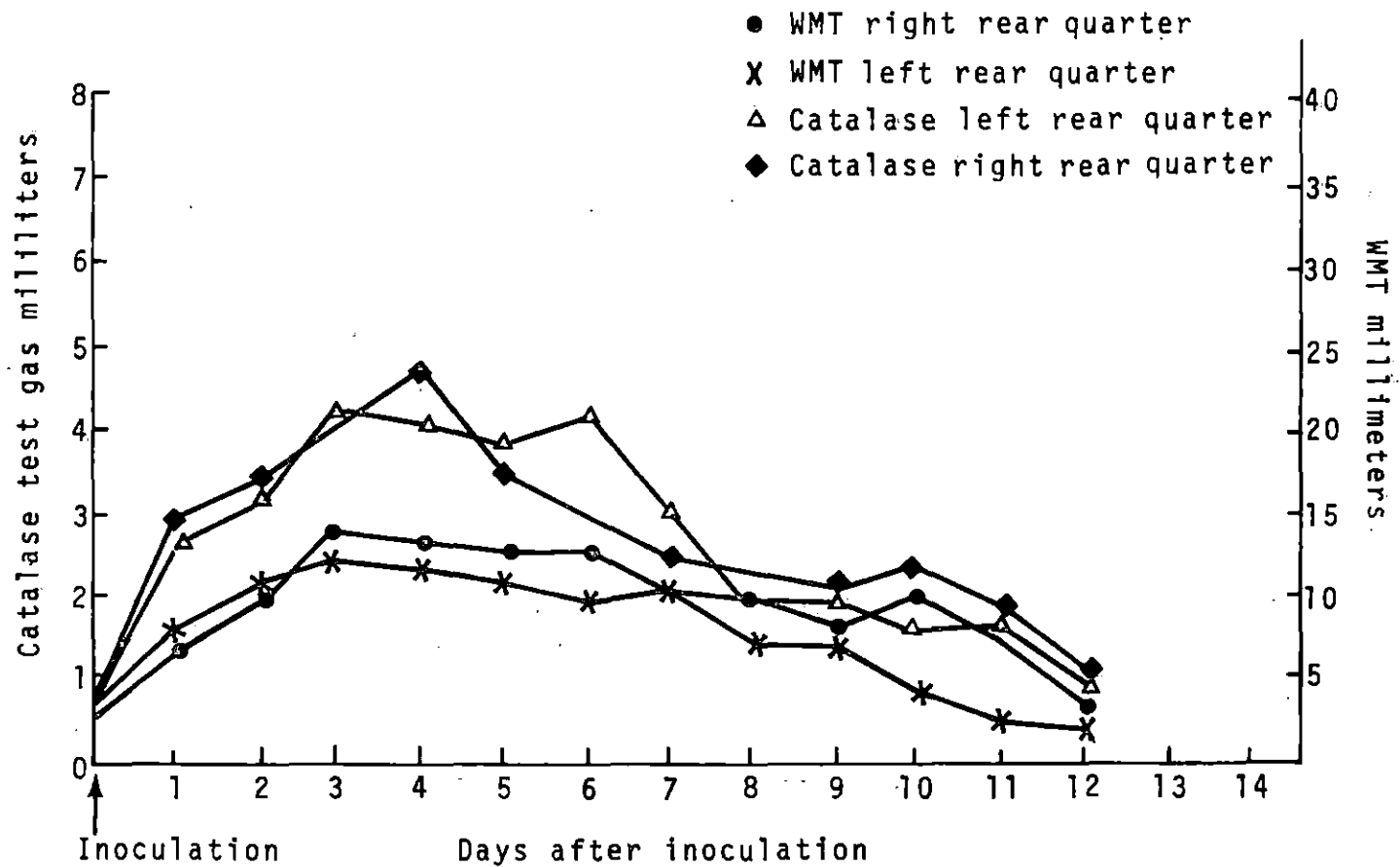


Figure 16. Catalase test results from milk obtained from right rear quarter after inoculation of fresh rabbit serum and left rear quarter after inoculation of inactivated rabbit serum.



