IMMUNIZATION OF PREGNANT GUINEA PIGS WITH STAPHYLOCOCCAL MATERIALS AND THE CLINICAL RESPONSE TO CHALLENGE DURING LACTATION

Thesis for the Degree of M. S.

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ABSTRACT

IMMUNIZATION OF PREGNANT GUINEA PIGS WITH STAPHYLOCOCCAL MATERIALS AND THE CLINICAL RESPONSE TO CHALLENGE DURING LACTATION

By

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Using the guinea pig mammary gland as a model for staphylococcal mastitis, the efficacy of several staphylococcal vaccines was tested. Pregnant animals (primiparous, 3 months old, 500 to 600 g) were vaccinated via subcutaneous or intramammary route with staphylococcal materials. The vaccine preparations contained various mixtures of purified staphylocoagulase, staphylolipase, and staphylococcal polysaccharide antigen (SPA). All vaccines contained 1 X 10^8 washed, heat-killed, whole cells of Staphylococcus aureus (Slanetz strain UNH 10). Nontreated animals and those receiving saline placebos served as controls. Challenge on day eleven post-partum was made intramammarily with 2 X 10^9 c.f.u. of S. aureus (homologous strain UNH 10). Initial drop in milk secretion was seen in response to all intramammary injections. Rapid, sustained elevation of leukocyte counts

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in milk were correlated with an increase in the DNA concentration of mammary gland tissue. The challenged glands remained apparently unchanged in outward appearance, but at necropsy eight days post-challenge they had atrophied. The RNA/DNA ratio, as an indicator of protein synthesizing activity, decreased. S. aureus was isolated from milk samples of challenged glands up to the termination of the experiment, and was isolated from 80% of the excised challenged mammary glands. In both serum and whey, precipitin antibodies could be detected to coagulase, lipase, but not to SPA. Under our experimental conditions the strain of S. aureus used was probably not sufficiently virulent. Although the guinea pig mammary gland was susceptible to staphylococcal infection and constituted a good model for study of the mastitic syndrome, in terms of clinical manifestations, no protection could be confirmed in this study by using the aforementioned parameters.

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By

Steven Glenn

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INTRODUCTION

Bovine mastitis continues to be a problem of economic importance. With the advent of intensive antibiotic therapy the etiologic agent of this disease has shifted from the streptococci to Staphylococcus aureus. Because S. aureus possesses the ability to overcome antibiotic treatment by emergence of antibiotic resistant strains, nonchemotherapeutic approaches to treatment and prevention have been explored. Munch-Petersen (1938) stated that the idea of an anti-bacterial vaccine as a possible immunizing agent has been studied since the early part of this century. Along with these studies has come the realization of the complexity of staphylococcal immunity which has yet to be satisfactorily understood.

Early workers were concerned with the diffusible bacterial substances known to be toxic, in particular the alpha and beta toxins. Although many reports have supported the efficacy of toxoid treatment in immunizing the test animal, no satisfactory prophylaxis, with respect to mastitis, could be established. More recently the somatic antigens of S. aureus have been investigated as possible

immunizing agents. These antigens, a variety of substances, have proved to be very complex. Morse (1960) and Fisher et al. (1963) have reported a surface antigen from the diffuse variant of the Smith strain, a highly virulent organism for mice, which, when administered to mice, will protect these animals from challenge with viable Smith diffuse cells. Ekstedt (1963c) isolated a protein somatic antigen which also protected mice from a similar challenge.

Other diffusible staphylococcal products have been isolated and studied for their possible roles in the pathogenicity of the organism. Our laboratory has studied two in particular, staphylocoagulase and staphylolipase. San Clemente (1970) incorporated these purified antigens into a vaccine containing the immunizing surface antigen from the Smith diffuse strain (staphylococcal polysaccharide antigen--SPA), as well as heat-killed whole cells. Choosing two routes for vaccination, intramuscular and intramammary, he obtained some encouraging results with respect to protection from intramammary challenge, especially with SPA and staphylocoagulase.

In the following study we have chosen the guinea pig as a model for staphylococcal mastitis and report the preliminary results of the effects of several vaccine preparations.

REVIEW OF THE LITERATURE

Mastitis Etiology and Experimental Mastitis

Staphylococcus aureus as the causative agent of mastitis, an inflammatory infection of the mammary gland, was reported as early as 1887 by Nocard in a study of gangrenous mastitis in ewes. Minett (1939) reviewed the literature on the occurrence of staphylococcal mastitis in ewes. He cited Gilruth[1] as noting the high virulence of staphylococci, especially those isolated from cases of mastitis, when injected into the udders of nonmastitic sheep. Rosati and Bertolina[2] and Klesov[3] reported a high mortality in sheep with staphylococcal mastitis. In his review of the literature previous to 1935 on mastitis in cows, Munch-Petersen (1938) cited studies by Plastridge

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[1]Minett (1939) gave the following incomplete reference: Gilruth, J. A. 1910. Vet. J. 66:648.

[2]*Ibid.*: Rosati, T. and P. Bertolino. 1938. Clin. Vet. 61:465.

[3]*Ibid.*: Klesov, M. D. 1936. Sovyet Vet. 5:53.

et al.[4] and Little and Foley (1935) who gave evidence that the staphylococci were serious pathogens in the bovine udder; skin abscesses, as well as both chronic and peracute mastitis, were noted.

Before the intensive use of antibiotics in the late 1940's to treat bovine mastitis, the ubiquity and severity of streptococcal mastitis overshadowed the importance of the staphylococcus as an udder pathogen. In a survey of cases of mastitis, Carpenter (1925) isolated staphylococci in only a very few instances, and the predominant infecting organism was a streptococcus. Lamont (1925) studied a small herd and reported 30% of the cows with mastitis were infected with staphylococci. He therefore proposed staphylococcus as an agent of mastitis. With the advent of antibiotic therapy and subsequent intensive eradication programs, the predominant organism has shifted from the streptococcus, which proved to be highly susceptible to antibiotics, to the staphylococcus from which antibiotic resistant forms rapidly emerged.

A survey of several studies on the epizootiology on mastitis reveals the ubiquity of the staphylococcus. In a six-year study Packer (1952) reported that in Iowa

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[4]Munch-Petersen (1938) gave the following incomplete reference: Plastridge, W. N., E. R. Spaulding, G. D. Brigham. 1933. Proc. Soc. Amer. Bact. (J. Bact.).

S. aureus was found in 70% of the positive samples taken from cases of bovine mastitis. Similarly, Niksch et al. (1960) isolated S. aureus from 50% of the cases of bovine mastitis. More recently, Nurmi and Koiranen (1967) found a 28% frequency of S. aureus in mastitic cows. In all of these studies the staphylococcus was predominant over any other organism. Edwards and Smith (1966) found that antibiotic therapy in a herd of cows reduced the incidence of streptococcus mastitis from 61% to a stable level of about 3 to 5%. Comparing two herds over a 13-year period, these authors found a steady increase in the incidence of staphylococcal mastitis.

Slanetz and Bartley (1953) carried out studies on staphylococci isolated from cases of bovine mastitis. The organisms produced alpha, alpha and beta, or beta hemolysins, and coagulase. Plastridge et al.[5] had previously associated coagulase activity with highly virulent strains of S. aureus from mastitic cows. The use of coagulase activity as an indicator of staphylococcus pathogenicity, first proposed by Darányi (1935), has become well established (Elek, 1959). Reid and Wilson (1959) concurred with this observation while studying staphylococci isolated from acute and chronic infections, as well as apparently normal udders. In addition, these

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[5]Munch-Petersen (1938) gave the following incomplete reference: Plastridge, W. N., F. J. Weirether, and L. F. Williams. 1935. Proc. Soc. Amer. Bact. (J. Bact.).

authors claimed a correlation with the "egg-yolk factor"--lipase--in that positive strains were most often associated with acute infections, while negative strains were seen in chronic and normal udders. Hajeck and Marsalek (1968) likewise found low lipase producers associated with a carrier state and high producers from acute infections; isolates from acute infections were more highly coagulase positive than those from a carrier state. Flemming and Paton (1961) also isolated a high percentage of coagulase positive strains of S. aureus from cases of bovine mastitis. All the strains studied for toxin activity (50%) were found to have some alpha toxin activity. Loken and Hoyt (1961) were able to correlate production of beta toxin with strains known to be pathogenic for cows.

Stabenfeldt and Spencer (1966) and Spencer et al. (1968) have described a mild but progressive udder infection in the cow from which coagulase negative staphylococci were isolated. Similarly, 44.3% of the strains of staphylococci isolated from cases of bovine mastitis by Kastli (1967) were coagulase negative, nonhemolytic, and did not utilize mannitol. Sompolinski et al. (1969) reported isolating a most unusual strain of S. aureus from cows with mastitis. This strain was a Ca-pantothenic acid auxotroph, showing a G-variant dwarf colony on

enriched medium, and it did prove to be coagulase positive and highly pathogenic for cows.

Flemming (1960), after surveying the literature on phage types of S. aureus strains isolated from bovine mastitis, concluded that the predominant phage types were 42D, 44A, and 81. Slanetz and Bartley (1962) found 85% of the strains isolated from cases of bovine mastitis could be typed by combinations of the Blair-International and Seto-Wilson phage sets. Solomon et al. (1961) studied a large number of staphylococcal strains isolated from cows' udders, but no single widely distributed phage type of either the Seto-Wilson or Blair-International phage sets was found in the various herds studied. Quantitative coagulase activity was also found to vary greatly even among isolates of similar phage types. The authors noted that although many of the Blair-International set phages lysed the bovine isolates, the patterns were often unlike those seen in human isolates. Loken and Hoyt (1961) also found that in twenty-six out of thirty-three herds studied, each herd had a predominant phage pattern type of S. aureus. Parisi and Baldwin (1963) reported that the phage pattern using a combination of the S-W and B-I phage sets demonstrated predominant phage type of S. aureus within each given herd. However, when more than one quarter on a cow was shedding organisms, the bacteria isolated from each quarter were usually of different phage types.

Drury and San Clemente (1962) experimentally exposed cow udders to the human "epidemic strain" of S. aureus 80/81. They were able to establish an infection which proved, as in human infections, to be refractory to many antibiotics. Wallace et al. (1962) reported a naturally occurring "epidemic strain," 80/81, in a herd of milk cows. The pathogenicity of this organism among cattle appeared to be no greater than any other infecting strain.

With regard to the heterogeneity of phage types of S. aureus causing mastitis, Flemming and Paton (1961) maintain that any vaccine should be composed of either a common protective antigen or be polyvalent with respect to somatic antigen composition. Similarly, Norcross and Stark (1969) mentioned that a vaccine against bovine mastitis should contain toxins or toxoids, coagulase, and large numbers of killed organisms representing the many strains of staphylococci commonly found in mastitic infections.

Numerous reports of experimentally induced staphylococcal mastitis are found in the literature. Cows given intramammary injections of S. aureus by Carpenter (1922) developed systemic infections. Little and Foley (1935) placed as few as 200 to 900 viable S. aureus cells into the teat canal of cows and found acute mastitis appeared in only one of the experimental cows. Minett (1939) was also able to establish mastitis in goats by intramammary

infusion of strains of S. aureus isolated from previous cases of mastitis. Miller and Heishman (1943b) found a great variation in the virulence of strains of S. aureus isolated from milk or udder secretions. Some cows required multiple exposures of the organism to establish mastitis. They also noted variable susceptibilities among herds exposed to the same strain of organism. In a study similar to that of Little and Foley (1935), Klastrup (1956) used identical numbers of organisms to infect cows' udders and saw the same low incidence of acute mastitis. Although most of the infections were subclinical many of the cows did not exhibit any signs.

Obiger (1962) used toxigenic strains of S. aureus isolated from cases of bovine mastitis to infect the udders of nonmastitic cows. There were, in the milk from these cows, transient cell elevations, and increased catalase and chloride levels. The manifestations usually disappeared by one month post-injection, and none of the cows showed any systemic reactions. Kastli (1967) used infusions of 10^3 to 10^6 toxigenic and coagulase positive staphylococci to establish mastitis in cows. While most cows required repeated injections before they became infected, those with a previous nonspecific inflammation of the udder reacted rapidly. The authors stress the importance of factors predisposing the udder to infection, such as trauma to the glandular mucosa.

Slantez et al. (1963) claim that experimental infections should be performed with organisms suspended in a nonirritating fluid to reduce early stimulation of leukocyte infiltration which would reduce the infectivity of the organism. They found that skim milk was ideal as a nonirritant, and an acute infection could be established with as few as 10^3 organisms in some cows. Derbyshire (1958) injected various numbers of S. aureus, isolated from cases of bovine mastitis, into goats' udders. All the goats except one developed mastitis of varying degrees of severity from mild to gangrenous. At post-morten examination of the infected udders S. aureus was isolated from the challenged gland and its supramammary lymph node, but not from the uninoculated quarters.

Systemic Vaccination and Staphylococcal Immunity

The exact nature of the role played by virulence factors in the pathogenicity of Staphylococcus aureus has not yet been clearly defined. Extensive studies have proved that various extra-cellular products account for the initiation and maintenance of infection (Elek, 1959). Most early work with vaccination against staphylococcal infection was done with the diffusible toxins, both alpha and beta, which caused tissue necrosis in vivo and lysed blood cells in vitro. As early as 1887 Nocard reported a gangrenous mastitis in ewes and attributed the clinical

manifestations to bacterial substances released into the infected tissue. Vaccination with bacterial cells or products, or combinations of these, especially the toxins, was a popular method of treatment for and prophylaxis against mastitis (Munch-Petersen, 1938). However, much of this old work is difficult to interpret because of questionable controls (Derbyshire, 1962).

This review by Munch-Petersen included a report by Reid,[6] who used a complex but poorly defined vaccine prepared from a heat-inactivated whole culture filtrate of streptococci. Gerlach and Kralicek[7] and Louros and Gessler[8] used similar whole culture filtrates to vaccinate against streptococci. A staphylococcal culture filtrate containing inactivated toxin was used by Parish et al. (1934) to raise the circulating antitoxin levels in animals and humans.

Forssman (1936a,b) induced active immunity to staphylococci in mice by vaccination with formolized whole cells or formolized culture filtrate. Protection from challenge with viable staphylococci was achieved

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[6]Munch-Petersen (1938) gave the following incomplete reference: Reid, H. A. 1910. N. Z. J. Agric. 1:515.

[7]Ibid.: Gerlach, F. and E. A. Kralicek. 1927. Dtsch. tierarztl. Wschr. 35:331.

[8]Ibid.: Louros and Gessler. 1927. Zbl. Bakt. I. (Orig.) 104:356.

with both vaccines. Because no antitoxin could be measured in mice vaccinated with the whole cell vaccine, the author concluded that antitoxin is not necessary for protection. Specific immune responses involved antidermonecrotic factors and usually antihemolysins. Thus, the protection was from an immune substance and an unidentified serum factor. A series of experiments by Ramon et al. (1936a,b) and Djourichitch (1936) established the importance of elevated levels of antitoxin in the serum for survival of intravenously challenged animals. The animals with antitoxin also showed diminished reactions to challenge.

Kitching and Farrell (1936) demonstrated that vaccination with staphylococcal toxoid reduced the severity of lesions in animals injected with toxin, while vaccination with whole cells alone did not. The serum from the toxoid vaccinated animals, when given to nonimmune animals, conferred similar protection which was correlated with antitoxin titers of the injected serum. Downie (1937) similarly found that no protection could be induced by whole cell vaccination. However, while no protection was observed from either the whole cell or toxoid vaccination in this study, agglutinins, precipitins, and complement fixing types of antibodies to S. aureus extracts were present in the serum of animals vaccinated with the heat-killed whole cells.

Purification of staphylococcal toxin from culture filtrates was performed by Kitching and Farrell (1938) by precipitation with potassium alum solution. This treatment enhanced the antigenicity of the toxoids and often did not effect the toxic nature of the preparation. Smith (1937) found the formolized toxin and the alum precipitated toxin were very effective in inducing antitoxin production. A correlation between serum antitoxin titer and protection from intravenous challenge was reported in this work, but the antitoxin did not protect the surviving animals from local staphylococcal lesions. Vaccination with alum precipitated toxin gave increased antitoxin levels in non-lactating ewes, as compared with levels following vaccination with formolized toxin. In this study by Minett (1939), with respect to both local and systemic signs, intramammary challenge with staphylococci resulted in less severe reactions in the vaccinated than in the nonvaccinated animals. Ramon and Richou (1939) were able to further heighten the antibody response to toxoid injection by adding certain stimulating substances such as tapioca. Later Richou et al. (1941) suggested adding alum of potassium with the toxoid to enhance antitoxin formation, while Ramon et al. (1952) preferred aluminum hydroxide. More recently, Skean and Overcast (1969) found that when Freund's incomplete adjuvant is added to injections of alpha and beta toxoids, a high serum titer of specific antitoxins was produced.

Richou and Holstein (1941) noted an especially rapid rise in antitoxin titer in some animals following vaccination. This enhanced titer occurred in those animals with pre-existing levels of antibody. Thus a series of injections over several weeks was recommended for administering a substance to elicit highest antibody levels. A similar reaction had been reported by Minett (1939), who screened his test animals for antitoxin titers using only those which had the lowest levels.

In order to test the therapeutic or prophylactic capacity of serum antitoxins against staphylococcal mastitis following vaccination with a commercially-prepared staphylococcal toxoid (Wellcome Physiological Research Laboratories), two field trials performed without controls were carried out by Gould (1942) and Wilson (1942). Gould treated cows from a herd with a high incidence of mastitis with intramuscular injections of the toxoid and reported consistent reduction in both systemic and local signs and a reduction in abnormal milk. Wilson claimed similar success with the toxoid and found that the pathogen was eliminated from the infected glands and the animal subsequently recovered. Kearney and Moloney (1943) vaccinated cows with a mixed autogenous vaccine composed of staphylococcal toxoids and streptococcal "anaculture." One year post-vaccination there was a reduction in the number of cows with clinical signs of mastitis and an

increase in the average milk yield. Murphy (1946) found staphylococcal toxoid effective as a treatment for staphylococcal mastitis with a 72.5% cure rate, but unfortunately he ran no controls for comparison.

Murphy (1946) and Collinson (1949) reported toxoid therapeutic when combined with intramammary infusions of penicillin to treat mixed staphylococcal and streptococcal infections. Gould (1942) also used antibiotics (sulfonamides), along with the toxoid, to treat bacterial mastitis. Later, in a controlled study, Maclay et al. (1946) established an experimental staphylococcal mastitis in lactating ewes by intramammary infusion of viable organisms. Treatment at the onset of clinical signs with sulfonamides or staphylococcal toxoid (Wellcome Laboratories) did not cure any of the infections. Similar results with the toxoid-antibiotic preparation were reported by Ramon et al. (1951).

In a carefully controlled study by Spencer et al. (1956), the complexity of establishing staphylococcal immunity with respect to several variables was explored. Used as test animals, sheep, rabbits, and cows were vaccinated with a pellet of formolized staphylococcal cells, cells suspended in either aluminum hydroxide or mineral oil, or a commercial toxoid. The formolized cells suspended in aluminim hydroxide effectively elicited antitoxin in rabbits and gave the best protection from

challenge with viable staphylococci. Cows vaccinated in the same manner also developed high antitoxin titers and reaction to subsequent intramammary challenge with staphylococci appeared less severe than in the nonvaccinated animals. Sheep vaccinated with a similar preparation neither produced antitoxin, nor were they protected against intramammary challenge. North (1958) reported the loss of protective capacity of S. aureus whole cell culture filtrates, especially when the materials were passed through a Seitz filter. Test animals injected with the filtrate often produced serum antibody titers as high as those animals given unfiltered culture. Because protection in the animals given the filtrate was not as complete as that seen in the animals given the unfiltered preparation, additional protection from the nonfiltered preparation may come from somatic antigens on cell debris which would be lost by filtration.

Slanetz et al. (1959) used a formolized staphylococcal bacterin-toxoid with potassium aluminum sulfate adjuvant for intramuscular vaccination. The vaccinated cows appeared to contain no udder infection, developed increased antitoxin levels, and underwent a marked reduction in the number of acute relapses. Although the vaccinated cows challenged with 1 to 5 x 10^9 viable staphylococcal cells showed some protection, all cows gave severe reactions when the challenge dose was increased

to 2 x 10^{10} cells. In this study vaccination did not cure established cases of mastitis. Using a commercially-prepared vaccine (S. aureus Toxoid, American Cyanamid Co.) administered intramuscularly, Slanetz et al. (1963) found similar results. The vaccinated animals were highly resistant to intramammary infusions of a minimum infective dose of most of the challenge strains suspended in a nonirritating milk solution. One highly virulent strain was able to overcome the resistance in many of the vaccinated cows. The vaccinated cows were also resistant to spread of udder infection.

Flemming (1960) described an antistaphylococcal vaccine prepared with toxin from a highly toxigenic strain of S. aureus, and used it alone or incorporated it into a preparation of cells or adjuvant. Comparing the serum titers of rabbits injected with these preparations inactivated with either formaldehyde or beta-propionolactone (BPL), the later treatment of toxin with an adjuvant proved most effective. Gel diffusion studies with the rabbit antitoxin and toxin, or toxoid, showed heaviest and most numerous lines of precipitation when the antiserum was reacted with BPL inactivated toxin. Thus the author recommended a vaccine prepared from BPL inactivated whole cells of a variety of known pathogenic strains, alpha and beta toxins, and an adjuvant such as aluminum hydroxide.

Jensen-Salsbery Labs, Inc., marketed a standardized staphylococcal vaccine composed of beta-propionolactone inactivated alpha and beta toxins, and "staphylococcal cellular substance of multivalent origin" (Jensen-Salsbery Labs., 1960). The manufacturer claimed a ten-fold increase in the serum antitoxin titers following inoculation of cattle with this vaccine. Later, Philpot (1962) administered this vaccine to cattle at 1 week, 2 week, and six month series. Over an eighteen month post-vaccination observation period, he noted an increase in the numbers of quarters in the herd which were positive to the California Mastitis Test. Similarly, using an unidentified commercially prepared staphylococcal vaccine Oehme and Coles (1967) found it worthless in conferring protection from infection.

Thorne and Wallmark (1961) found treatment with cell-toxoids ineffective in treating established staphylococcal udder infections. The old vaccinated cows gave high antitoxin titers while the young cows which had no previous exposure to S. aureus antigens responded to vaccination with low titers and subsequently developed new infections of S. aureus at a rate similar to that of the nonvaccinated cows.

Pearson (1959) vaccinated cows with a staphylococcal cell-toxoid preparation. During the first eighteen months post-vaccination there was a significant

difference between the number of flare-ups of mastitis of the vaccinated and nonvaccinated groups, but during the last twelve months of the two and a half year study, the difference disappeared. A drop in antitoxin production over the observation period was correlated with the decreased protection.

Derbyshire (1960a) compared the effectiveness of vaccination in goats injected with formolized cell-toxoid with aluminum hydroxide adjuvant, formolized cell-toxoid alone, or formolized whole broth culture. The injections of formolized cell-toxoid alone, or formolized whole broth induced staphylococcal antibodies (agglutinins, precipitins, antitoxins, coagulase neutralizing, and mouse protective) better than the other preparations. This vaccine also established a high level of protection to intramammary challenge of 10^9 viable cells of the homologous strain in goats and cows, with only mild transient reaction, as compared with gangrenous reactions in nonvaccinated animals.

Schultze et al. (1963) found no reduction in the incidence of staphylococcal mastitis in groups of cows vaccinated with an unidentified commercially-prepared bacterin-toxoid. Wallace (1963) performed vaccination on cows with a bacterin-toxoid of unspecified composition and claimed a significant difference in the number of new cases between the vaccinated and nonvaccinated cows. Thorne et al. (1963) reported intramuscular vaccination in cows

with alpha and beta staphylococcal toxoids was most effective in producing high levels of antitoxin. Since no challenge was performed, no protection could be claimed by the author.

Derbyshire and Edwards (1963) vaccinated cows antepartum with an intramuscular injection of a cell-toxoid-adjuvant mixture, and then proceeded to observe the incidence of new cases of staphylococcal mastitis during the following two years. Although subsequent antitoxin levels of the vaccinated cows were twenty times higher than those of the nonvaccinated controls, the difference of incidence of mastitis between the two groups was not significant. In those cows developing mastitis, vaccination via the intramuscular route did not reduce the severity of symptoms.

Slanetz et al. (1965) tested several vaccines prepared from disintegrated cells, toxoid inactivated with formaldehyde and with BPL, whole cells and toxoid with aluminum hydroxide, and toxins concentrated by precipitation with alum. The vaccine prepared from formaldehyde inactivated polyvalent cells and toxin was the most effective in enhancing the resistance of cows to intramammary challenge with a number of homologous strains of S. aureus. A particularly virulent strain (570) was able to overcome the protection in some of the vaccinated cows, but when the vaccine did not contain strain 570 no protection from challenge by this strain was seen in any of the cows. In

this study, the vaccines inactivated with BPL were not as effective as those inactivated with formaldehyde.

Derbyshire (1961) established staphylococcal infections in the skin and udders of cows. There was an increase in serum antitoxin titers in the cows as a result of both types of infection. In goats, intramammary infusion of organisms regularly elicited precipitins while skin infections gave erratic results; however, neither administrative procedure produced mouse protective antibodies to the homologous strains. Three of four goats with skin abscesses who were challenged intramammarily with viable homologous staphylococci reacted with mild, transient mastitis and eventually recovered, while the control animals suffered gangrenous mastitis after the challenge with 10^9 cells. Intramammary challenge of the goats which had received the series of intramammary infusions of increasing numbers of viable staphylococci showed mild reaction and eventual recovery. These vaccine and challenge infusions were given in the left quarters only. The protection was only local in that later challenge to the right side of the gland produced severe reaction. Both of the control goats developed gangrenous mastitis.

Greenberg and Cooper (1960) and Greenberg et al. (1961) lysed whole cells with dornase, centrifuged the particulate matter, and used the supernatant fluid as the vaccine. The authors claimed that the somatic antigens

released during the enzyme lysis were responsible for the protection seen in vaccinated rabbits from challenge doses, which otherwise caused skin abscesses or death in the controls. Agglutinins to staphylococcal cells were formed in response to the vaccination indicating the participation of somatic antigens. The vaccine appeared to protect against a broader span of staphylococcal phage types than whole cell preparations. Further studies by Greenberg and LeRiche (1961) showed that in vaccinated animals no anticoagulase, antileukocidin, or antitoxin could be found; therefore, he concluded that these antigens did not participate in the immunization process.

Barnum (1962) vaccinated cows with somatic antigens, prepared after Greenberg and Cooper (1960), supplemented with staphylococcal alpha toxoid. The number of cases of mastitis seen in the vaccinated animals stabilized, while in the nonvaccinated cows there was a steady increase in the number of new cases. Cows with chronic mastitis did not respond to this treatment. A commercially prepared somatic antigen (Somastaph; Stevenson, Turner and Boyce, Ltd.) was used by Lepper (1967) to vaccinate goats intramuscularly. Challenge with a known pathogenic staphylococcus was performed by intramammary infusion of 10^3 to 10^4 colony-forming units suspended in fresh goat's milk. Following challenge, all vaccinated animals developed mastitis of varying degrees of severity,

which did not differ from the reactions seen in the non-vaccinated control goats. Other parameters, such as milk volume or colony count and leukocyte count per ml of milk, were similar for both experimental and control groups. Sawar et al. (1964) vaccinated human patients suffering from dermal infections caused by S. aureus type 80/81 with a polyvalent somatic staphylococcal vaccine prepared after Greenberg and Cooper (1960). There was no measurable protection from recurring infection, and no correlation could be made between agglutinin titers in the serum, which varied greatly, and any change in the incidence of infection.

Derbyshire and Helliwell (1962), finding unsatisfactory results with the simple cell-toxoid-adjuvant preparations, used a vaccine composed of the basic cell-toxoid adjuvant (Derbyshire, 1960a) supplemented with formolized alpha toxoid, beta-propionolactone toxoided coagulase and leukocidin. Protection from infection by an intramammary infusion of viable staphylococci of the homologous strain was seen, but at no higher level than that seen with the simple cell-toxoid-adjuvant vaccine. However, variation in protection occurred when a different strain, S. aureus Compton strain BB, was used as challenge strain. In this experiment the complex vaccine appeared more effective in conferring protection. Although the authors could not explain this variability, it might be

due to the difference in specificity of the protection developed by vaccination, in that the BB strain is an heterologous strain with respect to the strain used to prepare the vaccine, the Compton strain 201. Antitoxin (both anti-alpha toxin and antileukocidin), anticoagulase, and mouse protective antibodies were found in the sera of animals vaccinated with the complex vaccine. Differences in the protective capacity of the vaccines could not be correlated with the presence of specific serum antibodies.

Blobel and Berman (1962) prepared a vaccine, similar to that of Derbyshire and Helliwell (1962), composed of bacterin-toxoid with the addition of coagulase and "egg-yolk factor." Specific antibodies to the vaccine antigens were found in the serum: alpha and beta antitoxins, staphylococcal cell agglutinins, antileukocididin, and both coagulase and egg-yolk factor neutralizing antibodies. Although there was substantial variation in the titers of these antibodies in individual cows, all the vaccinated cows showed increased resistance to intramammary challenge with the homologous strain seen as reduced clinical symptoms. In agreement with Derbyshire and Helliwell (1962), they did not see any resistance in vaccinated cows to the heterologous challenge strain.

The possible role of leukocidin in the establishing of protection has been further explored. Souckova-Stepanova et al. (1966) subcutaneously vaccinated rabbits

with a mixture of purified staphylococcal leukocidin and a formolized Wood strain 46 culture filtrate (anatoxin), or each material alone, and then challenged them intravenously. Those vaccinated with either the anatoxin or leukocidin had antibodies to alpha toxin and both the F and S fractions of the leukocidin. However, in one specific group a commercially available alpha toxoid (Wellcome Laboratories) induced no antileukocidin activity. When survival to a lethal dose of staphylococci was used as the criterion of efficacy, the animals immunized with the leukocidin and the anatoxin showed some protection which was not, according to the authors, related to the presence of anti-alpha toxin because protection could not be correlated with corresponding antibodies. Mudd et al. (1962) found that a commercial alpha toxoid also did not elicit high antileukocidin titers in vaccinated human subjects, and recommended enhancement of the antileukocidin to confer protection in the patient. The action of leukocidin is controversial because Gladstone and van Heyningen (1957) found leukocidin from three different strains of staphylococci to be inactive against sheep polymorphonuclear leukocytes. But Szmigielski et al. (1968) demonstrated that in rabbits a series of intravenous infections of staphylococcal leukocidin impaired the reactivity of their leukocyte system, as indicated by a reduced response to subsequent intravenous injection of alpha toxin.

Despite the large amount of work with staphylococcal vaccines designed to induce high levels of serum antitoxins, the participation of these antitoxins in the protective mechanism has not been firmly established. Brown (1960) reviewed the literature concerning the role of antitoxin in immunity and concluded that the antitoxin levels had no ability to effectively prevent infection of the udder with staphylococci, but they did diminish the clinical manifestations. This effect he attributed to the neutralization of necrotizing toxin produced by the infecting bacteria. Also the elevation of such antitoxin levels did not eliminate an established staphylococcal infection. Edwards and Smith (1959) correlated stimulation of serum antitoxin titers with the presence of pathogenic staphylococci in the cattle. Brown (1962a) reported that in cows chronically infected with staphylococci, the serum agglutinins and alpha and beta antitoxin levels increased with the number of infected quarters, but within limits characteristic for each cow. Later, in a study of the levels of these antitoxins in cows with acute mastitis, Brown (1962b) found that the levels peaked in a few cows one or two weeks after the onset of the infection. In some there was measurable antitoxin present before the acute relapse. In most of these cases the causative bacterium was highly toxigenic. Finally,

Brown and Scherer (1963) observed a direct relationship between increased levels of staphylococcal antitoxin in the serum of cows free from obvious staphylococcal infection and their age.

In a study of the course of mastitis in cows, LeGall and Plommet (1965) outlined four phases of infection: (1) the lag phase, (2) the growth of bacteria, (3) reaction by the host, and (4) clinical phase in which the lesion develops. The authors contended that the growth of bacteria did not depend upon previous vaccination with toxoid, but the severity of lesions in phase four was related to the number of bacteria at the maximum growth period, volume of milk removed from the udder at milking, and the presence of alpha and beta antitoxin.

Intramammary Vaccination and Staphylococcal Immunity

Ehrlich (1892) recognized the presence of antibody in the colostrum. The role of this antibody, aside form its passive immunization of the nursing young (Smith and Little, 1922) and its protecting the lactating cow from udder infection, has not been consistently demonstrated. Jones and Little (1927) and Jones and Simms (1930) found a high bactericidal activity in raw milk from normal cows present in herds with a high incidence of udder infection. The authors proposed an active substance different from antibody, because they found no significant

difference in antibacterial activity between immune and nonimmune cows. This substance, named lactenin, was heat labile and precipitated with the protein fraction of whey. However, more attention has been focused on the identifiable antibodies in the milk. Jones and Little (1934) reported that bovine milk from infected quarters was less bactericidal than that from the quarters which were infection free. McEwen and White (1950) found that changes in the bactericidal activity of milk could not be correlated with changes in diet, season, or even inflammation of the gland whether bacterial or sterile. They did note a drop in this activity at drying off.

Miller and Heishman (1943a) were able to correlate high levels of blood serum antitoxin with high levels of whey antitoxin. Although in one case the whey level exceeded that of the serum, there was a great variability in the whey titer from one quarter to another. The observed levels drop significantly after the secretion of colostrum. Smith (1946) found that hyperimmunization of cows increased the immune component of the whey above the normal level of 10%, but the relationship was not consistent. Edwards and Smith (1959) found low antitoxin levels in milk of normal cows as opposed to very high levels seen in the colostrum and in cows with mastitis.

Thorne (1956) found lower staphylococcal anti-hemolysin titers in the serum of cows with no signs than in those cows with chronic mastitis. Some positive correlation between the level of antitoxin titer in the serum and that found in the whey was also noted. Lasmanis and Spencer (1954) found generally low antitoxin titers in the whey of a heterogeneous group of cows. A positive correlation existed between the level of antitoxin in the serum of the cows and the titers of antitoxin in the whey. At calving titers of antitoxin from colostral whey rose to near plasma levels (a few cows even showed higher levels), then dropped during the remainder of lactation except in those cows which developed mastitis when the titer in the whey continued to parallel antitoxin titer seen in the plasma (Edwards and Smith, 1959). Richou et al. (1962) did not find any change in the milk agglutinin titer following intramuscular vaccination with bacterin-toxoid, but intramammary vaccination resulted in very high titers in the whey and increased blood serum titers. Rivera-Anaya et al. (1966) were able to demonstrate a brief elevation in serum and whey antitoxin levels in cows following intramuscular vaccination with staphylococcal toxoid.

By using an intramammary infusion of heat-killed bacteria, Smith et al. (1923) stimulated a rise in the agglutinin titer of both milk and serum. Rather than

attributing the source of milk agglutinins to increased permeability of immune serum globulin via glandular endothelium or epithelium, these authors proposed local production of these antibodies. Kerr et al. (1959) infused the bovine udder with various bacterial whole cell antigens and recorded the appearance of agglutinins specific for each of these antigens. Infusion of staphylococcal antitoxin did not increase udder agglutinins.

Dixon et al. (1961) contended that the udder of the cow continually incorporates serum proteins into its secretions throughout lactation. This is especially true during colostrum formation when the acinar epithelium selectively transports the serum gamma-globulin across the epithelial barrier into the mammary gland secretory lumen. This results in a measurable equivalent loss of gamma-globulin from the serum accompanied by a hypogammaglobulinemia. The colostrum concentration of gamma-globulin can exceed the concentration of the gamma-globulin in the serum by 100-fold, and it is from the serum that this antibody is derived rather than the result of in situ formation. Mackenzie et al. (1966) also described an increased blood serum flow into the mammary gland acini during acute and peracute mastitis. Labeled gamma-globulin injected intravenously into the cow was found in high levels in the milk as well as the lymph;

such transfer would indicate a general increased capillary permeability during a mastitic infection.

In cows with high serum levels of antitoxin, Derbyshire (1960b) found that intramammary infusion of distilled water increased whey antitoxin. According to Derbyshire, irritation of the glandular epithelium altered its permeability to circulating immune globulins. Following intramammary infusions of sterile distilled water, Prafulchandra and Morse (1964) demonstrated in cows with S. aureus udder infection an increase in the blood serum albumin in the whey. However, Skean and Overcast (1969) did not find intramammary infusion of staphylococcal products sufficiently irritating to consistently induce blood serum globulin infiltration. The raised whey antibody titers were rare and sporadic, even when the antibody levels in the serum were very high.

Campbell et al. (1957) proposed a local source for antibody production in the udder because of the speed with which the udder responds with specific antibodies following the infusions of antigens. Sawar et al. (1964) reported specific agglutinins were found in cow's milk after intramammary boosters of whole cell vaccine. Agglutinins specific for the infused antigens appeared rapidly in the milk within two days post-vaccination. Reiter and Oran (1967) also found agglutinins in the milk specific for the infused antigens. When Porterfield et al. (1959) infused

the udders of cows with various antigens, they reported the whey antibody higher than that of the serum. This intramammary response was seen a few hours post-infusion while antibody levels in blood serum did not shift until 1 to 2 days post-infusion. Booster doses of antigen, when administered intramammarily, were found to be most effective in stimulating milk antibody. Derbyshire (1960b) did not find antitoxin in the whey of nonvaccinated cows until three days after experimental infection with S. aureus. However, in cows previously vaccinated with cell-toxoid-adjuvant preparations, antitoxin appeared in the milk six hours after intramammary challenge with viable staphylococcus organisms. In cows previously vaccinated with bacterin-toxoid, the degree of udder inflammation was correlated by Spencer et al. (1963) with high colostral and serum agglutinin levels.

Spencer et al. (1963) studied the bactericidal activity of milk with a growth inhibition test as an indicator of this activity. The inhibition was not consistently correlated with resistance of the udder to bacterial infection. Derbyshire (1964) compared the growth of S. aureus in cultures of milk from normal, mastitic, and cows which had been traumatized by means of intramammary infusions of irritants. The milk from mastitic cows inhibited the growth of the bacteria in vitro but milk from normal cows did not. Milk from the udders

traumatized with distilled water was as effective as the mastitic milk in growth inhibition. There was a heat labile humoral factor in the traumatized cows' milk which was apparently responsible for inhibiting bacterial growth, for when normal serum was added to normal milk, a heat labile factor from the serum conferred the ability of inhibition to the normal milk at the same level as milk from the traumatized udders.

Campbell et al. (1950) demonstrated histologically that an increase in milk antibody (which they hypothesized must be associated with a rise in the numbers of antibody secreting cells in the udder, if it is of local origin) was correlated with an increase in the number of plasma cells in the glandular tissue; this increase begins in late gestation and extends throughout the first four days of lactation. Lee and Lascelles (1969) infused ewe mammary glands, at weaning, with antigens, and measured high levels of pyroninophilic cells of this gland, especially in areas of degenerating glandular alveoli. By day sixteen post-weaning the highest number of pyroninophilic cells was recorded, and many of them resembled mature plasma cells. These cells may be responsible for a local allergic reaction which can be elicited in the gland during this time. Later, Lee and Lascelles (1970) used a fluorescent antibody technique to demonstrate that many cells in this same area of the mammary gland were positive

for producing anti-IgA antibody. They also found cells which specifically bound fluorescein isothiocynate (FITC) conjugated antibody for IgA, IgG_1, IgG_2, and IgM, indicating that these pyroninophilic cells are responsible for local antibody production; the IgA specific cells appeared to be most prevalent.

Willoughby (1966) reported that when he used staphylococcal antibody conjugated with fluorescein isothiocyanate, plasmacytes which took up this antibody were seen in the tissue from cows' udders infected with staphylococci. Early in lactation most of the conjugated antibody positive cells are found in the supramammary lymph node. Later, a few of these plasmacytes were found in the intra-alveolar stroma of the mammary gland, but only after the infection had persisted for fourteen days. Gupta et al. (1970b) found plasma cells in mastitic gland tissue of guinea pigs.

Mach et al. (1969) claimed to have found a true IgA species of gamma-globulin in the colostrum of cows. In mature milk, however, there was very little IgA, but the "secretory piece" of IgA was found in the milk. Outteridge et al. (1968a) found most of the immunoglobulin in post-partum colostrum was not associated with IgM or IgG_2, but with the IgG_1 and not IgA. Mach et al. (1969) also averred that most of the immune globulin in the cow milk is of the IgG_1 type which may have taken the place

of the IgA seen in man and rabbits, and that this IgG_1 is selectively secreted by the gland tissue.

Pillet et al. (1959a) administered staphylococcal antigens intramammarily in ewes and produced an effective local immunity as measured by an intramammary challenge. The vaccine was composed of alpha and beta toxoids and killed whole cells of selected staphylococcal strains. One to three weeks after the last of a series of three intramammary vaccinations, a significant level of protection to intramammary challenge with an homologous strain was demonstrated. Although a drop in protection was evident about one month after the final vaccination (and had completely ceased in one animal), a booster vaccination given after the initial protection had diminished rapidly increased the level of protection (Pillet et al., 1959b). A similar vaccine was used by Plommet (1960) to treat cows afflicted with staphylococcal mastitis, some of whom had been unsuccessfully treated with antibiotics. Following intramammary infusion of the vaccine, 50% of the animals recovered, although no correlation could be made between recovery and the age of the cow, state of lactation, or duration of previous infection. Of the strains of S. aureus isolated from the infected udders, those which produced beta toxin alone, or alpha and delta toxins, appeared to be most susceptible to the vaccine treatment.

Plommet and LeGall (1963a,b) found specific antitoxins and serum agglutinins in the milk of cows vaccinated intramammarily with a bacterin-toxoid (alpha and beta toxoid). Although no protection from infection was observed, the vaccinated animals showed less severe reactions to challenge than the controls. By intramammary vaccination of ewes with formalin-killed staphylococci, Fujikura (1966) could protect them from intramammary challenge with the homologous strain. However, this protection could not be demonstrated in any of the vaccinated animals thirty days post-vaccination.

Lascelles et al. (1966) infused bacterial antigens into the quarters on one side of a group of ewes' udders and found a slight increase in specific agglutinins only in the infused quarters. Plasma levels of the same agglutinin remained consistently higher than the whey levels. However, when similar infusions were made during mammary gland involution, a substantial increase in the titers of whey and afferent lymph was observed which, after two infusions ten days apart, reached a level many times that of the plasma. The authors averred these results would indicate local antibody production. Infusion of these antigens into dry glands elevated antibody titers in whey collected from the immunized quarters. Although the total fall in whey antibody after the colostral period secretion was great, whey antibody/plasma antibody ratios of the

infused gland remained above unity throughout lactation. During lactation the antibody titer of the mammary lymph was below that of the whey from the infused gland. This result indicates that the antibody forming cells lie close to the glandular epithelium.

Outteridge and Lacelles (1967) infused a mixture of staphylococcal alpha, beta, and leukocidin toxoids into the quarters of one side of a ewe's udder during the dry period. The specific antitoxin titers of colostral whey were consistently higher in the infused side than in the noninfused side of the same udder, suggesting possible local antibody production. Challenge on both sides of the udder with either virulent staphylococci or alpha toxin gave rise to an inflammation in all quarters which was less pronounced in the infused quarters. A rise in antitoxin was seen in the milk from the immunized quarters two days post-challenge, which peaked about 3 days later at a level eight times that of the plasma.

Outteridge et al. (1965) used a similar toxoid to vaccinate ewes intramammarily. An intramammary challenge of 10^6 viable staphylococci did not visibly affect the treated glands, but the noninfused quarters developed mild to acute inflammation. Agglutinins in the milk were not only increased in the infused quarters, but during the first three days post-challenge fewer bacteria were isolated from them. The nonimmunized quarters, when

compared to the immunized quarters at post-mortem, were severely indurated. Similarly, Derbyshire and Smith (1969) successfully raised serum levels of alpha antitoxin and staphylococcal agglutinins following intramammary infusion of staphylococcal cell-toxoid. Although the vaccinated groups were more resistant to challenge with 10^4 viable staphylococci suspended in milk, with respect to clinical signs and bacterial and total cell counts in the milk, infection was established in all challenged quarters.

Stepanov (1969) used a formolized culture filtrate to vaccinate cows. Following two intramammary infusions of vaccine, he reported maintenance of milk antitoxin titers for more than 60 days post-vaccination. San Clemente (1970) administered various staphylococcal antigens to cows via the intramammary route. The vaccines consisted of various combinations of heat-killed whole staphylococcal cells, purified staphylocoagulase and staphylolipase, and staphylococcal polysaccharide antigen. As indicated by a reduction in gross clinical symptoms, intramammary challenge with an homologous strain of staphylococcus was significantly resisted by those cows which had been vaccinated with the combination of polysaccharide antigen, whole cells, and staphylocoagulase. McDowell and Lascelles (1971b) immunized pregnant ewes 24 days ante-partum intramammarily with a

live staphylococcal cell vaccine or a staphylococcal cell-toxoid vaccine. Some protection to challenge with viable staphylococci was observed with both vaccines, as well as increased milk production in the vaccinated quarters over the nonvaccinated quarters, in addition to both reduced bacterial and total cell counts in the milk and general gross clinical signs.

One month ante-partum Lascelles and McDowell (1970) infused ewes intramammarily with brucellar somatic antigens. Immunelectrophoresis of whey and precipitation with anti-IgG_1, IgG_2, and IgM resulted in bands reacting with IgG_1 and a beta electrophoretic band seen in whey from glands infused with antigen. Further study of the beta electrophoretic antibody proved it to be distinct from the other immunoglobulins and the authors concluded that it was analogous to the IgA immunoglobulin observed in the milk of other species. McDowell and Lascelles (1971a) gave intramuscular injections of brucellar somatic and salmonella flagellar antigens to ewes which had been previously immunized intramammarily ante-partum and were producing antibody locally in their udders. These intramuscular injections resulted in increased blood serum levels of the specific antibodies, but corresponding whey antibody was almost imperceptible.