## DISCUSSION

The immunological response to antigen in lactating cows is determinable. It is of special importance as it relates to the disease mastitis and it can be assayed in a variety of ways which include both humoral and cell-mediated mechanisms. While the number of cows observed in this study is small, sufficient data was gathered to indicate that the techniques devised to assay cell-mediated responses are applicable to the bovine mammary gland secretions.

Freund's complete adjuvant, an established stimulator of cell-mediated immunity, was administered and used as a guide. Very limited work has been published which applies this procedure to immunological studies of the bovine mammary gland.

One of the most striking observations made in the present study was the apparent difference in responses of individual cows to the same antigen. Examination of Table 6 indicates that all cows responded to the Freund's adjuvant as determined by the MIF results using PPD as sensitizing antigen for the lymphocytes, although there were apparently some individual differences. It should be noted that cow 3 had the highest response to MIF using streptococcal protein, skin sensitivity and transfer factor, but did not produce agglutinin or react in the Hotis test. On the other hand, cows 2, 5 and 6 generally gave a poor response to the

cell-mediated tests but gave good reactions to the Hotis and agglutination tests. The only exception was the positive response of cow 2 to MIF using streptococcal protein.

From these limited observations it might be suggested that in an individual animal one type of response, i.e., either cell-mediated or humoral, tends to dominate.

#### Migration Inhibitory Factor

Only cow 6 was negative for MIF when her lymphocytes were cultured in the presence of streptococcal protein. This could be due to the fact that this cow, in general, followed a humoral response rather than a cell-mediated one. However, cow 6 was positive for MIF when milk cells were used in the test. This result could be partially due to the fact that Streptococcus agalactiae cells were inoculated into the udder to test it for hypersensitivity a few days before the MIF test. It could be possible that the bacterial cells enhanced, to some extent, the cell-mediated response of the mammary gland.

When the MIF test was performed with the supernatant of lymphocytes cultured in the presence of polysaccharides from Streptococcus agalactiae, only cow 2 showed a positive reaction. The other cows gave very inconsistent results. It may be that the polysaccharides are less antigenic than PPD in the stimulation of MIF or that the polysaccharides were toxic to the lymphocytes causing their death and thus



giving inconsistent results in the MIF test. It also may be that the use of guinea pig macrophages instead of those from cattle resulted in variations in the tests. Perhaps the use of macrophages from the same species as the sensitized animal would result in more consistent and reliable results.

### Skin Sensitivity

Cow 3 presented a delayed hypersensitivity type of reaction to the intracutaneous injection of streptococcal protein. In cow 5 the maximal intensity of the skin reaction was apparent by 20 hours postinoculation. This is not in agreement with the usual delayed hypersensitivity reactions which are described as appearing 6 to 12 hours postinoculation reaching a peak of intensity within 24 to 48 hours and then fading over a period of time that could last several days. An immediate type of tuberculin reaction in guinea pigs sensitized with horse serum and killed tubercle bacilli in paraffin oil or by BCG injection, had been reported by Peys (106b). Although the reaction of skin sensitivity presented by cow 5 is not a definite immediate type, it may tend more toward the immediate type than the delayed type.

Cows 2 and 6 were negative for skin reaction after inoculation of Streptococcus agalactiae, however, they gave good humoral responses to that organism. These results are similar to those reported by Bloom (4b) who stated that animals possessing only circulating antibody failed to

inhibit the migration of macrophages, a cell-mediated response. It is suggested that failure of an animal to produce MIF may also extend to other cell-mediated mechanisms such as delayed hypersensitivity reactions which take place in the skin. Therefore, the variability of the immunological response of each individual is a very important factor in considering the response to any antigen.