Two weeks ante-partum McDowell et al. (1971) injected pregant guinea pigs intramammarily with formalin

killed S. aureus, a crude preparation of staphylococcal protein A, or staphylococcal mucopeptide. Agglutinin titers and immunoelectrophoresis were performed on whey prepared from pooled milk samples collected at various times during lactation. Agglutination titers of serum and whey collected at parturition were substantially higher than samples collected before immunization, and the serum titers were higher than the whey titers. In two animals the whey titer from the immunized left gland was higher than whey titer from the nonimmunized right gland. In animals injected with mucopeptide, the agglutinin titers in the serum showed a 2 to 26-fold increase at parturition, and again the whey titers from the immunized glands were higher than those from the nonimmunized glands. Precipitin titers for protein A in the parturient serum was not significantly different from that sampled before immunization. Immunoelectrophoretic patterns of whey reacted with rabbit antiguinea pig serum showed lines corresponding to albumin, IgG, IgM, and a beta motility line which was neither detected in the serum of the animals nor in the whey of nonimmunized animals. The authors contended that this beta electrophoretic line may be locally produced IgA. The presence of a guinea pig IgA present in the milk of the animal has been identified and characterized by Vaerman and Heremans (1972) and Coe (1972).

The Role of Intramammary Leukocytes

Experimental leukocytosis was successfully established in the bovine udder following an intramammary infusion of saline by Blobel and Katsube (1964). This was a local response because untreated quarters in the same cow showed no such elevation in leukocyte count. Three of four quarters showing this elevated cell count resisted infection to infusion of 10^3 viable staphylococci, while all the untreated ones became infected with staphylococci. Streptococci, at the same challenge dose, were able to overcome the leukocyte barrier and establish infection; only a transient initial depression in the number of recoverable streptococci was seen. Schalm et al. (1966), however, were able to establish a leukocyte barrier to streptococci in the bovine udder by infusions of 100 to 350 organisms, singly or in repeated doses, and as the cellular reaction became intensified, resistance was effective against as many as 2.5×10^4 organisms.

Challenge with Aerobacter aerogenes, by Blobel and Katsube (1964), was similarly affected by an established leukocytosis, as was the staphylococcal challenge. Schalm et al. (1964a,b) using A. aerogenes, and Schalm and Lasmanis (1963), using Escherichia coli, confirmed the results of Blobel and Katsube. Reiter and Oram (1967) used several bacterial types including

staphylococci and streptococci and further confirmed bacterial inhibition by intramammary leukocytosis.

Newbould and Neave (1965a,b) introduced very few viable S. aureus cells into the bovine teat cistern and then stripped the milk out of the teat and performed viable counts. They discovered an inverse relationship between the number of staphylococci recovered and the number of leukocytes pre-existing in the milk from the udder. Often the recovery of organisms was possible only after centrifugation of the milk and cultivation of pellet. This may indicate the possible release of ingested staphylococci by disrupted leukocytes, a phenomenon which, if it occurred in vivo, would partially explain the chronicity of the staphylococcal infection. In a later study, Newbould et al. (1966) noted increased growth of staphylococci in a milk medium when udder derived leukocytes were added. The authors claimed a possible release of leukocyte lipase which broke down butter fat into useful nutrients. Brownlie (1971) on the other hand isolated a basic protein from milk neutrophils and, using immunodiffusion of the protein antibody and whey from cows with experimental mastitis, found a positive correlation between levels of this protein in the whey and the bactericidal activity of the milk.

Slanetz et al. (1965) demonstrated that a delayed cellular response would enhance bacterial invasion.

Using a nonirritating menstruum which delayed the infiltration of leukocytes into the udder, infection with staphylococci was possible with as few as 10^3 organisms. Confirmation of this phenomenon was achieved by Jain et al. (1967). By sensitizing a cow with egg albumin, then giving the animal an injection of the albumin to elicit a systemic anaphylaxis, they could maintain a leukopenia, and infection with Aerobacter aerogenes was easily induced. At the same time, however, one quarter of a leukopenic cow became infected with a normally nonpathogenic, coagulase negative strain of staphylococcus. Multiplication of the organism and acute local signs were observed until the delayed neutrophil infiltration began, after which the signs were reduced and the organism disappeared.

Katsube and Globel (1964) observed the phagocytosis of staphylococci in vitro. These leukocytes obtained by saline infusion of the bovine udder phagocytized the staphylococci, but most of the coagulase positive strains were able to survive this phagocytosis. Although addition of serum proteins to the in vitro system enhanced phagocytosis, further enhancement was not possible by including homologous antiserum or complement.

Small Animal Mammary Gland

In small animals like the rat, mouse, and guinea pig, correlation of several parameters had been used to

determine physiological state and general activity of the glandular tissue. Reece and Warbritton (1953) observed the greatest mitotic activity in the rat mamma during the middle of pregnancy. During lactation, however, the mitotic activity seen in histologic preparation was very low. Jeffers (1935) found mitosis in the gland tissue of rats 10 hours post-partum. By 24 or 48 hours and for the remainder of lactation, Maeder (1922) found no mitosis in the glands of rats. In guinea pigs Naito (1958) observed a similar increase in the number of glandular cells seen in the alveoli which had increased during gestation and peaked at parturition. He then correlated this increase with rise in DNA concentration of glandular tissue. Both cell numbers and DNA then remained relatively constant for the remainder of lactation.

Hesselberg and Loeb (1937) and Kuramitsu and Loeb (1921) reported that in the guinea pig gland the peak time of mitosis, as seen in histologic section, occurred during the peak growth period determined by changes in weight at about two days post-partum. Mitotic figues were absent immediately after and during the rest of lactation. The authors considered this an indication of decreased mitosis and cell turnover after the initial post-partum growth.

Davidson and Leslie (1950) proposed a method of studying tissue activity by considering its nucleic acid

content as an index of cell number. The validity of this index depends upon a uniform level of DNA/nucleus of cells analyzed. Tucker and Reece (1963a) demonstrated the stability of the DNA/nucleus values in the rat mamma during pregnancy and lactation. Although the concentration of DNA did fluctuate, it was associated with mitosis and increased cell numbers and thus no change in DNA/nucleus ratio. Kirkham and Turner (1953) claimed that RNA/DNA ratio of a tissue serves as an indicator of the condition of growth and activity of protein secreting glands. This ratio is an accurate measure of the protein synthesis.

Nelson et al. (1962) prepared a detailed study of the lactating guinea pig mamma with respect to weight changes, nitrogen levels, milk volumes, and nucleic acid concentrations. Gland weight, RNA/DNA ratios, and milk yield peaked at days 3 to 4 post-partum and then gradually declined. Tucker and Reece (1963b) found an increase in the total DNA concentration and the RNA/DNA ratio during the peak lactation period in the rat mamma. An early study by Hesselberg and Loeb (1937) claimed that milk secretion peaks immediately after parturition and, although gradually decreasing, will continue for three to four weeks post-partum.

Nelson et al. (1962) noted that the RNA/DNA ratio plotted against time follows the lactation curve. They also noted that nitrogen remained stable from day 2 to

day 28 of lactation and then dropped off. Likewise, DNA concentration was stable from days 2 to 10 and then it declined. Greenbaum and Slater (1957) saw an RNA rise during pregnancy in the rat which increased rapidly at parturition and then decreased during involution. The DNA rose during gestation, about doubled at parturition, and then remained fairly level for the rest of lactation. Kirkham and Turner (1953) also saw an increase in DNA during the early part of pregnancy which increased only slightly during lactation. The RNA was reported to increase throughout lactation and gestation. They saw a peak at day 21 or 22 post-partum. After involution began the RNA level dropped rapidly.

Nelson et al. (1951) published lactation curves for the guinea pig. The milk flow was greatest about day three post-partum, declined rapidly to day eight, and then decreased in volume more slowly. There was no difference in the volume of milk obtained, whether the animal was milked daily or every other day. When compared to the first lactation curve, however, there was a significantly higher milk volume and a higher sustained production seen in one animal during its second lactation.

Involution of the gland caused certain specific changes. Kuramitsu and Loeb (1921) and Hesselberg and Loeb (1937) observed a slight infiltration of leukocytes during natural involution and in those animals whose

mammae had been ligated to induce artificial involution. Kuramitsu and Loeb (1921) proposed an inverse relationship between the pressure within the gland as a result of reduced fluid withdrawal after weaning or ligation, and the level of infiltration of the leukocytes into the gland. Similarly, Slater (1962) reported a slight but significant increase in the DNA concentration in the glands of the animals undergoing involution. Invasion of the gland tissue by leukocytes, observed histologically, could partially account for the increase in DNA. Nelson et al. (1951) and Nelson et al. (1962) reported a drop in milk flow and RNA and DNA levels of the mammae at involution.

Gupta et al. (1971c) reported levels of leukocytes observed in milk samples from apparently normal, i.e., not infected, guinea pig mammary glands. He found variation as great as 1.0×10^4 to 6.0×10^6 cells per ml of milk. No difference was seen between the samples from the left or right glands. Bacteriological studies were not made.

Mastitis in Small Animals

Hesselberg and Loeb (1937) reported a bacterial infection of the mammary gland of a guinea pig, but did not identify the pathogen. In a study using nursing mice, Williams and Patnode (1948) found spontaneous infection of the mammae in 13% of those animals which were

prematurely weaned. Gupta et al. (1970b) reported bacterial mastitis in guinea pigs was associated with elevated somatic cell counts in the milk. An alpha-hemolytic streptococcus was isolated from the milk of both cases and from the tissue of one of the glands. Somatic cell counts in the milk ran as high as 2.4×10^7 cells per ml of milk.

Experimental mastitis in small animals has been reported. Said (1969) established mastitis in nonpregnant, nonlactating rabbits by means of an intramammary infusion of a viable coliform culture. Gupta et al. (1971b) induced a sterile mastitis in guinea pigs by intramammary infusions of bacterial endotoxin. This mastitis was associated with elevated somatic cell counts in the milk and increased levels of DNA, as compared to the controls, in the mastitis tissue. Gupta infused Smith diffuse strain Staphylococcus aureus into the glands of guinea pigs and established a mastitis similar to that observed with endotoxin (personal communication). Following intramammary infusion of a Pasteurella sp. isolated from a mouse with mastitis, Williams and Patnode (1948) reported a severe mastitis developed in the test mouse.

Chandler (1970), using S. aureus strains recently isolated from cases of bovine mastitis, infused approximately 2.75×10^2 organisms intramammarily into mice and established an acute mastitis. When higher numbers of organisms were used, the mastitis became severe enough to

cause death. Histologic section of the mastitic glands showed neutrophil infiltration, numerous cocci, and tissue necrosis. Similarly, Anderson (1971) screened several strains of S. aureus isolated from cases of bovine mastitis for pathogenicity in mouse mammary gland. Both the virulence and pathogenicity of the strains varied. Some were better able to establish a severe infection than others, and often mortality and sickness were not necessarily indicators of histopathologic changes in the tissue.

Staphylococcal Antigens

Capsular and Somatic Antigens

When injected intraperitoneally, encapsulated strains of Staphylococcus aureus which showed diffuse colony growth in serum or plasma soft agar, e.g., the Smith diffuse variant, are highly virulent for mice (Hunt and Moses, 1958). Blackstock et al. (1968) found that the encapsulated strains, which grew diffusely and did not show the clumping factor in plasma soft agar, did possess the clumping factor, but it was blocked by the presence of a capsule. The capsule appeared to be a phagocytosis inhibiting factor, because Cohn and Morse (1959) demonstrated phagocytosis of these encapsulated organisms required the presence of anticapsular opsonizing antibodies in the challenged animal. Koenig and Melly

(1965) described certain strains of encapsulated staphylococci which grew as diffuse colonies in serum soft agar and depended upon encapsulation for their virulence. Morse (1960) prepared a lyophilized, deproteinized alcohol precipitate from the culture fluid of the Smith diffuse strain and showed this substance, when incubated with anticapsular immune serum, removed both the capacities of the serum to enhance phagocytosis of bacteria and to agglutinate.

Derived surface antigens from encapsulated strains have been isolated and characterized. Morse (1962) characterized a culture extract of Smith diffuse strain staphylococci, and found it to be a polysaccharide which he called the Smith surface antigen (SSA). This SSA was identified as the antiphagocytic factor reported earlier by Morse (1960). Chemical analysis of the substance showed it to be a 2-deoxy-2-amino-glucuronic acid. Later, Fisher et al. (1963) independently isolated and characterized an acid extractable substance from the Smith diffuse strain which he called staphylococcal polysaccharide antigen (SPA). Haskell and Hanessian (1963, 1964) chemically characterized SPA as a polysaccharide D-glucosaminuronic acid and L-alanine with O- and N-acetyl substitutions. Morse (1963) contended that this SPA is identical with his SSA.

Fisher et al. (1963) found SPA to be immunogenic in mice, dogs, and man. Later San Clemente et al. (1966) and

San Clemente (1970) reported production of anti-SPA antibodies which, when administered to mice, protected them from challenge with the Smith strain (Fisher, 1959). These antibodies were only found after SPA injection in cows but not in rabbits. Morse (1963) found the SSA antiserum precipitated with extracts from other non-Smith diffuse strains of encapsulated staphylococci. This reaction was not seen with pathogenic, nonencapsulated strains of S. aureus.

Other encapsulated strains of S. aureus have been studied as well as other Smith diffuse strains extracts. Wiley (1961) hyperimmunized rabbits with an encapsulated strain and induced protective antibodies which were capsule specific. Wiley and Wonacott (1962) used an extract of a highly mucoid, encapsulated strain of S. aureus to adsorb the protective factor out of immune, anticapsular serum. This extract, unlike SPA or SSA, had a polyglycerol fraction and may be related to teichoic acid. A capsular extract stimulated antibody production in animals whose serum could passively protect chick embryos from staphylococcal challenge.

Ekstedt (1963a) gave mice a single intraperitoneal injection of either formalin or heat-killed cells of Smith diffuse strain as well as a sub-lethal dose of live cells. Protection from challenge by the homologous strain was established and peaked at two weeks post-vaccination and

then dropped off. There was no correlation between protection and serum levels of antitoxin or agglutinins, but protection could be correlated with enhancement of the phagocytic-bactericidal activity of the immune serum. Cross-protection with a different challenge strain was not present.

Following vaccination of mice with cell wall extracts, crude culture supernatant, cell extracts, and a substance similar to the SSA of Morse, Ekstedt (1963b) found none of these preparations protected against a challenge with saline-suspended injections of the homologous strain. However, when the infective dose was reduced and the organisms were suspended in mucin, a high level of protection could be seen. Therefore, Ekstedt (1963c) proposed a two-phase immunity to staphylococcal surface antigens: a protein substance which confers immunity to saline suspensions of cells but not mucin suspensions, and a heat stable polysaccharide which only will protect from challenge with mucin-suspended organisms. Morse (1962) previously stated that SSA does not protect mice from challenge with cells suspended in saline, because the challenge organisms produce alpha toxin too rapidly for the host to overcome. Koenig et al. (1962b), however, found that mice with high serum levels of antitoxin were killed by the saline-suspended cells.

Angyal et al. (1967) administered to human patients with chronic and relapsing staphylococcal skin diseases the following vaccines: an autogenous vaccine, heterogenous vaccine, Smith diffuse and Smith compact strains, and a commercially available polyvalent vaccine. The best response, indicated by a rise in the phagocytic index and mouse protective titers of the patients' sera, was found in those subjects vaccinated with the Smith diffuse and autogenous vaccines.

Ekstedt and Yoshida (1969) were able to demonstrate increased protection in mice which had been vaccinated with a living vaccine of a non-Smith diffuse strain of staphylococci to a subsequent challenge with the Smith diffuse strain, but not in those mice vaccinated with heat-killed organisms. This protection could be transferred passively by the immune serum, and certain non-Smith strains, when grown in a special medium designed to induce capsule formation and diffuse growth, could then adsorb the protective factor out of the immune serum.

Fisher (1959) and Fisher (1961) have reported that normal human serum could passively immunize mice against the Smith diffuse strain. Rogers and Melly (1962) found Smith strain anticapsular opsonins in normal human sera. Analyzing S. aureus from human infections, Yoshida et al. (1970) found that 4.2% were encapsulated

forms. These encapsulated types reverted to nonencapsulated, compact forms after one or two transfers on synthetic medium. Mice vaccinated with heat-killed encapsulated cells were protected from challenge infection with a known Smith diffuse strain. Similarly, the encapsulated cells removed the protective antibodies and neutralizing capacity of rabbit Smith diffuse strain antiserum. The authors concluded that these naturally occurring strains were apparently identical to the Smith diffuse strain.

Wiley and Maverakis (1968) described an encapsulated strain, the Wiley wound strain, which was truly encapsulated but not virulent for mice. Because they found agglutinating serum antibodies to the wound strain in mice, they concluded that the mice must carry this strain, despite encapsulation and developed protective antibodies against it.

Coagulase

Coagulase production associated with pathogenic strains of Staphylococcus aureus has been well established (Elek, 1959). Its precise role, if any, in the pathogenicity of the staphylococcus organisms is still in question. However, coagulase production by S. aureus in vivo has been demonstrated by Gladstone and Glencross (1960); Tager (1948) found it was lethal to animals

administered large doses of a purified preparation by intravenous injection. Ekstedt and Yotis (1960) found staphylocoagulase enhanced the virulence of coagulase negative staphylococci when coadministered intracerebrally into mice. The coagulase, without which these same bacteria were harmless, was probably bound to the bacterial cell and protected it from host defenses.

Following treatment of staphylococcal cells with either antiserum to coagulase or whole cells, Borchart and Pierce (1964) found both antisera enhanced phagocytosis and intracellular destruction. As antiserum to whole cells did not neutralize coagulase, but was very effective in stimulating phagocytosis, the authors concluded that another factor aside from coagulase must account for resistance to phagocytosis. Sawai et al. (1965) reported that clearance of virulent or avirulent staphylococci from the peritoneal cavity of mice was inhibited by the presence of coagulase. Similarly, mice infected with the coagulase positive strains showed more abscess formation than those infected with coagulase negative staphylococci. The authors averred that coagulase enhanced abscess formation and facilitated the invasiveness of the bacteria.

Vaccinating rabbits with relatively crude coagulase preparations, Boake (1956) found they survived intravenous challenge with viable staphylococci longer

than the nonvaccinated rabbits. Lominski and Roberts (1946) recognized a staphylocoagulase inhibiting substance in human plasma but did not identify it as antibody. Duthie and Haughton (1958) first prepared highly purified and concentrated coagulase with which they induced measurable amounts of anticoagulase in rabbits.

Yotis (1962) studied a water soluble globulin fraction of serum which was antibacterial. S. aureus cells previously incubated with this serum fraction, when injected intracerebrally into mice, gave a 0 to 30% mortality. When this fraction was incubated with coagulase and then cells and finally injected intracerebrally into mice, a 60 to 90% mortality was seen. A similar result of coagulase overcoming the protective capacity of immune serum was reported by Ekstedt (1965).

Lominski et al. (1962) immunized rabbits with coagulase rich preparations of culture supernatants and observed the formation of coagulase neutralizing antibodies. They claimed that the coagulase conferred protection to challenge and this protection followed the phage type specificity of the coagulase first noted by Barber and Wiley (1959). Coagulase prepared from a strain of phage type I protected animals from challenge only with the homologous strain, and no cross protection was observed. This homologous protection held for all the coagulase types studied. Derbyshire and Helliwell (1962) used

coagulase to supplement a toxoid preparation to vaccinate goats. Since no detectable coagulase neutralizing antibodies were seen, the authors proposed that the coagulase may have been chemically modified and lost its specific antigenicity (Derbyshire, 1962b).

Based on intraperitoneal challenge with viable staphylococci, Ekstedt (1963b) found no protection conferred on mice which had been vaccinated with coagulase and Freund's incomplete adjuvant. Harrison (1964) vaccinated rabbits intramuscularly with staphylocoagulase toxin and toxoid from two different phage type strains of S. aureus. Subsequent intravenous challenge with viable cells of the homologous strain showed a level of protection in both cases. The absence of cross protection was not absolute because coagulase from one strain did offer some cross protection to a heterologous challenge strain.

Using methods devised by Zolli and San Clemente (1963) for preparing highly purified staphylocoagulase, San Clemente et al. (1966) injected these substances into rabbits and cows. Both animals produced coagulase neutralizing antibodies. In a later study, San Clemente (1970) vaccinated cows with a combination of several staphylococcal materials including staphylocoagulase and found the latter gave some degree of protection, as seen by reduced local reaction of the udders to intramammary challenge with heterologous staphylococci.

Lipase

Lipase, a diffusible product of S. aureus, has been shown to be widely distributed among the staphylococci (Davies, 1954). The enzyme is antigenic and is found in 99.5% of isolated coagulase positive staphylococci (Elek, 1959). Elek further maintains, however, that there is no evidence for a role in the pathogenicity of S. aureus. Gillespie and Adler (1952) reported a positive correlation of lipase production with coagulase activity in S. aureus and that lipase is only produced by coagulase positive strains. Kimler (1962), however, was unable to link the egg-yolk agar positive strains with coagulase positive strains at a high frequency, while Lowburg and Collins (1964) reported close association with penicillinase and egg-yolk activity.

Bruni (1961) avers that lipase is not only a good index of virulence, but is an important virulence factor. Passage through rabbits of lipase positive strains increased their virulence and lipolytic activity. Burns and Holtman (1960) also found high lipolytic activity in cultures of S. aureus isolated from a lesion. O'Leary and Weld (1964) demonstrated possible uncoupling of oxidative phosphorylation in mammalian cells by formation of free oleic acids, which were seen in cultures of lipase positive S. aureus. The importance of lipolytic activity in mastitis was indicated by Vadehra and Harmon

(1964), who found that strains of S. aureus grown in milk were more highly lipolytic than the same strains grown in nutrient broth. Stewart (1965) proposed that the ability of lipolytic strains to utilize lipid substances found on the epidermis of humans enables the staphylococci to maintain colonization of the skin and sebaceous glands.

In 1967, Renshaw and San Clemente reported a method of obtaining highly purified lipase from staphylococci. The authors reported that it was antigenic for rabbits, cows, and mice, inducing precipitating, neutralizing, and agglutinating antibodies. San Clemente et al. (1966) also found lipase neutralizing antibodies in cows and rabbits given mixtures of staphylococcal antigens, which included lipase. An antistaphylococcal vaccine (San Clemente, 1970) with lipase as a constituent did raise the antilipase serum titer of cows vaccinated either intramuscularly or intramammarily.

MATERIALS AND METHODS

Materials

Animals and Their Care

The animals used in this study were all female, primiparous, short-haired, albino guinea pigs of the Connaught strain. They were obtained through the Michigan Department of Public Health at approximately four weeks of age. The animals were kept in groups of four in metal cages (2' x 2' x 2') with wire grid floors allowing waste to fall out of the cage. Ambient temperature was approximately 80°F. and eight hours of light were provided each day. Food consisted of Wayne Guinea Pig Chow (Allied Mills, Inc., Chicago, Illinois), which contained a vitamin supplement and was given along with water, ad libitum. The animals were about twelve weeks old and weighed approximately 500 to 600 g at impregnation. Males were introduced at this time and allowed to remain for two weeks. Late in gestation when a thumb could be inserted into the pubic symphysis, the pregnant animals were isolated in smaller cages (8" x 8" x 2'). Again food and

water were given ad libitum. At whelping the litter size was standardized to one pup per teat.

If death of a nursing young occurred, an extra animal could be shifted from one mother to another up to ten days post-partum; the mothers will accept new young during this time with little hostility. However, during the experiments no young were transferred after they had been exposed to the challenge dose of S. aureus, for fear of contaminating the new mother.

Bacterial Strain

The organism used was Staphylococcus aureus, UNH 10, obtained from Dr. L. W. Slanetz, University of New Hampshire. The strain, originally isolated from bovine mastitis, was highly virulent for dairy cattle when injected intramammarily. It is mannitol, coagulase, DNase, and protease positive, alpha hemolytic on ox blood agar, and reduced potassium tellurite. Phage typing yielded the pattern S_2, 107, (102, 1363/14), and it possessed the clumping factor showing compact colony morphology in serum soft agar. For the experimental studies the organism was grown in Trypticase Soy Broth (BBL, a Division of Bio-quest, Cockeysville, Maryland), and was kept in storage at 4°C on Trypticase Soy Agar (BBL) slants. Transfers to fresh agar slants were made every four weeks, at which time the organisms were tested for DNase, coagulase, hemolysis, and tellurite reduction.

Vaccine Preparation

The vaccine preparations were modeled after those used by San Clemente (1970), but the quantities of antigens were proportionately reduced for use in guinea pigs. From the following antigens combinations were prepared as shown in Table 1:

1. Whole cells. A 0.1 ml inoculum of a 24 hour Trypticase Soy Broth (TSB)(BBL) culture of Staphylococcus aureus UNH 10 was placed in a fresh tube of TSB and incubated for 18 hours at 37°C. After incubation the culture was centrifuged at 10,000 x g for 15 minutes, the supernatant fluid discarded, and the packed cells resuspended in 10 ml of a commercially prepared nonpryogenic 0.85% saline (Normal Saline, Cutter Laboratories, Inc., Berkely, California). This washing was repeated three times with the cells finally suspended in 5 ml 0.85% saline. Colony counts per ml of the cell suspension were determined by standard plate count methods and correlated with a spectrophotometric (Spectronic 20, Bausch and Lomb, Inc., Rochester, New York) optical density at 650 nm. The calibrated suspension was then heat-killed at 60°C for one hour. Sterility of the heated suspension was tested in fluid thioglycollate medium (Difco Laboratories, Inc., Detroit, Michigan).

TABLE 1.--Experimental Protocol of Various Groups of Guinea Pigs Given Staphylococcal Materials or Saline Solution by Two Different Routes.

Groups of Guinea Pigs[a]	Composition of Material (mg) Administered[b]
Administered subcutaneously (SC)[c]	
A	Cells and SPA (0.25) and lipase (0.12)
B	Cells and SPA (0.25)
C	Cells and lipase (0.12)
D	Cells and SPA (0.25) and coagulase (0.60)
E	Cells and coagulase (0.60)
F	Saline
Administered intramammarily in both glands (IM)	
A	Cells and SPA (0.25) and lipase (0.12)
B	Cells and SPA (0.25)
C	Cells and lipase (0.12)
D	Cells and SPA (0.25) and coagulase (0.60)
E	Cells and coagulase (0.60)
F	Saline
Control group	
	No treatment

[a]Each group initially consisted of seven guinea pigs, primiparous, about 3 months old, and averaged 500 to 600 g.

[b]Cells, heat-killed, 1 x 10^8, were prepared from Staphylococcus aureus UNH 10 (Slanetz strain). SPA, lot X9221, staphylococcal polysaccharide antigen was supplied by Dr. Myron Fisher of Parke, Davis and Co. Both lipase and coagulase were purified staphylococcal enzymes.

[c]The materials administered to animals groups A through F were supplemented with equal volumes of Freund's adjuvant, incomplete.

2. Staphylocoagulase. This antigen was prepared by the method of Stutzenberger and San Clemente (1967). The stock antigen contained 90 ug of protein per ml with a titer of 1/256.

3. Staphylolipase. This antigen was prepared by the method of Renshaw and San Clemente (1967). The stock antigen contained 1880 ug of protein per ml and contained 1.5 enzyme units per ml.

4. Staphylococcal polysaccharide antigen (SPA). This antigen was supplied by Dr. M. W. Fisher of Parke-Davis Laboratories. The stock antigen contained 3770 ug per ml of purified polysaccharide.

5. Freund's incomplete adjuvant (Difco).

All vaccine preparations were finally tested for sterility by inoculation into a tube of fluid thioglycollate broth (Difco), incubated for 24 hours at 37°C, and observed for any growth.

Experimental Procedures

The experimental animals were divided into three groups as indicated in Table 1. The intramammary group (IM) and the subcutaneous (SC) group were further divided into subgroups (A through F) according to vaccine composition. Each of these vaccine subgroups and the Control Group initially consisted of seven animals. As the

experiment proceeded several of the animals in various subgroups were eliminated as a result of either death or naturally occurring mastitis.

Vaccination

SC Group.--The animals in this group received three injections of the indicated vaccine preparations at day one, day twenty and day forty of gestation (estimated). The suprascapular area of the right side of each animal was shaved close to the skin and swabbed vigorously with 70% isopropyl alcohol. The injections of 0.2 ml of the vaccine or saline placebo were given subcutaneously in the shaved area, with a 22 gauge x 1/2 inch hypodermic needle (Yale Hypodermic Needle, Beckton, Dickinson, and Co., Rutherford, New Jersey) and glass tuberculin syringe (B-D Yale Luer-Lok Hypodermic Syringe, Beckton, Dickinson, and Co., Rutherford, New Jersey).

IM Group.--The animals in this group received 0.5 ml of the respective vaccine preparations or saline placebo intramammarily in both the left and right glands, on day four post-partum. Previous to vaccination the animals were milked dry, and then shaved close to the skin from the xiphoid cartilage to the inguinal region of the abdomen, making sure to remove all hair near the teat.

For vaccination it was necessary to lightly anesthetize the animal. A large dessicating jar with cotton balls, saturated with ether vapor (Ethyl Ether for Anesthesia, Mallinkrodt Chemical Works, St. Louis, Missouri) was used for the anesthetizing chamber. An animal was introduced into the chamber and observed until it lost consciousness and did not respond to a sharp noise. It was then removed, placed on its back, and the shaved area vigorously scrubbed with 70% isopropyl alcohol. Although Gupta et al. (1970a) recommended the use of a disinfectant detergent followed by a water wash, we found the alcohol preferable as it was adequately disinfectant and dried without residue.

After the teats were dry, the leg on the side of the gland to be inoculated was gently pulled away from the body to force the gland up. The teat was rolled between the thumb and forefinger until a small drop of milk could be seen at the tip, indicating the location of the teat opening. A 27 gauge x 1/2 inch needle (B-D Yule Leur-Lok Hypodermic Needle; Beckton, Dickinson, and Co.; Rutherford, New Jersey) was inserted by a gently twisting motion the length of the teat cistern into the lactiferous sinus. After inoculation was made, the gland was gently massaged for a few moments to assure dispersion of the inoculum. The animals were then returned to their cages and isolated

from the nursing young for four hours to prevent loss of the antigen by the action of nursing.

Control Group.--This group received no treatment.

Milking of the Animals

This technique was performed according to Gupta et al. (1970b). To reduce the possibility of contamination, the animals were shaved, unless they had been previously, in the same manner as described for vaccination; similarly, disinfection was performed. After the alcohol had evaporated, the animal was held in one hand across the upper abdomen and lower chest, with the fingers of the hand securing the animal's forelegs. The back of the animal was supported against the researcher's body. In this position the animal felt relatively secure and did not need to be further restrained or anesthetized. The mother guinea pigs were isolated from their young six hours prior to milking, allowing the gland to fill with milk. Isolation was done with a small wire cage which could be placed in the larger cage, so the mother could still see, hear, and smell them, which aided in stimulation of milk; this technique was recommended by Nelson et al. (1951).

The milking procedure used a vacuum collection device described by Gupta et al. (1970a). The teat was inserted into the small glass end of the device and vacuum was gently supplied in a pulsating rhythm until the desired milk volume was obtained or the gland was milked

dry. At peak milking times the volume per gland would often exceed 1.0 ml. To standardize the volumes and attempt to reduce dilution of somatic and bacterial cells in the sample, no more than 1.0 ml of milk was taken for analysis. The time required for the milking was about 2 minutes. We found that gentle massage of the gland before and during milking stimulated milk "let down." Often a small milk clot had formed in the distal end of the teat, requiring manipulation to remove and allow rapid milk flow.

By day five post-partum the volumes which could be collected by the vacuum method were too small to process. At this time the gland was massaged until small amounts of milk would appear at the opening. The first few drops were wiped away with an alcohol dampened swab. As the next few drops appeared they were withdrawn with a sterile calibrated pipette. Measurements of volume could be taken directly and milk transferred to plates and slides for further analysis.

The milk samples from separate glands were assayed for volume, total somatic cell count, and bacterial identification and colony count. The left gland and right gland samples from all the animals within a particular subgroup were pooled after bacterial analysis at each day of milking and then stored at -28°C until assayed for antibody.

Determination of Lethal Dose

Trial A.--Six guinea pigs differing in age, stage of lactation, and lactation number were chosen to be tested. Dilutions of a suspension of S. aureus (prepared as outlined in the section concerning test challenge) were made at 10^2, 10^3, 10^5, and 10^7. Plate counts of the undiluted suspension serially diluted in a standard plating technique were made. The animals were inoculated intramammarily with 0.1 ml of the organism preparation in each gland. Observations were made at 12, 24, and 48 hours post-inoculation. Dilutions and suspensions were made in nonpyrogenic physiological saline.

Trial B.--This determination was performed on a similar group of animals with all the preparations identical, with the exception of the 10^2 dilution which was eliminated. The final suspension of organism, however, was into sterile 5% hog gastric mucin. Intramammary inoculations of 0.1 ml of the suspension were made.

Trial C.--Identical procedures were carried out in this determination, with the saline used as diluent, and the organism used was a diffuse colony variant of the Smith strain of S. aureus. Intramammary inoculations of 0.1 ml of the saline suspended organism were made.

Trial D.--The final run was performed as Trial A, with the exception of the exclusion of dilution 10^2. In this case a larger inoculum, 0.5 ml, was administered intramammarily.

Analysis of the Guinea Pig Milk

Bacteriologic Examination.--Depending upon the volume of the original collection, fresh milk samples of 0.04 or 0.025 ml were plated with a glass spreader on Trypticase Soy Agar (BBL). The inoculated plates were incubated at 37°C for 24 hours. After incubation, colonies, if any, were counted with a Quebec Colony Counter (American Optical Co., Buffalo, New York). If the number of colonies on the plate was at least 50 and one type predominated, or if a colony resembled S. aureus, a Gram stain was performed and further identification was done by subculturing the suspicious colony on the media listed below. If, however, the colony count was less than 50 and there was no predominant colony type, no further examination was carried out. The various test media included:

1. Trypticase Soy Agar (BBL) for general purpose subculturing
2. DNase Agar (Difco) for detection of diffusible deoxyribonuclease

3. Vogel and Johnson Agar (BBL) with 1% potassium tellurite for detection of mannitol fermentation and tellurite reduction

4. Brain Heart Infusion (Difco) for coagulase and catalase determinations

5. Trypticase Soy Agar (BBL) with 5% defibrinated fetal ox blood (Colorado Serum Company Laboratories, Denver, Colorado)

6. Mitis-Salivarius Agar (according to Difco formula) for presumptive identification of certain streptococci

7. Methylene Blue Skim Milk (according to Difco formula) for presumptive identification of Streptococcus faecalis

Total Somatic Cell Count.--The method for determining the total somatic cell count of milk samples was that outlined by Carter (1967):

1. Fresh milk samples were thoroughly mixed to ensure adequate dispersion of the cells.

2. An aliquot of 0.01 ml was removed and spread on a microscope slide, which was positioned over a template of 1 cm^2 squares and allowed to dry at room temperature.

3. After bacteriological examination if the milk production was below normal, a sample of 0.025 ml was taken directly from the teat. The gland was gently massaged and as drops of milk were brought to the opening they were drawn into a calibrated pipette. This sample was spread on a 1 cm^2 area on a microscope slide and allowed to air dry.

4. After drying, the slides were gently heat-fixed and transferred to Coplin jars containing Newman's stain prepared from the formula outlined in Carter (1967). They were stained for two minutes, removed and set at an angle, and allowed to dry. After thorough drying, the slides were gently dipped in three washes of distilled water until the stain ceased to run and allowed to air dry.

5. The slides were read under oil immersion. Forty fields on each of two smears from one sample were counted and the total cell count was calculated by the formulas in Carter (1967).

Antibody Analysis of Whey

Preparation of Whey.--The pooled guinea pig milk samples were centrifuged at 2,000 rpm (International Equipment Co., Boston, Massachusetts) for five minutes in order to separate the milk fat. The fat was drawn off and the sample recentrifuged to maximize removal of

the fat. The skimmed milk was then acidified with 1N HCl to pH 4.6 to precipitate the casein (McDowell et al., 1971). The precipitated casein was centrifuged as described above and the supernatant fluid, the whey, was collected. The whey was neutralized to pH 7.0 with 1N NaOH and then analyzed for agglutinin titer and presence of corresponding precipitating antibodies to coagulase, lipase, and SPA.

Agglutinin Determinations.--The method used was that of Boger et al. (1960); the antigen was Staphylococcus aureus UNH 10 strain grown as described for vaccine preparation. The cells were centrifuged for 15 min at 10,000 x g with an ss-34 head (Ivan Sorvall, Inc., Norwalk, Connecticut), the supernatant fluid discarded, and the cells resuspended in 1/5 M sodium phosphate buffer pH 7.4 in 0.85% NaCl. To ten wells of a disposable Microtiter "U" plate (Cooke Engineering Co., Alexandria, Virginia) was added 0.05 ml of the phosphate buffer. Serial two-fold dilutions of the whey were made with a 0.05 ml Microtiter Micro-diluter (Cooke Engineering Co., Alexandria, Virginia). Then 0.05 ml of the antigen suspension was placed in each well; the wells were gently agitated and placed in a moist 37°C incubator for 18 hours. After incubation the results were read by a Test Reading Mirror (Linbo Chemical Co., Inc., New Haven,

Connecticut) and interpreted as recommended by Boger et al. (1960).

Precipitin Determinations.--Purified agar, "Iona-gar" No. 2 (Colab Laboratories, Inc., Glenwood, Illinois) to give a concentration of 1% (w/v) was added to 1/15 M sodium phosphate buffer pH 7.4 in 0.85% NaCl and the mixture brought to boiling to melt the agar. Ouchterlony slides were prepared by dispensing 2 ml of the molten agar onto frosted end microscope slides 2.5 cm x 7.5 cm. After the agar solidified the slides were placed in a sealed, humid chamber at 4°C until used.

Wells were cut into the gel with a cutter. Antiserum, approximately 5 ul, was placed in the outer well, and purified lipase, coagulase, or SPA was placed in the center well. A known antiserum positive for the particular antigen being tested was placed in the well adjacent to the unknown serum to observe identity between precipitation lines formed by the two antisera and the antigen. The wells were filled three times to assure adequate concentrations of reactants. The charged system was then placed in a sealed, humid chamber and incubated at room temperature for two weeks, and periodically observed.

Antibody Analysis of Serum.--Serum samples from the animals of each vaccine subgroup were pooled. These pooled

samples were inactivated at 56°C for 30 min. Procedures for the antibody analysis of the serum samples were identical to those performed for the whey analysis.

Analysis of Mammary Gland Tissue

Bacteriological Examination.--Immediately after the death of the animal, the mammary glands were removed and samples of tissue taken aseptically for both nucleic acid analysis and for bacterial culture. Trypticase Soy Agar (BBL) plates inoculated by pressing slices of tissue onto the surface were incubated for 24 hours at 37°C and subsequently observed for bacterial growth. Suspicious colonies were further studied as outlined in the section on bacteriological examination of milk.

Nucleic Acid Analysis.--

DNA determination: Immediately after death the whole mammary glands of the animal were removed and trimmed of any extra-parenchymous tissue, washed in 0.25 M sucrose, and weighed. A representative sample of glandular tissue was taken (approximately 1 g), weighed, placed in 0.25 M sucrose, and frozen at -28°C. These frozen samples were stored until analyzed.

For analysis, the glands were thawed and washed again in 0.25 M sucrose. Distilled water (20 ml H_2O/g of tissue) was added to the tissue and the mixture

homogenized in a Waring Blender at high speed for two minutes at 4°C. This homogenate then represented 0.05g tissue/ml of the homogenate. Four ml of this homogenate were simultaneously processed. The outline of the nucleic acid extraction, taken from Tucker (1964), is seen in Figure 1.

Supernatant fluids 4 through 6 were pooled and analyzed for DNA content at 268 nm on a Beckman DB-G Scanning Spectrophotometer (Beckman Instruments, Inc., Fullerton, California). A standard curve was prepared by dissolving measured amounts of highly polymerized calf thymus DNA (Sigma Chemical Co., St. Louis, Missouri) in 5% perchloric acid.

RNA determination: Supernatant fluids 1 through 3 were pooled and analyzed for RNA content by the colorimetric method of Mejbaum (1939).[9] A standard curve was prepared by dissolving measured amounts of highly polymerized yeast RNA (Calbiochem, Los Angeles, California) in 5% perchloric acid. Tests were read on a Spectronic 20 (Bausch and Lomb, Inc., Rochester, New York) at 660 nm.

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[9]Technique outlined by W. C. Schneider in S. P. Colowick and N. O. Kaplan, eds. 1957. Methods in Enzymology, Vol. III. Academic Press, New York. pp. 680-684.

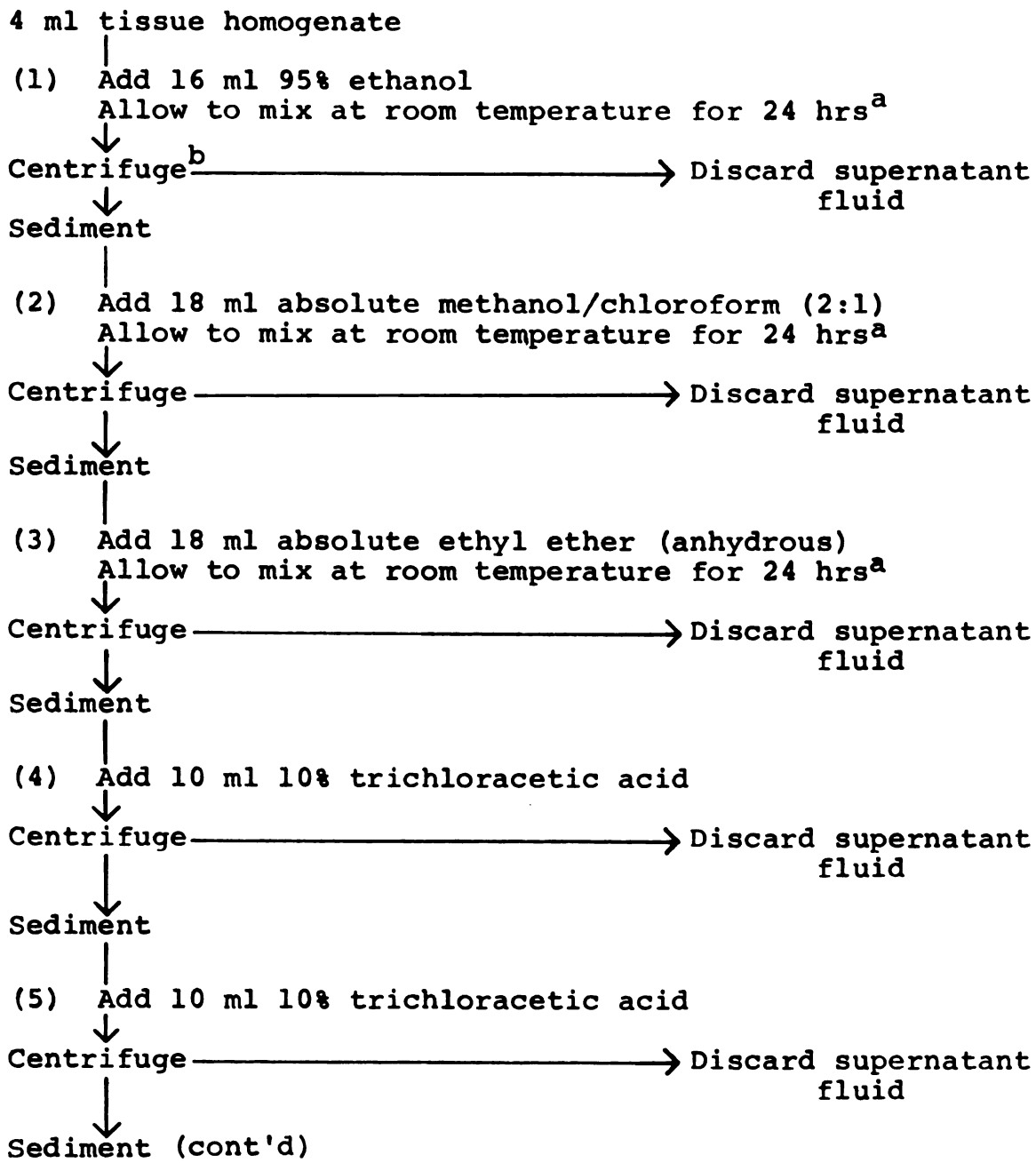

Figure 1.--Flow sheet for the extraction of nucleic acids from mammary gland tissue.

[a]During this mix time the tubes were gently shaken on a reciprocating shaker.

[b]All centrifuging was done in a Sorval table top centrifuge with a ss-34 head at 10,000 x g for 15 min at 4°C.