#### Transfer factor of sensitivity

Cows A and B were given subcutaneous injections of lysed lymphocytes taken from cows previously sensitized with a mixture of streptococcal cells and Freund's adjuvant. Intradermal injections of streptococcal protein were made 96 hours later in cows A and B. Cow B responded within 24 hours at the site of injection with a small circumscribed area of redness and edema. Cow A responded at the site of injection with an extensive area of redness and edema. The interval from injection to a noticeable reaction in cow A was approximately 12 hours with the peak of response reached by 20 hours. No published work could be found which describes the reactions in cattle resulting from the transfer of sensitivity by lymphocytes. The only basis of comparison are those reported for the guinea pig and humans (64). The reaction of cow B to challenge following the transfer by lysed lymphocytes was very similar to that described in the guinea pig.

The possibility that reactions observed in cows A and B may have been due to their sensitization by the first injection of streptococcal protein must be considered. However, the fact that no reaction occurred in the other two cows which also received a similar sequence of injections in tests for the transfer by intact lymphocytes, would argue against such an occurrence.

There is also a possibility that the cows responded in different ways because of their individual immunological capacities. Cow A was apparently more responsive to the transferred cells than cow B which resulted in cow A's stronger reaction. The idea that transfer of sensitivity, at least in part, is based on the inherent responsiveness of an animal can be found in the report by Pick (108).

Whole lymphoid cells were not effective in transferring the skin sensitivity reaction. This result was not in agreement with Lawrence (65a) who stated that whole cells are as effective as lysed cells in the transfer of sensitivity.

#### Hotis Reaction and Agglutinins for Streptococcus agalactiae

The cows that initially were negative for the Hotis reaction became positive to the test when a live culture of Streptococcus agalactiae was added to samples of their milk drawn three to four weeks post sensitization with Streptococcus agalactiae cells and Freund's adjuvant.

Following the streptococcal injection, there was a rise in the level of specific antibodies for Streptococcus agalactiae as the level of agglutinins in the serum was in direct correlation with the positive Hotis reactions. It could be said that the Hotis reaction is an indication of the level of the host's humoral response to the micro-organism.

Cow 4, which was not inoculated with Streptococcus agalactiae, developed positive reactions to the Hotis test and specific agglutinins were demonstrated in serum. This was not expected and there is no satisfactory explanation.

While no previous history of infection was available for cow 4, it was definitely established on the basis of repeated cultures and mastitis screening tests that no infection existed at the beginning of the experiments or was acquired during the period of observation. The fact that the Hotis reaction was negative at the beginning is an indication that this cow had not had previous contact with Streptococcus agalactiae. The agglutinin-stimulating factor in this case could not be determined.

The role of agglutinins in the Hotis reaction suggested by McCulloch and Fuller (73) is confirmed by findings in this study. The ability of IgG to convert Hotis-negative milk to positive was expected. However, it was anticipated IgM might also be able to function in this way. Failure of IgM separated from whey and serum to produce positive tests may have been

due to a lowered quantity of the IgM carrying a specificity for Streptococcus agalactiae. The whey and serum yielding the IgM fraction was collected six weeks after the last injection of antigen by which time the level of any IgM response may have waned.

#### Intramammary Inoculation of Streptococcus agalactiae

The gradual increase of body temperature following the inoculation of killed streptococcal cells into the udder of sensitized cows is a demonstration of an existing state of hypersensitivity in those cows that received Streptococcus agalactiae cells. Likewise, a lack of a thermal response by cow 1 that had received only Freund's adjuvant, indicates non-sensitivity. This evidence is supported by the results in Table 12.

Another indication of the hypersensitive state is the increase in the number of somatic cells in milk after the inoculation of killed cells of Streptococcus agalactiae into the udder. All cows had a sharp increase in the number of cells in milk except cow 1 which had a slight increase. This slight increase would be anticipated since it has been reported (52, 122) that any substance inoculated into the udder will cause an increase in the number of cells.