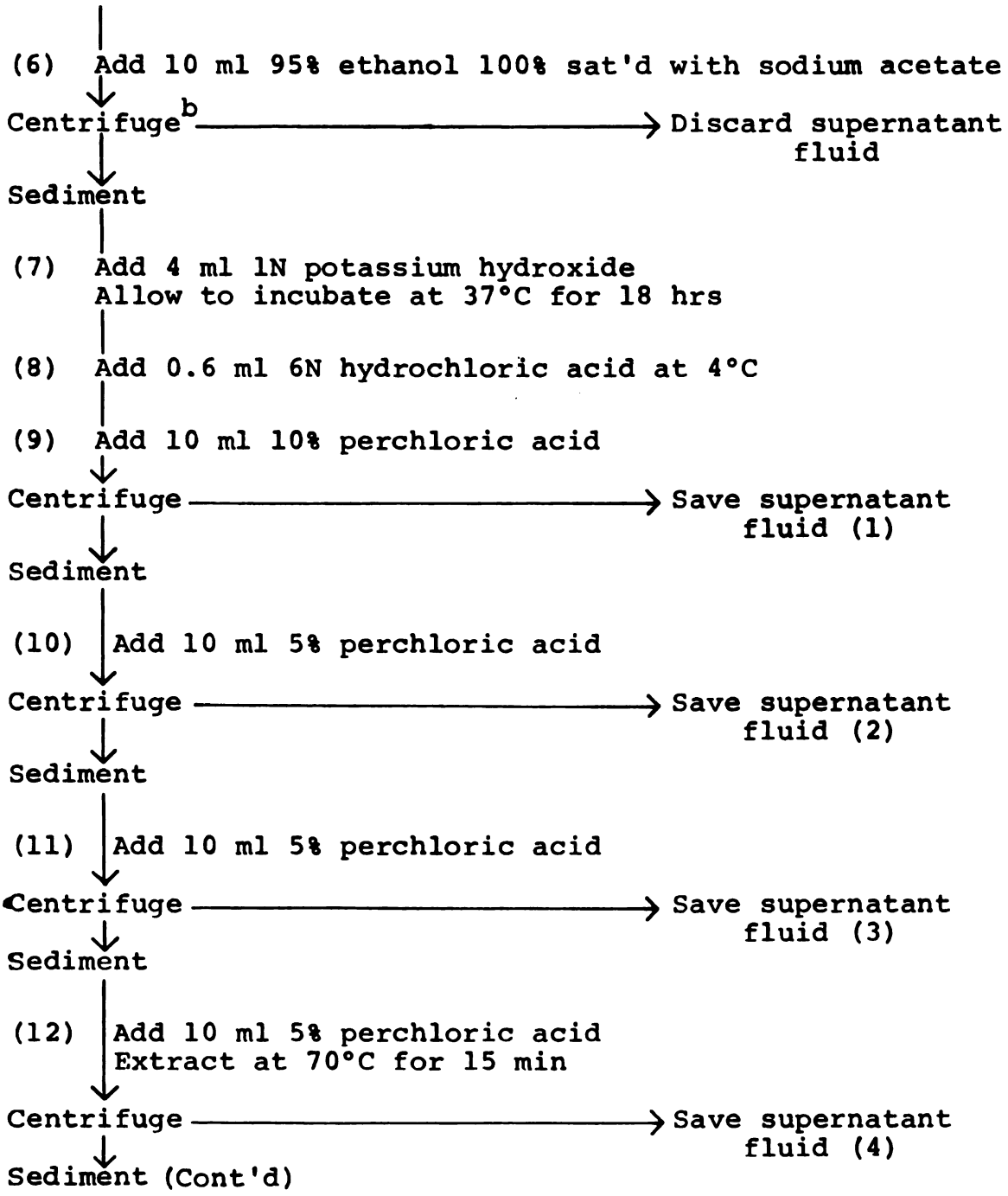

Figure 1.--Continued.

[b]All centrifuging was done in a Sorval table top centrifuge with a ss-34 head at 10,000 x g for 15 min at 4°C.

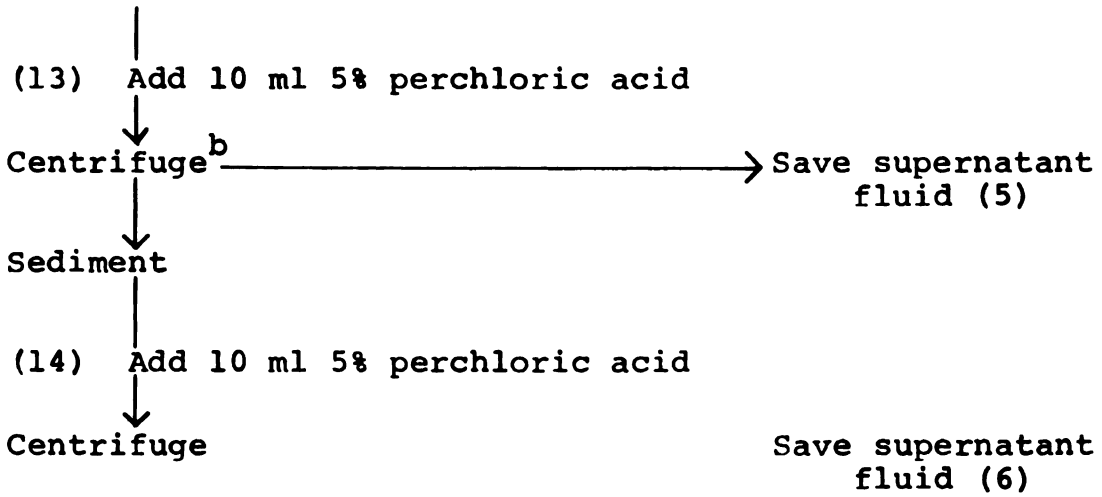

Figure 1.--Continued.

[b]All centrifuging was done in a Sorval table top centrifuge with a ss-34 head at 10,000 x g for 15 min at 4°C.

RESULTS

Experimental Mastitis in Guinea Pigs

The initial attempt to determine the LD_{100} of the staphylococci, Trial A, failed (Table 2). Injections of as many as 7.0×10^8 colony-forming units of S. aureus UNH 10 suspended in saline did not induce an acute mastitis. Instead, a chronic infection was established, indicated by elevation of total somatic cell counts in the milk, an overall reduction of milk production in the infected gland, and shedding of staphylococci in the milk. This condition could be established with as few as 70 organisms. When the organisms were suspended in 5% hog gastric mucin, Trial B, or Smith diffuse colony variant staphylococci substituted for the UNH 10 strain, Trial C, a similar chronic condition resulted.

When the inoculum volume was increased from 0.1 to 0.5 ml, Trial D, using the UNH 10 strain, positive reactions were seen. By means of an inoculum of 7.0×10^7 organisms, at least one of the inoculated glands became greatly swollen, and the teat area turned a deep purple color. The animal became immobile showing great

TABLE 2.--Determination of LD_{100} of Staphylococcal aureus When Instilled into the Lactiferous Sinus of the Mammary Gland via the Sinus Papillaris.

Animals	Dilution	c.f.u. of S. aureus	Response
Trial A (saline, total vol. 0.1 ml, Slanetz strain UNH 10)			
1	0	7.0×10^8	
2	10^{-2}	7.0×10^6	
3	10^{-3}	7.0×10^5	All
4	10^{-5}	7.0×10^3	Survived
5	10^{-7}	70	
6	-	0	
Trial B (5% hog gastric mucin, total vol. 0.1 ml, Slanetz strain UNH 10)			
1	0	1.4×10^9	
2	10^{-3}	1.4×10^6	
3	10^{-5}	1.4×10^4	All
4	10^{-7}	1.4×10^2	Survived
5	-	0	
Trial C (saline, total vol. 0.1 ml, Smith diffuse strain)			
1	0	1.3×10^9	
2	10^{-3}	1.3×10^6	
3	10^{-5}	1.3×10^4	All
4	10^{-7}	1.3×10^2	Survived
5	-	0	
Trial D (saline, total vol. 0.5 ml, Slanetz strain UNH 10)			
1	0	7.0×10^9	Died
2	10^{-2}	7.0×10^7	Died
3	10^{-4}	7.0×10^5	
4	10^{-6}	7.0×10^3	All
5	0	0	Survived

pain with death ensuing within 24 hours post-inoculation. When the number of organisms was increased to 7.0×10^9, both glands responded as above followed by death of the animal.

Milk Volumes

The milking technique described by Gupta et al. (1970a) proved to be an easy and rapid method for milking the animals. The animals could easily be controlled with one hand and apparently suffered little discomfort during the procedure. Figure 2 represents the difference observed between the mean values of the milk volumes from the right glands, which received the bacterial challenge, and the left glands, given the saline placebo, for the SC Group. A daily random fluctuation in the volume from all the glands was seen pre-challenge. After challenge, milk production in both glands dropped to low levels, but the control gland not receiving any organisms usually maintained a slightly higher output than the gland receiving the organisms. By the final two or three milkings both glands were secreting milk at very low volumes, less than 0.1 ml, and the difference between the glands was not significant; in some animals there was no difference between the output of the two glands.

Similar data for the IM Group can be seen in Figure 3. The random fluctuation in milk production

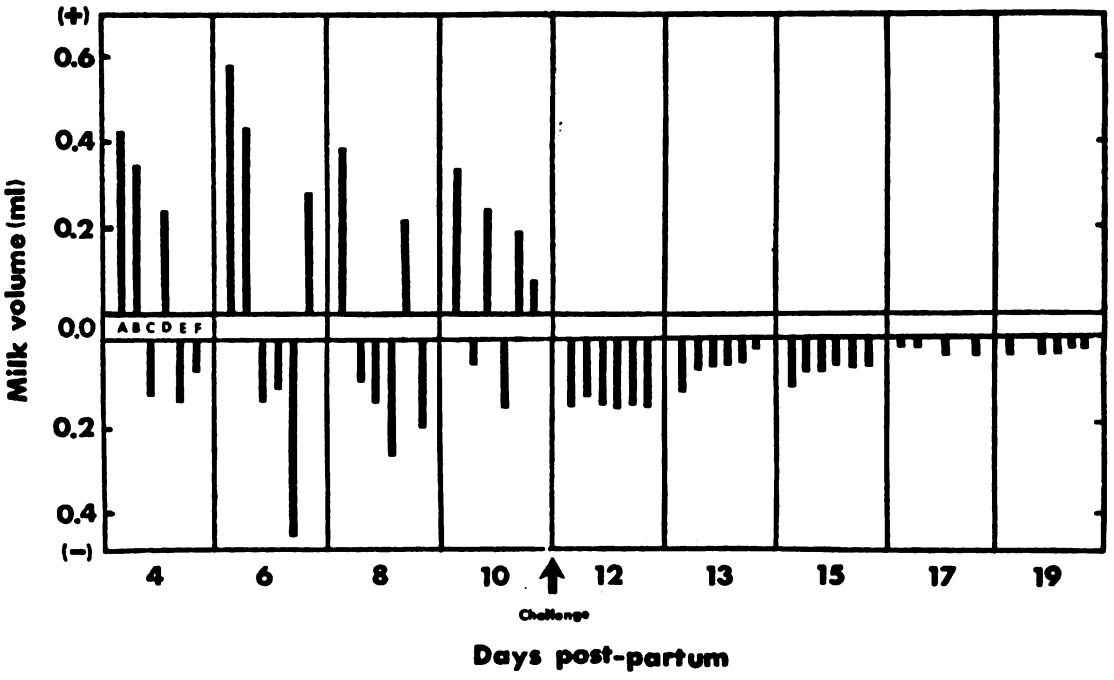

Figure 2.--SC Group: The difference between the right (challenged) and the left (nonchallenged) mammary glands from animals of each vaccine subgroup in terms of the mean volumes of milk collected at two-day intervals. This group of animals was vaccinated subcutaneously 21 days prior to challenge, 14 days ante-partum.

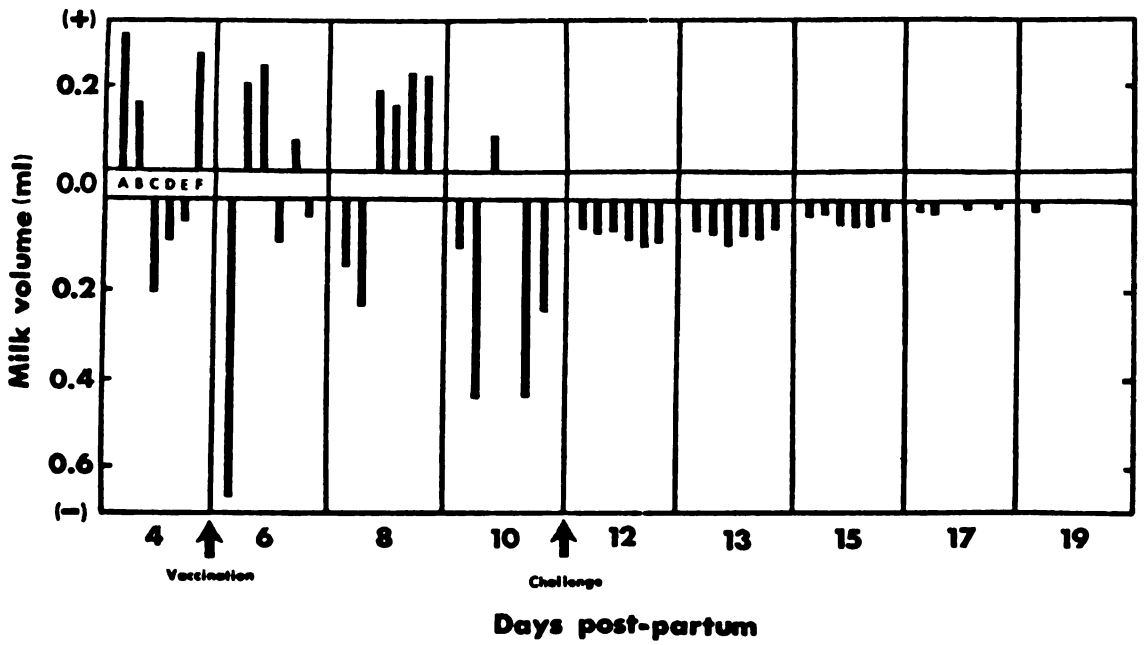

Figure 3.--IM Group: The difference between the right (challenged) and the left (nonchallenged) mammary glands from animals of each vaccine subgroup in terms of the mean volumes of milk collected at two-day intervals.

pre-challenge is also found in these animals. The intramammary challenge given on day 5 post-partum does not significantly affect the pattern of difference between the glands pre-challenge. Response to challenge is identical to that seen in the SC Group.

The milk production data for each vaccine subgroup of both the SC and IM Groups are represented in Figures 12 through 17 and Figures 18 through 23, respectively. The data for the Control Group are seen in Figure 24.

Total Somatic Cell Count

As with the milk output, the pre-challenge values of somatic cell count per ml of milk showed random fluctuations for the challenged and nonchallenged glands of each vaccine subgroup from the SC Group and IM Group. Although this pattern was maintained in the SC Group up to the day of challenge, a reaction to intramammary vaccination in the IM Group can be seen in Figures 18 through 23 by a rise in the number of somatic cells in milk samples collected following this vaccination.

At challenge the difference between the challenged and nonchallenged glands increased greatly, which continued throughout the milk collection. This difference was seen in both the SC and IM Groups in Figures 4 and 5.

The total somatic cell counts for milk samples from each vaccine subgroup of both the SC and IM Groups are

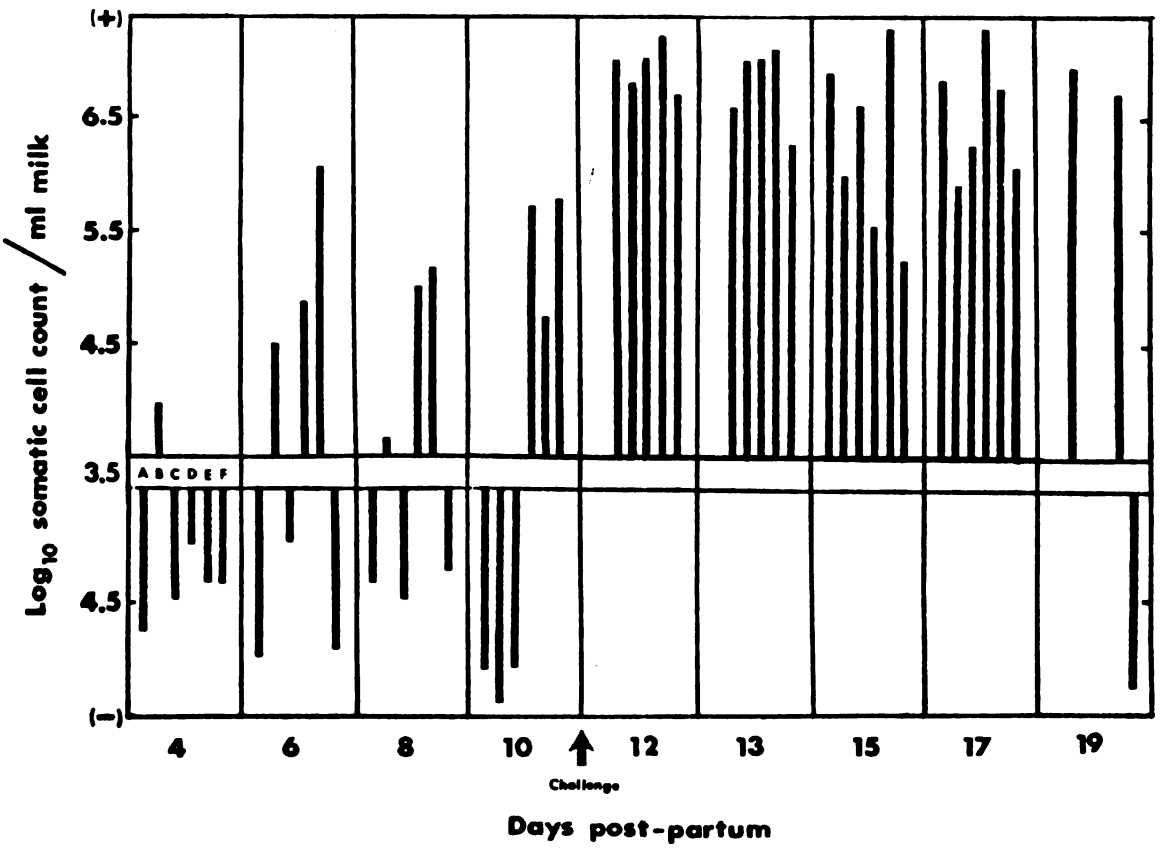

Figure 4.--SC Group: The difference between the right (challenged) and the left (nonchallenged) mammary glands from animals of each vaccine subgroup in terms of the $\log_{10}$ of the mean values of the total somatic cell count per ml of milk collected at two-day intervals. This group of animals was vaccinated subcutaneously 21 days prior to challenge, 14 days ante-partum.

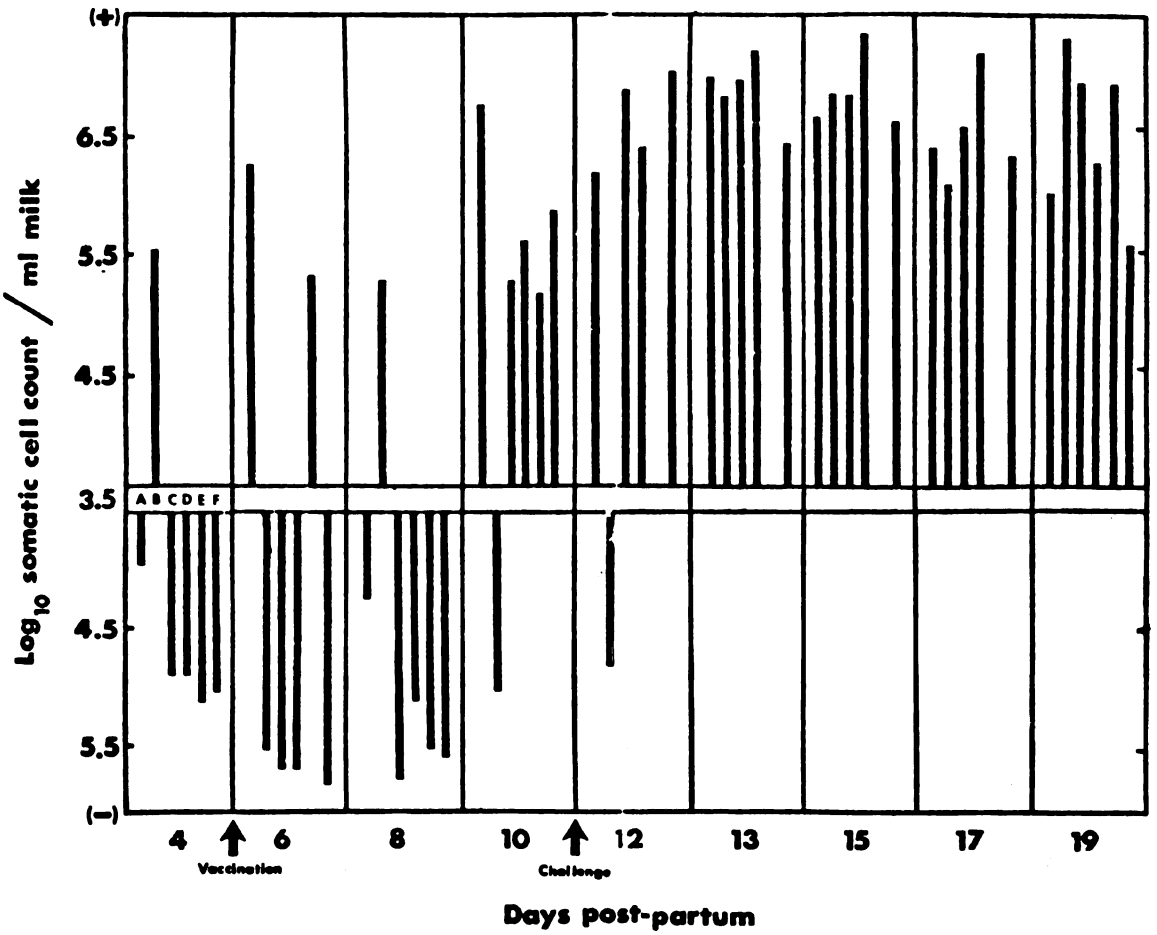

Figure 5.--IM Group: The difference between the right (challenged) and the left (nonchallenged) mammary glands from animals of each vaccine subgroup in terms of the $\log_{10}$ of the mean values of the total somatic cell count per ml of milk collected at two-day intervals.

represented in Figures 12 through 17 and Figures 18 through 23, respectively. The data for the Control Group are seen in Figure 24.

Bacterial Colony Count

Before challenge there were few bacteria isolated from the milk samples. When organisms were isolated, however, Micrococcus was the predominant genus. In addition, occasional Gram negative enteric types were found, as well as several unidentified Gram positive rods, which were very few in number, and often alpha-hemolytic streptococci. No Staphylococcus aureus was isolated from any of the milk samples during the pre-challenge period. Occassionally a transient bacterial infection of the mammary gland was found, accompanied by elevated somatic cell and bacterial colony-forming unit counts.

In one animal a Bacillus sp. was found, but it disappeared by the next milking. Often these transient infections were associated with isolation of large numbers of alpha-hemolytic streptococci in the milk sample. When this condition was noted and the inflammation did not subside within one milking period, the animal was removed from the experimental group.

Post-challenge all the glands receiving injections of S. aureus began to shed large numbers of the organism (Figures 12 through 17, 18 through 23, 6 and 7). Although

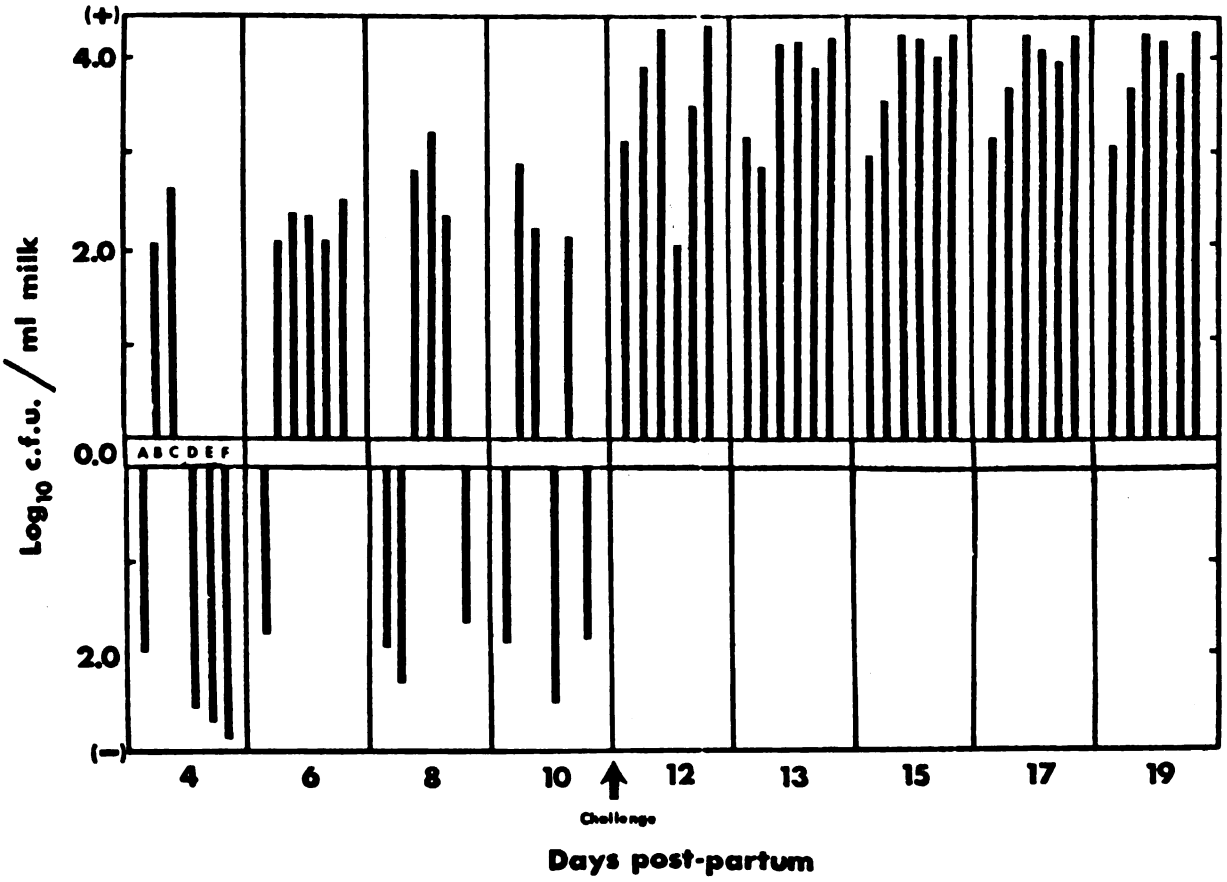

Figure 6.--SC Group: The difference between the right (challenged) and left (nonchallenged) mammary glands from animals of each vaccine subgroup in terms of the $\log_{10}$ of the mean values of the colony forming units (c.f.u.) per ml of milk collected at two-day intervals. This group of animals was vaccinated subcutaneously 21 days prior to challenge, 14 days ante-partum.

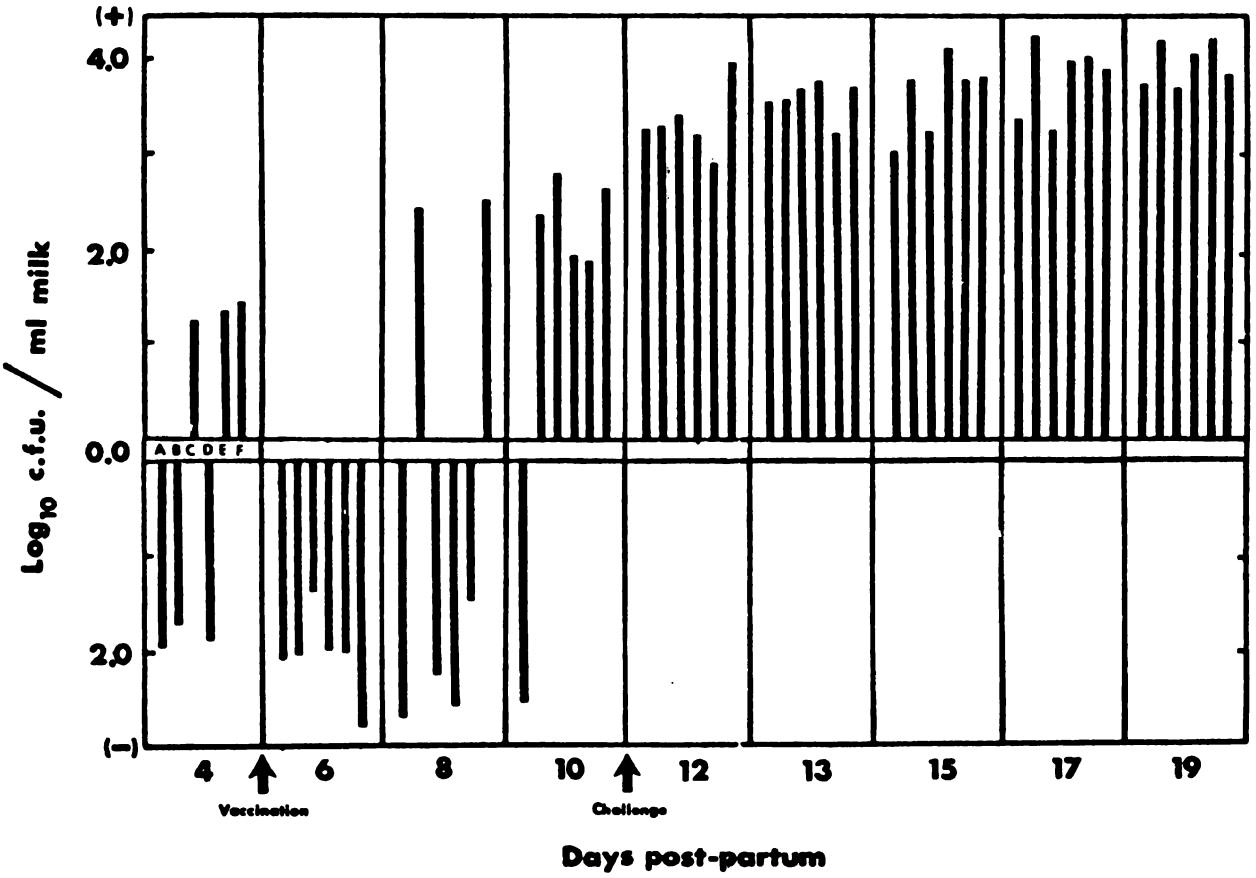

Figure 7.--IM Group: The difference between the right (challenged) and the left (nonchallenged) mammary glands from animals of each vaccine subgroup in terms of the $\log_{10}$ of the mean values of the colony forming units (c.f.u.) per ml of milk collected at two-day intervals.

the predominant organism in the milk samples was S. aureus, alpha-hemolytic streptococci were also occasionally found.

The colony-forming units for milk samples from each vaccine subgroup of both the SC and IM Groups are represented in Figures 12 through 17 and Figures 18 through 23, respectively. The data for the Control Group are seen in Figure 24.

Antibody Analysis of Whey

Agglutinin titers recorded for whey samples are seen in Table 3. The titer of samples from the subgroups SC-1, SC-D, and IM-A peaked at milk collection 2 and then returned to or dropped below the titer levels of milk collection 1 by milk collection 4. Samples from subgroup IM-B peaked at collection 2 and remained at this peak level to collection 4. Titers of the whey samples of the other subgroups fluctuated at random. With respect to its ability to raise the whey agglutinin titer, no correlation could be observed between the particular vaccination preparation and the route of administration. Likewise, no route of administration appeared to be more efficacious than the other in stimulating agglutinin titers in the whey.

A correlation between the antigens infused intramammarily and those given subcutaneously and the appearance of precipitating antibody in the whey is seen in Table 4. All the animals responded to staphylolipase and

TABLE 3.--Determination of the Agglutinin Titers of Both Pooled Whey and Pooled Serum Samples from the Animals of Each Vaccine Subgroup.[a]

	Reciprocal of Titer				
	Milk Collection[b]				Serum
	1	2	3	4	
SC Group					
A	8	8	16	8	32
B	4	4	4	4	32
C	8	16	4	4	ND[c]
D	8	16	8	8	ND
E	8	8	8	8	ND
F	4	4	16	8	64
IM Group					
A	8	16	16	8	ND
B	8	16	16	16	ND
C	8	4	4	4	ND
D	8	4	8	4	32
E	16	8	8	8	16
F	8	4	8	8	32
Control Group	4	4	4	8	32

[a]Antigen used was formalin killed cells of S. aureus UNH 10.

[b]Milk collections used included only 1 to 4, as beyond this time the volumes of milk were too small for whey preparation.

[c]Serum samples were not available for these subgroups and thus titers could not be determined (ND).

TABLE 4.--Determination of the Presence of Precipitins in Both Pooled Whey and Pooled Serum Samples from the Animals of Each Vaccine Subgroup.

	Reaction[a]					
	Whey[b]			Serum		
	Lipase	Coagulase	SPA	Lipase	Coagulase	SPA
SC Group						
A	+	-	-	+	-	-
B	-	-	-	+	±	-
C	+	-	-	ND[c]	ND	ND
D	-	+	-	ND	ND	ND
E	-	+	-	ND	ND	ND
F	-	-	-	-	±	-
IM Group						
A	+	-	-	ND	ND	ND
B	-	-	-	ND	ND	ND
C	+	-	-	ND	ND	ND
D	-	+	-	±	+	-
E	-	+	-	±	+	-
F	-	-	-	±	±	-
Control Group	-	-	-	-	-	-

[a]Positive reaction was seen as a single line of precipitation between antigen and antibody wells.

[b]These samples were all taken post-vaccination and pre-challenge.

[c]Serum samples were not available for these subgroups and thus titers could not be determined (ND).

staphylocoagulase by production of specific precipitating antibodies to these antigens. All the tests run with SPA were negative. In the controls, the internal controls SC-F and IM-F, and the Control Group animals, no corresponding antibodies were found to the specific staphylococcal antigens tested.

Antibody Analysis of Serum

Of those samples tested, serum agglutinin titers were consistently higher than those titers observed in the whey samples (Table 3). Vaccination did not appear to greatly enhance the titer over the control groups, as the highest titer observed was from the control group SC-F, and the lowest of vaccine subgroup IM-E. The nontreated controls had serum titers at least as high as most of the vaccine subgroups tested. The sera were consistently positive for the particular antigen to which they had been exposed (Table 4), and samples from three vaccine subgroups were weakly positive for the second staphylococcal antigen, either lipase or coagulase, to which they had not been experimentally exposed. All the sera were negative for SPA precipitating antibodies.

Bacteriological Analysis of Mammary Gland Tissue

Staphylococcus aureus was isolated from 80% of the excised and cultured right glands of all vaccine subgroups,

including the SC-F and IM-F subgroups, but not from any of the left glands. Both the glands of the Control Group were consistently negative. Those glands of the vaccine subgroups which were culturally negative, or S. aureus negative, did not fall in any particular group and this was an apparently random phenomenon. During the course of the experiment, three apparently random deaths from bacterial mastitis occurred, and at necropsy S. aureus was isolated from the glands.

Mammary Gland Weight

The mammary glands which had been challenged with the bacteria showed a consistently, although slightly, lower weight than the nonchallenged glands. Figure 8 represents the difference in weight between the challenged right glands and the nonchallenged left glands for each vaccine subgroup. The mean weights for nontreated glands of the Control Group at day 19 post-partum were determined from values in the Control Group and compared to the mean values of the left (nonchallenged) glands of both the SC and IM groups. The difference between these values is shown in Figure 11. The IM Group with the greater difference in mean weight was shown to have lower mean weight than the SC Group.

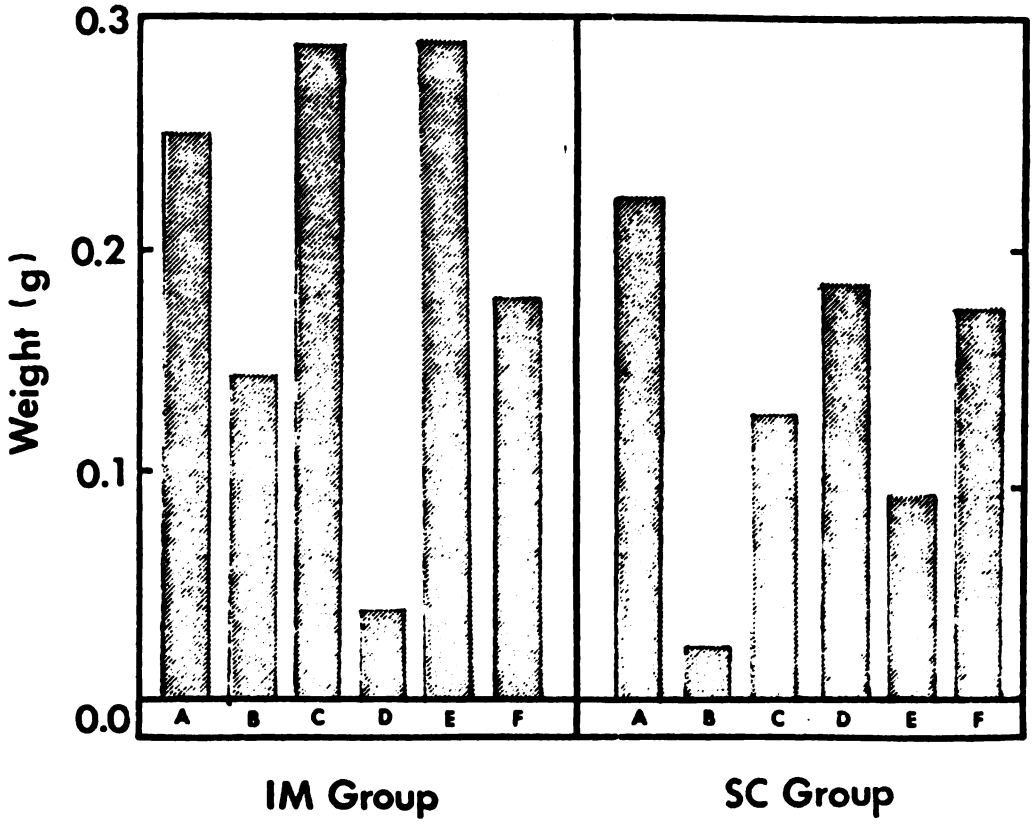

Figure 8.--Differences between the right (challenged) and left (nonchallenged) mammary glands of the animals in each vaccine subgroup expressed in terms of the mean weights of the glands determined 19 days postpartum.

Nucleic Acid Levels of Mammary Gland Tissue

DNA Levels

Consistently higher DNA concentrations per gram of mammary gland tissue (Table 5) were found in those glands which had been challenged with the staphylococci than in tissue from the glands which received the saline placebo. Figure 9 represents the differences in mean DNA concentrations per gram of tissue between the left and right glands for each vaccine subgroup. Although the Control Group, which was not treated, did not show any significant difference between the DNA concentrations of the left and right glands, the mean value for this group did differ from the mean values of the nonchallenged glands from both the SC and IM Groups (Figure 11). The left gland mean value from the IM Group was much higher than for either the Control Group or SC Group values.

RNA Levels

RNA levels of the challenged glands were lower than the RNA levels of the nonchallenged glands for all vaccine subgroups (Table 6). In a comparison of the mean values of the nonchallenged glands of the IM and SC Groups with the mean value for the Control Group (Figure 11), the Control Group value was higher in both cases, with the IM value being slightly less than the SC Group.

TABLE 5.--Mean Concentration of DNA per Gram of Mammary Gland Tissue for Animals of Each Vaccine Subgroup Determined 19 Days Post-partum.

	Milligrams of DNA/gram of Mammary Gland Tissue ± S.E.	
	Left Gland	Right Gland
SC Group		
A	3.89 ± 0.51	4.51 ± 1.49
B	3.91 ± 0.19	5.33 ± 0.92
C	5.19 ± 1.06	6.73 ± 1.23
D	4.16 ± 0.09	4.48 ± 0.24
E	4.36 ± 0.48	5.26 ± 0.57
F	4.37 ± 0.40	6.54 ± 0.25
IM Group		
A	4.45 ± 1.03	5.26 ± 0.17
B	4.83 ± 0.84	5.70 ± 1.48
C	4.75 ± 0.20	6.57 ± 0.62
D	4.35 ± 0.12	6.57 ± 0.65
E	5.23 ± 1.29	6.39 ± 1.97
F	5.19 ± 0.95	6.22 ± 0.87
Control Group	4.77 ± 0.21	4.98 ± 0.55

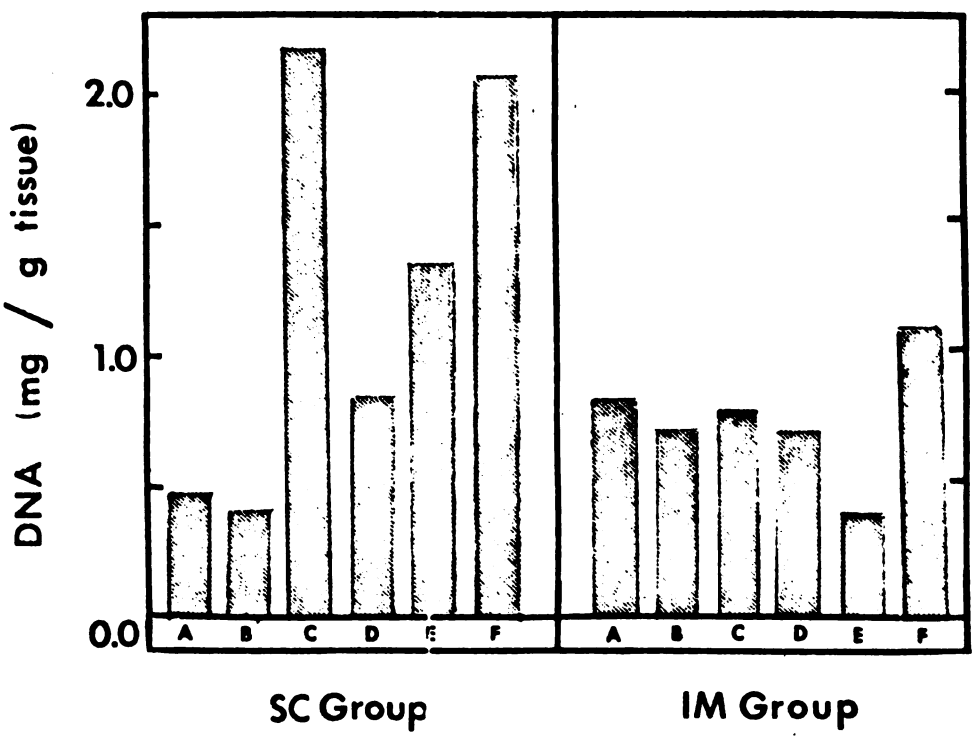

Figure 9.--Differences between the right (challenged) and the left (nonchallenged) mammary glands of the animals in each vaccine subgroup expressed in terms of the mean values for mg of DNA per gram of mammary gland tissue determined 19 days post-partum.

TABLE 6.--Mean Concentration of RNA per Gram of Mammary Gland Tissue for Animals of Each Vaccine Subgroup Determined 19 Days Post-partum.

	Milligrams of RNA/gram of Mammary Gland Tissue ± S.E.	
	Left Gland	Right Gland
SC Group		
A	10.43 ± 0.70	6.02 ± 0.66
B	12.45 ± 2.97	4.25 ± 2.89
C	14.88 ± 4.57	8.27 ± 3.24
D	14.46 ± 4.33	12.11 ± 3.25
E	14.29 ± 1.65	6.19 ± 0.81
F	11.98 ± 3.34	7.57 ± 2.30
IM Group		
A	11.56 ± 2.11	5.09 ± 2.64
B	13.46 ± 1.08	8.70 ± 2.40
C	15.26 ± 2.85	11.60 ± 3.95
D	11.85 ± 4.33	8.92 ± 2.81
E	11.68 ± 3.53	10.28 ± 1.88
F	11.59 ± 3.20	8.40 ± 1.70
Control Group	15.74 ± 3.69	16.21 ± 2.21

RNA/DNA Ratios

For all vaccine subgroups the RNA/DNA ratios (Table 7) for the challenged glands were lower than ratios calculated for the nonchallenged glands. The difference between the mean values are seen in Figure 10. The mean RNA/DNA ratio of the Control Group was compared with the mean values of the left glands of the SC and IM Groups. Figure 11 represents the difference, indicating the higher values for the Control Group.

TABLE 7.--Mean Values for RNA/DNA Ratios of Mammary Gland Tissue for Animals of Each Vaccine Subgroup Determined 19 Days Post-partum.

	RNA/DNA ± S.E.	
	Left Gland	Right Gland
SC Group		
A	2.22 ± 0.06	1.45 ± 0.65
B	2.38 ± 0.97	1.22 ± 0.64
C	2.45 ± 1.40	1.29 ± 0.62
D	2.67 ± 0.63	1.66 ± 0.62
E	2.79 ± 0.55	1.34 ± 0.39
F	2.30 ± 1.17	1.31 ± 0.07
IM Group		
A	2.54 ± 0.71	1.34 ± 0.68
B	2.56 ± .150	1.58 ± 0.60
C	2.46 ± 0.92	2.60 ± 0.66
D	2.39 ± 0.78	1.62 ± 0.77
E	2.39 ± 1.13	1.71 ± 0.25
F	2.31 ± 0.82	1.22 ± 0.46
Control Group	3.47 ± 0.39	3.63 ± 0.65

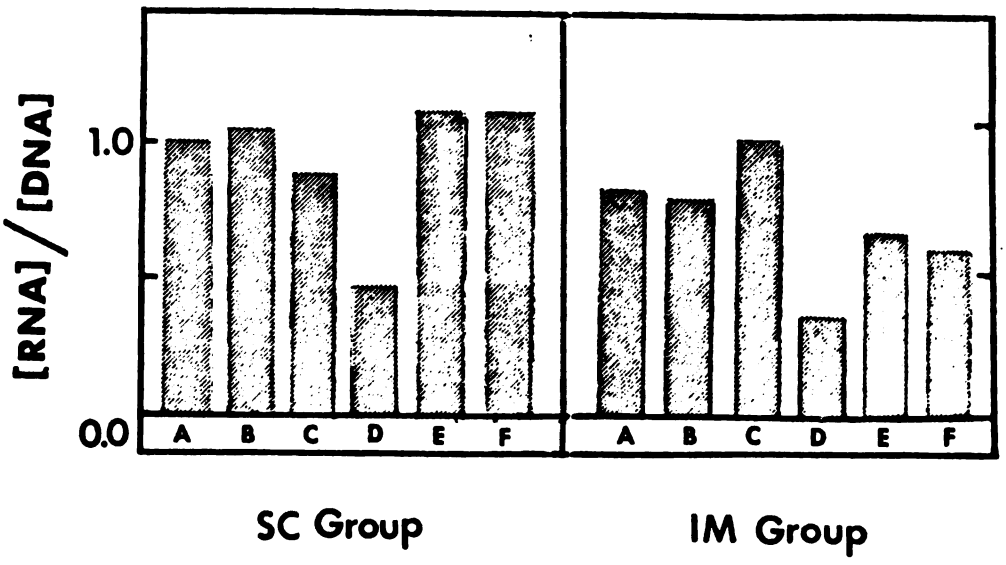

Figure 10.--Differences between the right (challenged) and the left (nonchallenged) mammary glands of the animals in each vaccine subgroup expressed in terms of the mean values of the RNA/DNA ratios of mammary gland tissue determined 19 days post-partum.