The higher number of cells in milk is an indication that there is an infiltration of cells from the blood as a defensive response from the udder to the presence of

Streptococcus agalactiae.

## Inoculation of Antigens

The reaction presented by the mammary gland after infusion of Salmonella pullorum is possibly due to the irritation produced by the infused particulate antigen that causes the mammary gland epithelium to become highly permeable, allowing the passage of circulatory components into the gland ducts. The same irritation might indirectly affect the non-inoculated quarter.

The agglutinin level of the infused gland was very high in relation to the non-infused gland. After this level decreased, it was not as low as it was before inoculation. Possibly some antigens remain for a long time within the mammary gland which in time will elicit the local production of antibodies.

The infusion of soluble antigen into the mammary gland produces a local inflammatory response that was demonstrated by the high number of cells in milk and the presence of swelling and pain in the quarter inoculated with rabbit or horse serum. When inoculated with horse serum, the reaction lasted a shorter time than when inoculated with rabbit serum. It is possible that rabbit serum is more foreign to the cow than horse serum and therefore it could elicit a stronger and longer reaction. The fact that the udder of one cow responded in a different way to the intramammary infusion

of fresh and inactivated rabbit serum, could be due to a particular type of response of the cow itself and not to the composition of the antigen. The same trial was repeated in two other cows and in both of them the response was similar for both fresh and inactivated serum.

Tests for the presence of antibodies to rabbit and horse serum by the agar double-diffusion method were negative. Failure to stimulate precipitin formation may have been due to lack of retention of the small amount (2 ml) of these soluble antigens by the mammary gland in comparison to particulate ones such as Salmonella pullorum.

## SUMMARY AND CONCLUSIONS

Two groups of three cows each were sensitized with Freund's complete adjuvant and in four (two in each group) the adjuvant was mixed with a suspension of killed Streptococcus agalactiae cells. After sensitization, they were tested for MIF, skin sensitivity and transfer factor as well as for levels of agglutinins for Streptococcus agalactiae and the Hotis reaction.

The MIF test in all cows was positive when their lymphocytes were cultured in the presence of PPD but in cows 1 and 2 the reactions were only slightly positive. Three of the four cows sensitized with Streptococcus agalactiae were positive to the MIF test when an extract of that organism was used as the antigen in the test.

Only one cow gave a positive reaction (slight) when her lymphocytes were cultured in the presence of polysaccharide extracted from Streptococcus agalactiae. The transfer of sensitivity was positive in two out of a total of six cows tested.

The level of agglutinins for Streptococcus agalactiae in four out of six cows which showed titers, ranged from 1:40 to 1:160. The inoculation of the milk from each of the six cows with a live culture of Streptococcus agalactiae failed to produce a Hotis reaction before sensitization. Hotis reactions became positive by this procedure a few



weeks following sensitization in four cows.

The reactions to intrammary inoculation of killed, whole cells of Streptococcus agalactiae were observed. The three sensitized and one non-sensitized cow showed a rise in body temperature, local inflammatory reaction of the inoculated quarter and high numbers of somatic cells in milk.

Other antigens, Salmonella pullorum, rabbit and horse serum, were inoculated into the mammary gland of another group of cows. Salmonella pullorum produced an elevation of the agglutinin titer 48 hours after the inoculation. Fresh horse serum and fresh and inactivated rabbit serum produced a localized inflammatory response when injected into the udder but precipitins were not detected by the agar double-diffusion test.

The observations made in this study lead to the following conclusions:

1. Subcutaneous injection of Streptococcus agalactiae in adjuvant into cows stimulated the development of an immune response in cows of either humoral or cell-mediated type or both, varying from individual to individual.
2. The MIF test results from lymphocytes cultured in the presence of PPD or Streptococcus agalactiae protein were satisfactory, although the number of individuals tested was not large.
3. Hotis reactions correlated with the humoral responses as represented by the level of agglutinins for Streptococcus

agalactiae.

4. IgG plays an important role in the positive result of the Hotis reaction.

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