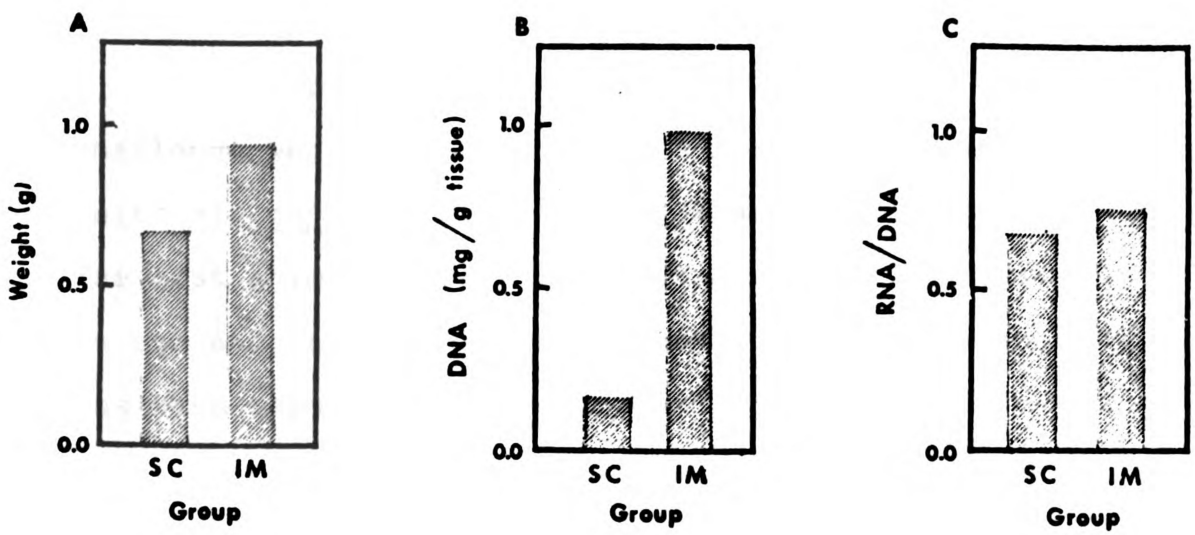

Figure 11.--Differences between the left (non-challenged) mammary glands from animals of both the SC and IM Groups, and the mammary glands from animals of the Control Group expressed in terms of (A) the mean values for gland weight, (B) mg of DNA per gram of gland tissue, and (C) the gland tissue RNA/DNA ratios. All values determined on day 19 post-partum.

DISCUSSION

In preliminary tests the LD_{100} for intramammary challenge was determined to be 2×10^9 colony forming units of Staphylococcus aureus Slanetz strain UNH 10. The test animals were a heterogenous group with respect to age and lactation number; many were multiparous. It has been reported (Brown, 1962a; Rogers, 1966) that as animals which are susceptible to staphylococcal infection become older and encounter numerous minor episodes, they would be better able to resist such an infection, because the specific humoral resistance factors increase with age. Therefore, we assumed that a challenge dose which killed the older animals should also kill the younger, primiparous animals.

In our primiparous animals, however, instead of a peracute infection in response to challenge, a subacute, chronic type of infection was established. The animals showed no outward signs of malaise and in all of the animals, milk production continued, although at a greatly reduced level. S. aureus was isolated from the milk throughout lactation and, at necropsy, from the glands

of most of the animals. A similar response was seen in the lethal dose test animals when they were given a sublethal challenge. At this sublethal dose, the multiparous females were able to maintain a higher volume of milk output than the challenged primiparous ones. Thus, the reason for the failure of the predetermined LD_{100} may be the heterogeneity of the test group as mentioned above.

In a staphylococcal model system, the advisability of using LD_{100}, or a similarly overwhelming dose, is questionable. It has been proposed (Slanetz et al., 1963; MacLeod et al., 1963) that an infection resulting from such a dose is not an accurate parallel to what happens during initiation of natural infection. Under these circumstances death or pathological changes may not be due to infection but to toxin production by the large numbers of bacteria injected (Fisher, 1962). Thus, a more suitable approach would be to use a minimal infective dose.

Because staphylococcal immunity may be considered an antitoxic (or anti-extracellular product) or antibacterial immunity, we considered both approaches in our vaccine preparation. The staphylococcal capsular antigens can induce formation of capsule specific antibodies which, when injected into mice, will protect them from intraperitoneal challenge with the highly mouse virulent Smith diffuse strain of S. aureus. Cows, in response to intramuscular injections of SPA, form similar anticapsular

antibodies which can passively protect mice from intraperitoneal challenge with the Smith diffuse strain (San Clemente et al., 1966; San Clemente, 1970). These encapsulated strains appear to be widely distributed, as indicated by anticapsular antibodies observed in normal human serum (Rogers and Melly, 1962), and by Yoshida et al. (1970), who isolated 4.2% encapsulated strains of staphylococci from human clinical sources. Therefore, we included the capsule specific antigen as one vaccine constituent.

Heat-killed whole cells were included in the vaccine to add a high number of staphylococcal somatic determinants. Cohen et al. (1958) demonstrated that mechanical or chemical manipulation of the bacterial cells can result in the loss of a number of antigenic determinants, thus our whole cells were simply heat-killed. Ekstedt (1963a) and Yoshida et al. (1970) have reported that mouse protection from challenge with the Smith diffuse strain could be induced by vaccination with heat-killed cells. Addition of these whole cells might also serve to intensify the local cellular activity, which in turn increased humoral response. This combination of events is analogous to those with the mycobacterial cells in Freund's complete adjuvant.

Coagulase has long been implicated in the pathogenicity of the staphylococcal infection, but definite proof of its precise role has not been established. As

purified preparations have been immunogenic (Tager and Hales, 1948; Duthie and Haughton, 1958) and protective (Harrison, 1964; Lominski et al., 1962), coagulase was tested in our model system as a probable factor. Although discounted as a virulence factor by many authors (Elek, 1959; Cameron, 1963), the presence of lipase in pathogenic strains of S. aureus has been correlated with coagulase activity (Elek, 1959; Gillespie and Adler, 1952). It is immunogenic in cows and rabbits (San Clemente et al., 1966; San Clemente, 1970), has been associated with lesions (Burns and Holtman, 1960), and is very active in staphylococci grown in milk (Vadehra and Harmon, 1964). However, because its protective capacity had not been extensively studied, it was included in our preparations.

Our vaccine preparations, when administered subcutaneously, did not result in any outward systemic reactions. Locally, however, those vaccines containing coagulase gave rise to an inflammation after twenty-four hours, followed by local necrosis and formation of a cutaneous lesion, which eventually healed. Similarly, intramammary vaccination did not result in a systemic reaction, but a local response by leukocyte infiltration into the gland was measurable with all infusions.

The mammary glands of several animals are known to be sensitive to irritants, and will respond to infusions of these irritants with an infiltration of leukocytes.

Many researchers (Blobel and Katsube, 1964; Schalm et al., 1964a,b; Reiter and Oram, 1967; Derbyshire and Berman, 1968) have induced leukocytosis in milk by infusion of saline, and Gupta et al. (1971a) noted a similar response in guinea pigs. A pre-existing elevated milk leukocyte count has been shown to reduce the infectivity of a dose of viable organisms introduced into the mammary gland (Blobel and Katsube, 1964; Schalm et al., 1964a,b; Slanetz et al., 1963). In the case of low numbers of organisms as a challenge, the pre-existing leukocytes can protect the gland from infection by eliminating the bacteria (Newbould and Neave, 1965a,b).

All of the vaccine preparations in our study, as well as the saline placebo, when administered intramammarily, stimulated a transient rise in total somatic cell count in the milk. However, because the counts did return to prevaccination levels by the following milk collection, they did not interfere with the rest of the experiments. To assure the return of somatic cell counts in the milk to prevaccination levels, the vaccinations should be given early in lactation and be composed of materials mild enough to minimize irritation. Ideally, the vaccination should be given ante-partum (McDowell et al., 1971), which minimizes the interference with gland function during lactation. Unfortunately, we found that the teat orifice of the primiparous guinea pigs was too

small to be safely manipulated without causing some trauma to the gland.

Comparing the post-challenge somatic cell infiltration of milk in both the SC and IM Groups, the reactions were similar, indicating that the intramammary vaccination of the IM Group did not sensitize the glands any more than the subcutaneous vaccination of the SC Group. However, the pre-exposure of both of these groups to staphylococcal antigens may have sensitized them to the subsequent challenge of staphylococci. The subgroups SC-F and IM-F showed a lessening of total somatic cell count in milk samples taken in the last days of lactation, a response not seen in the animals given staphylococcal antigens where the cell counts remained elevated and stable. Thus, if total somatic cell count is to be an accurate index of the severity of inflammation and infection in these glands, then the effect of gland sensitization following exposure to antigens must be considered.

The productive capacity of guinea pig mammary glands is severely reduced by the introduction of irritating substances and is, therefore, a less satisfactory index of infection than somatic cell count. In addition, as milk production normally fell throughout lactation, it was difficult to differentiate between the effect of the infection on production from that of natural gland retrogression. By day 11 post-partum the glands were

sensitive even to saline infusions and as a result, such an infusion, as well as the bacterial challenge, caused a severe drop in production of milk. Subsequently, post-challenge differences between milk production in vaccinated and nonvaccinated glands and challenged and non-challenged glands were too small to be significant.

The numbers of bacterial colony forming units (c.f.u.) isolated from the milk samples remained stable in all animals up to day 11 post-partum. While the count rose rapidly in the challenged glands, the numbers of c.f.u. from nonchallenged and nontreated glands increased only slightly over the period of observation. Because the mammary glands are continually exposed to the oral bacteria of the nursing young, bacteria, especially alpha hemolytic streptococci, are found in most milk samples throughout lactation. Whether the streptococci isolated from the nonchallenged and untreated glands were actually infecting the glands as reported by Gupta et al. (1971a), or were merely flushed into the milk during nursing was not determined. The continual presence of bacteria in the milk of these glands was evident in the stable c.f.u. count and might be responsible, in part, for the steady increase in the somatic cell count of milk collected from these glands late in lactation. Kuramitsu and Loeb (1921), Hesselberg and Loeb (1937), and Slater

(1962), however, observed a slight infiltration of leukocytes into the mammary gland during normal involution. This may indicate that the elevated somatic cell count observed in the Control Group and nonchallenged glands cannot be correlated with the presence of bacteria in the milk and may represent a normal condition in an involution gland.

The DNA concentration and RNA/DNA ratios of the gland tissue may be a more accurate indication of the intensity of gland infection. That the mitotic activity of the mammary gland in small animals peaks before or at the onset of lactation has been well documented histologically (Reece and Warbritton, 1953; Jeffers, 1935; Maeder, 1922; Hesselberg and Loeb, 1937; Kuramitsu and Loeb, 1921). At the same time the DNA concentration of the mammae also peaks and then remains stable throughout lactation (Naito, 1958; Nelson et al., 1962; Greenbaum and Slater, 1957; Kirkham and Turner, 1953). Gupta et al. (1971b) found that leukocyte infiltration during sterile mastitis is also associated with increased concentration of DNA in that tissue. Therefore, if mitosis had ceased during lactation, the increase in DNA must be due to the diapedesis of leukocytes into the gland tissue. DNA values from our study did show this increase in concentration in the infected glands. Although our values for the nonchallenged and untreated glands were higher than those published by

Gupta et al. (1971b) for saline injected glands, this increased DNA may be the result of low grade irritation and subsequent leukocyte infiltration of the glands by bacteria, saline, or from natural involution processes. Gupta's study was done on glands removed from animals early in lactation and 24 hours post-treatment.

According to Kirkham and Turner (1953) the RNA/DNA ratio is an accurate indication of mammary gland activity with respect to protein synthesizing activity. Gupta et al. (1971b) showed that in guinea pigs this ratio fell during mastitis. Our studies showed similar data; the infected glands had consistently lower ratios than the noninfected glands. Our values for RNA were somewhat higher than those reported by Gupta, which may be due to the presence of some leukocyte RNA in the final assay, in addition to the mammary tissue RNA, giving a total higher reading. This source of error then affects the interpretations which can be made from the RNA/DNA ratios, for a decreased ratio may be due to increased DNA or decreased RNA, and conversely for an increased ratio. In an involuting gland where the synthesizing capacity is decreasing, a stable or slightly increased DNA level would result in a reduced ratio, but in an actively secreting gland, an infiltration of leukocytes, increasing the DNA concentration of that gland, could show a reduced ratio even in an actively secreting gland. Thus, these analyses should

be correlated with analysis of the milk obtained from the gland in terms of somatic cell counts and some index of the amount of milk being secreted.

Agglutinin titers of whey samples did not appear to be increased by vaccination. These titers were generally low and may indicate an unsatisfactory or faulty assay which was not sensitive enough to detect the shift in titer for, in a study in guinea pigs McDowell et al. (1971), following intramammary injection of staphylococcal antigens, recorded high agglutinin titers in whey and serum samples collected post-partum. Because these authors pooled many milk samples to give a large working volume of whey, their technique for preparation of whey by acidifying milk was adequate. However, addition of acid to our smaller samples served to dilute the antibody present and may have contributed to the low titers observed. Serum titers in our study were higher than those of the whey samples but, because serum was collected on day 19 post-partum, the elevated titer may be the result of the staphylococcal infection rather than vaccination, because some agglutinin was seen in subgroups SC-F and IM-F, which had only been infected with staphylococci.

Precipitating antibodies were in whey samples from animals vaccinated with the homologous antigen. In addition to the vaccine induced antibodies, weak precipitin lines to other antigens (either lipase or

coagulase, or both) were seen in the serum samples. Again, because the serum samples were obtained 8 days post-infection, these animals were exposed to numerous staphylococcal antigens, including lipase and coagulase. These weak precipitation lines were detected in samples from the nonvaccinated animals, SC-F and IM-F, which were challenged, but not in the Control Group animals. As expected, no precipitating antibodies to SPA were found, because guinea pigs do not respond immunologically to purified polysaccharide (Kabat, 1968).

A comparison of the results of these studies, with respect to any effects of vaccination, did not show any enhanced protection. Where one parameter appeared to indicate a reduced infection, it could not be correlated with the other parameters and must be considered a random phenomenon. Thus, although the guinea pig model is potentially suitable for mastitis studies, a more sensitive combination of parameters must be chosen to adequately measure a change in infection due to vaccination.

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APPENDIX

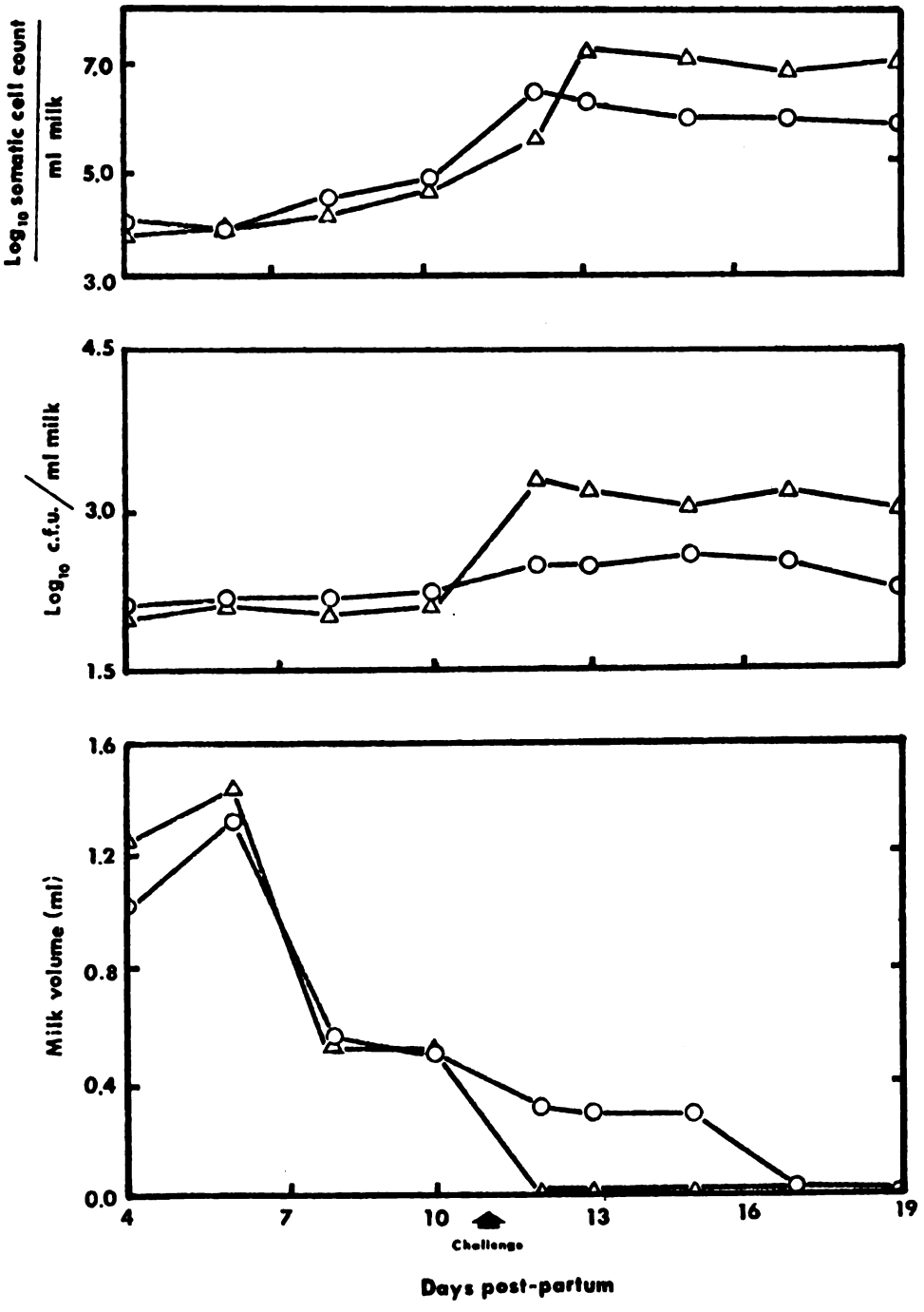

Figure 12.--Subgroup SC-A: Determination of the mean values of total somatic cell count per ml of milk, colony forming units (c.f.u.) per ml of milk, and milk volume of the right (challenged) (-Δ-) and left (non-challenged) (-O-) mammary glands at each collection.

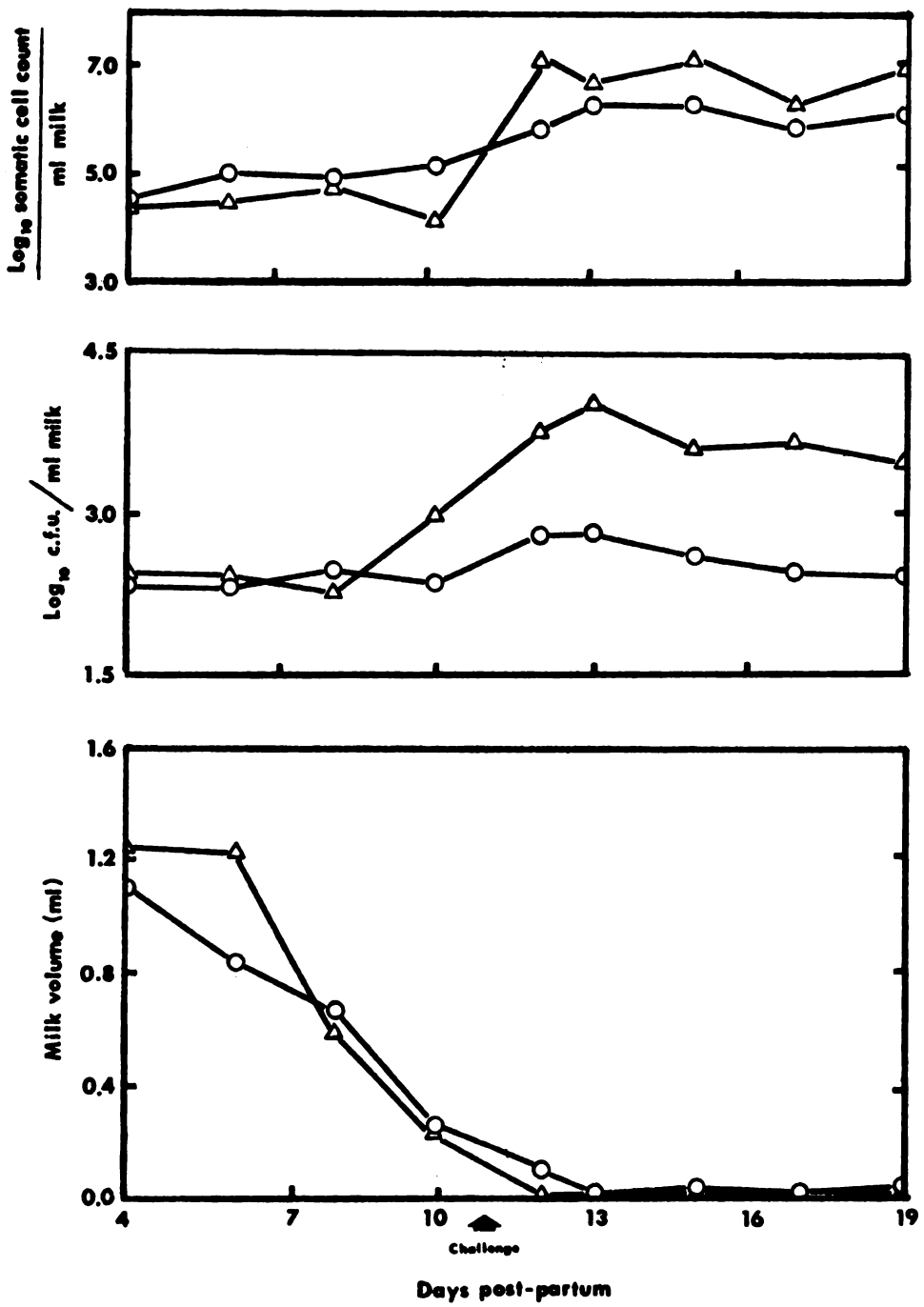

Figure 13.--Subgroup SC-B: Determination of the mean values of total somatic cell count per ml of milk, colony forming units (c.f.u.) per ml of milk, and milk volume of the right (challenged) (-Δ-) and left (non-challenged) (-O-) mammary glands at each collection.

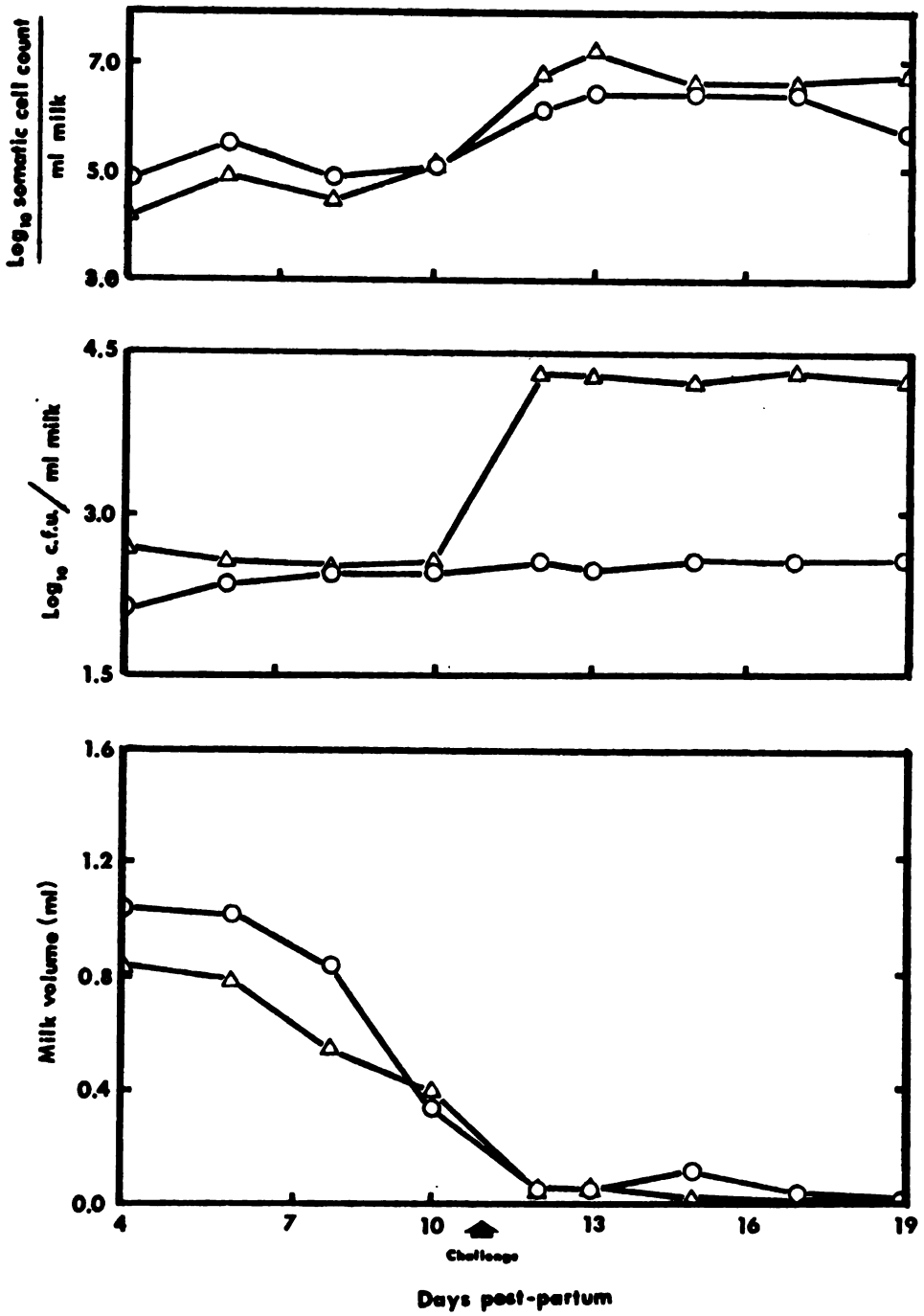

Figure 14.--Subgroup SC-C: Determination of the mean values of total somatic cell count per ml of milk, colony forming units (c.f.u.) per ml of milk, and milk volume of the right (challenged) (-Δ-) and left (non-challenged) (-O-) mammary glands at each collection.

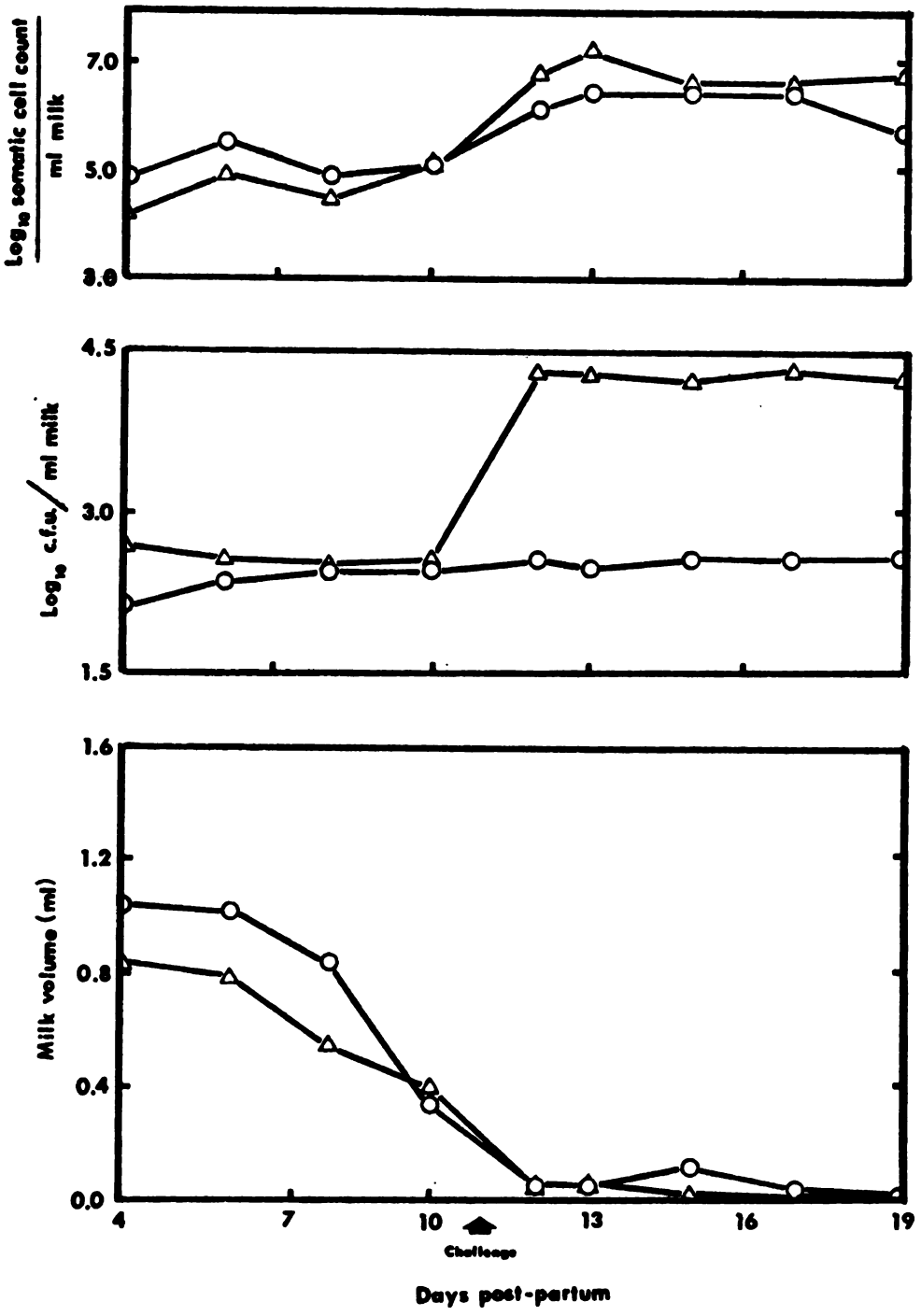

Figure 14.--Subgroup SC-C: Determination of the mean values of total somatic cell count per ml of milk, colony forming units (c.f.u.) per ml of milk, and milk volume of the right (challenged) (-Δ-) and left (non-challenged) (-O-) mammary glands at each collection.

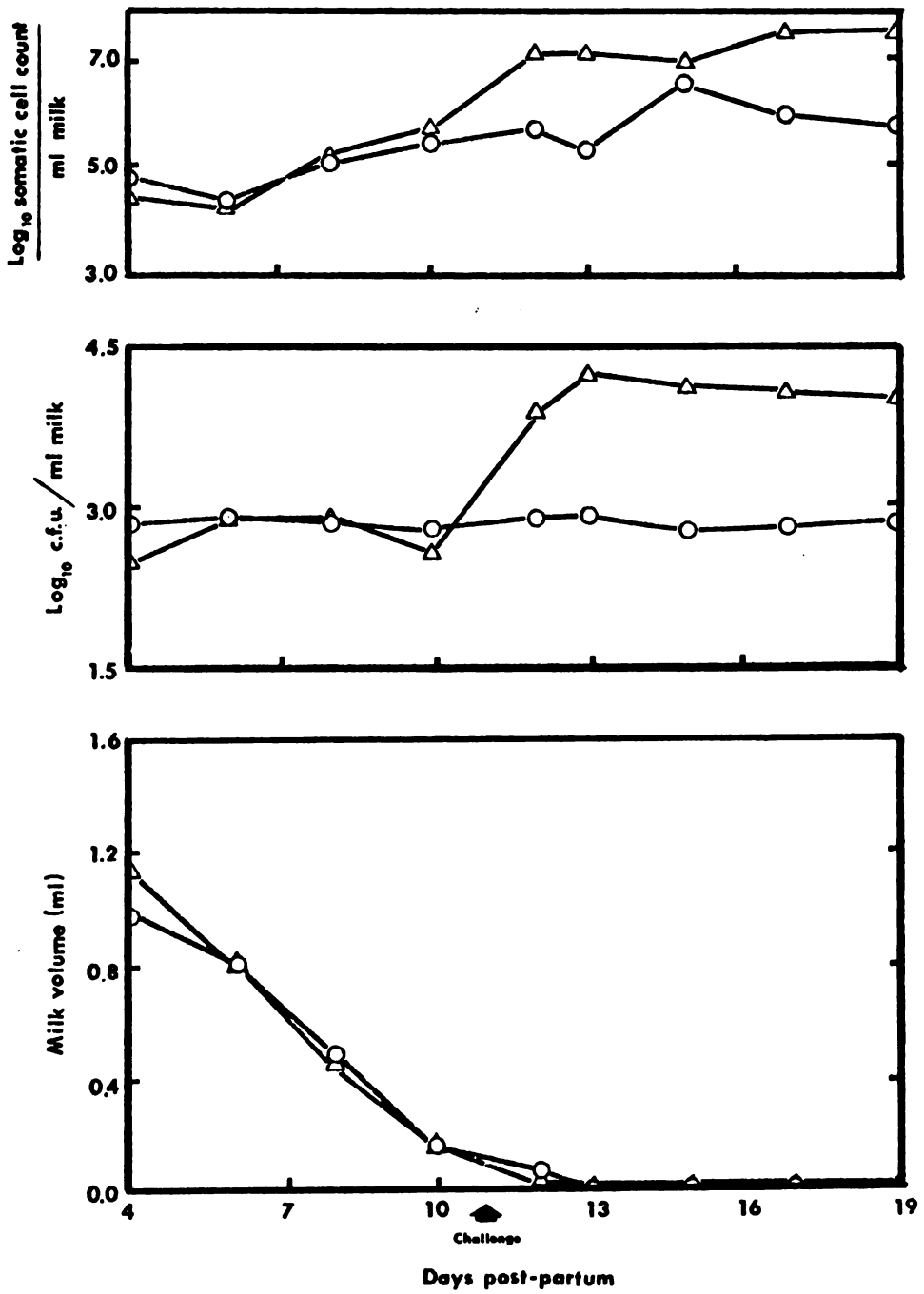

Figure 15.--Subgroup SC-D: Determination of the mean values of total somatic cell count per ml of milk, colony forming units (c.f.u.) per ml of milk, and milk volume of the right (challenged) (-Δ-) and left (non-challenged) (-O-) mammary glands at each collection.

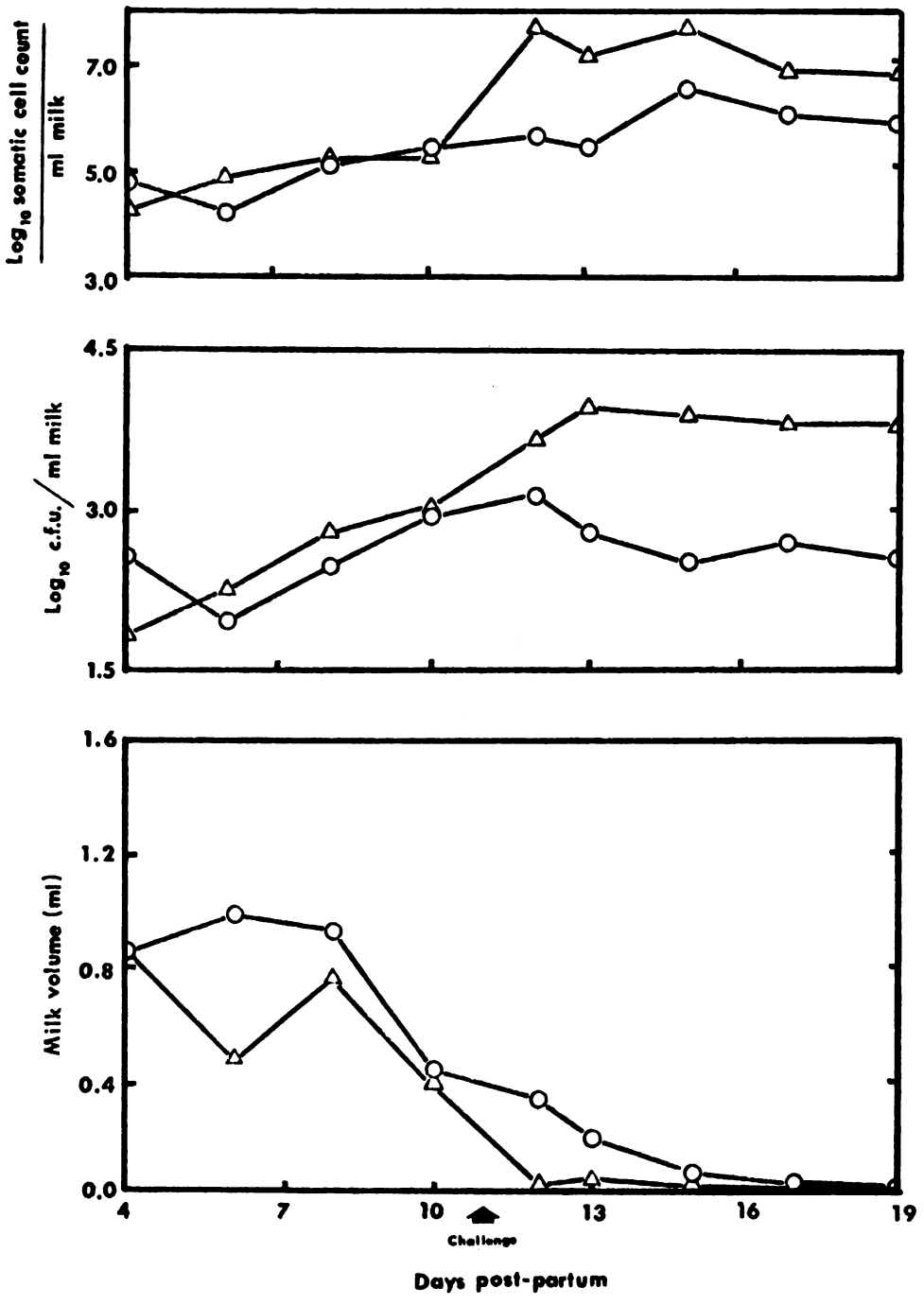

Figure 16.--Subgroup SC-E: Determination of the mean values of total somatic cell count per ml of milk, colony forming units (c.f.u.) per ml of milk, and milk volume of the right (challenged) (-Δ-) and left (non-challenged) (-O-) mammary glands at each collection.

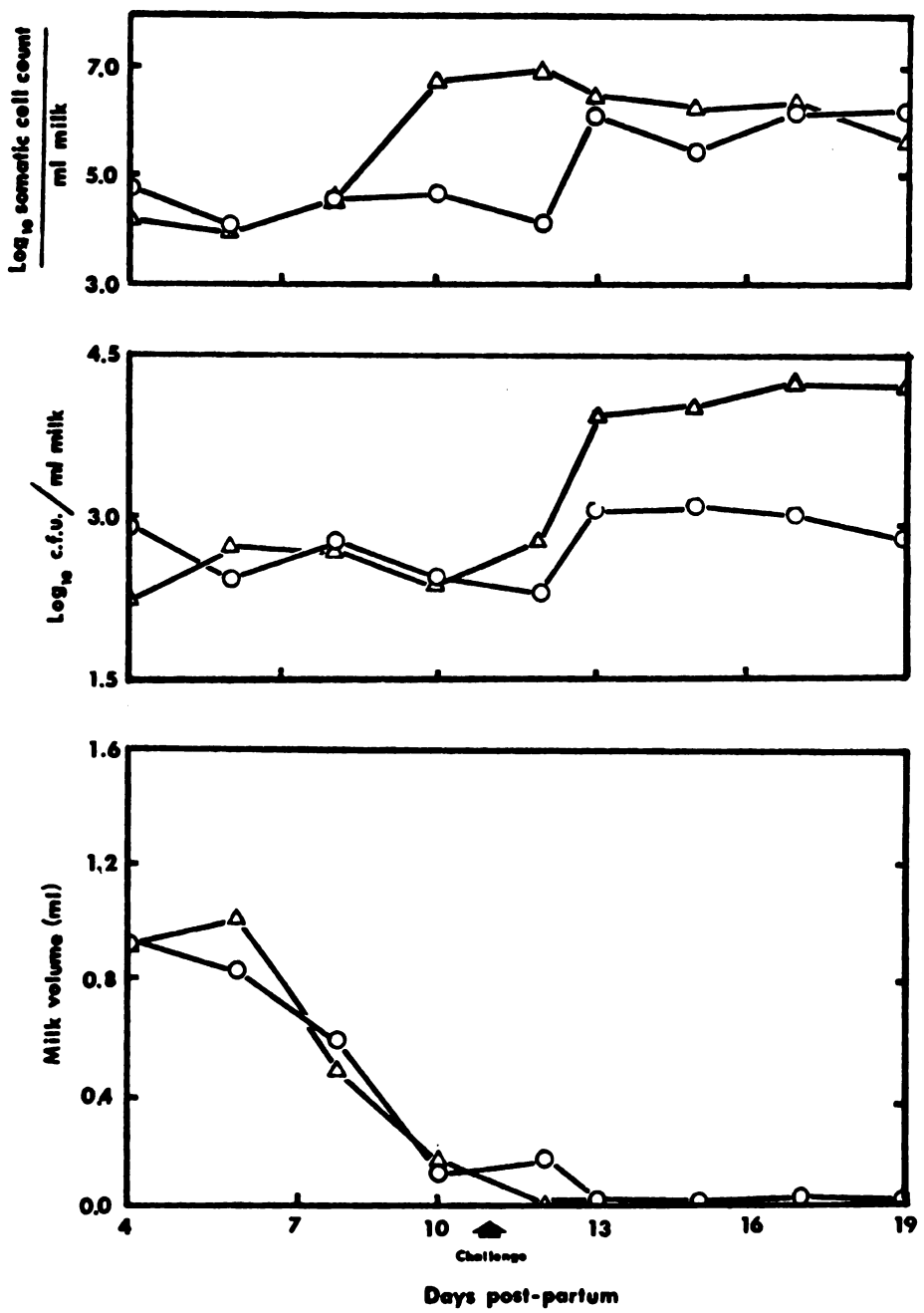

Figure 17.--Subgroup SC-F: Determination of the mean values of total somatic cell count per ml of milk, colony forming units (c.f.u.) per ml of milk, and milk volume of the right (challenged) (-Δ-) and left (nonchallenged) (-O-) mammary glands at each collection.

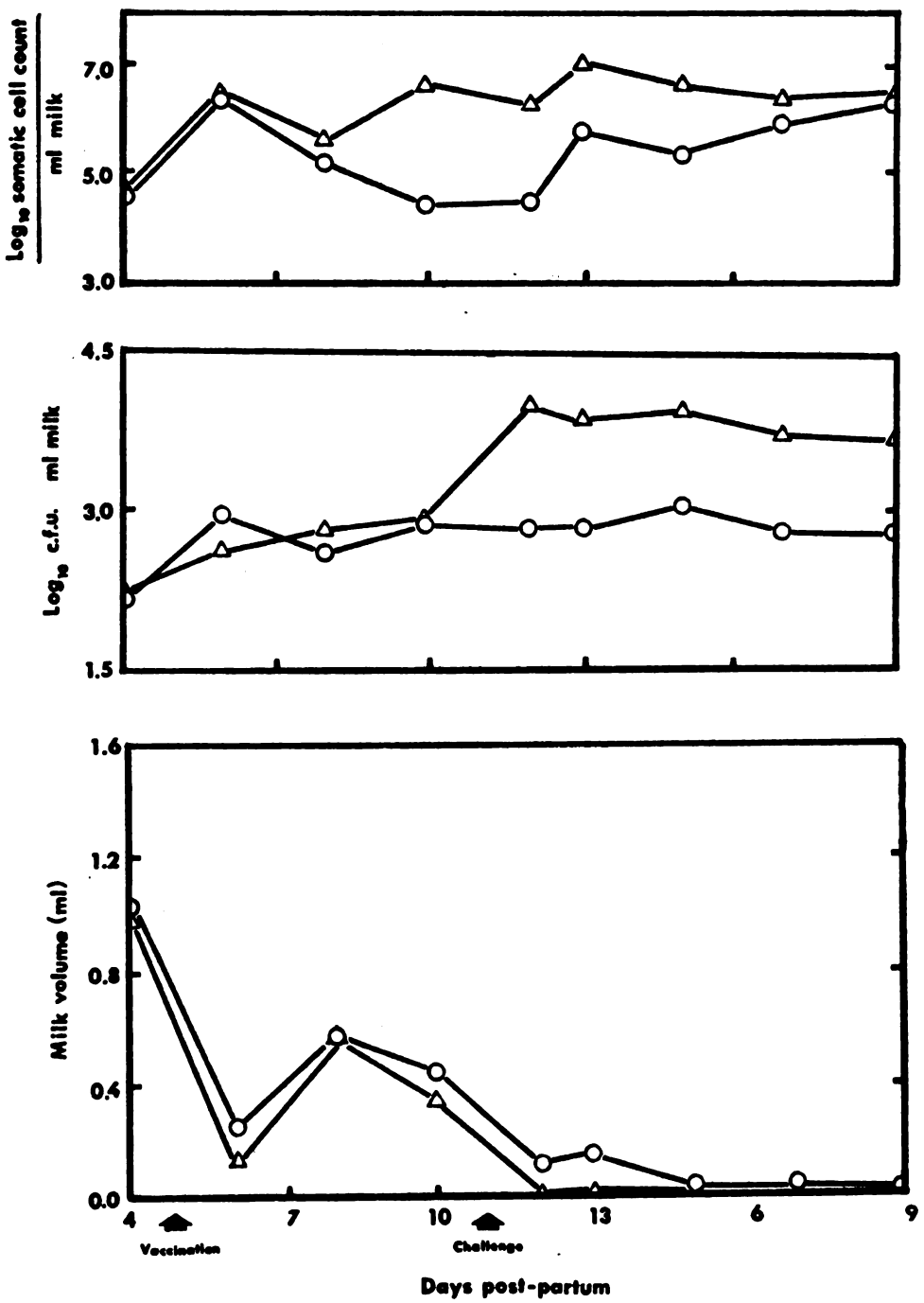

Figure 18.--Subgroup IM-A: Determination of the mean values of total somatic cell count per ml of milk, colony forming units (c.f.u.) per ml of milk, and milk volume of the right (challenged) (-Δ-) and left (non-challenged) (-O-) mammary glands at each collection.

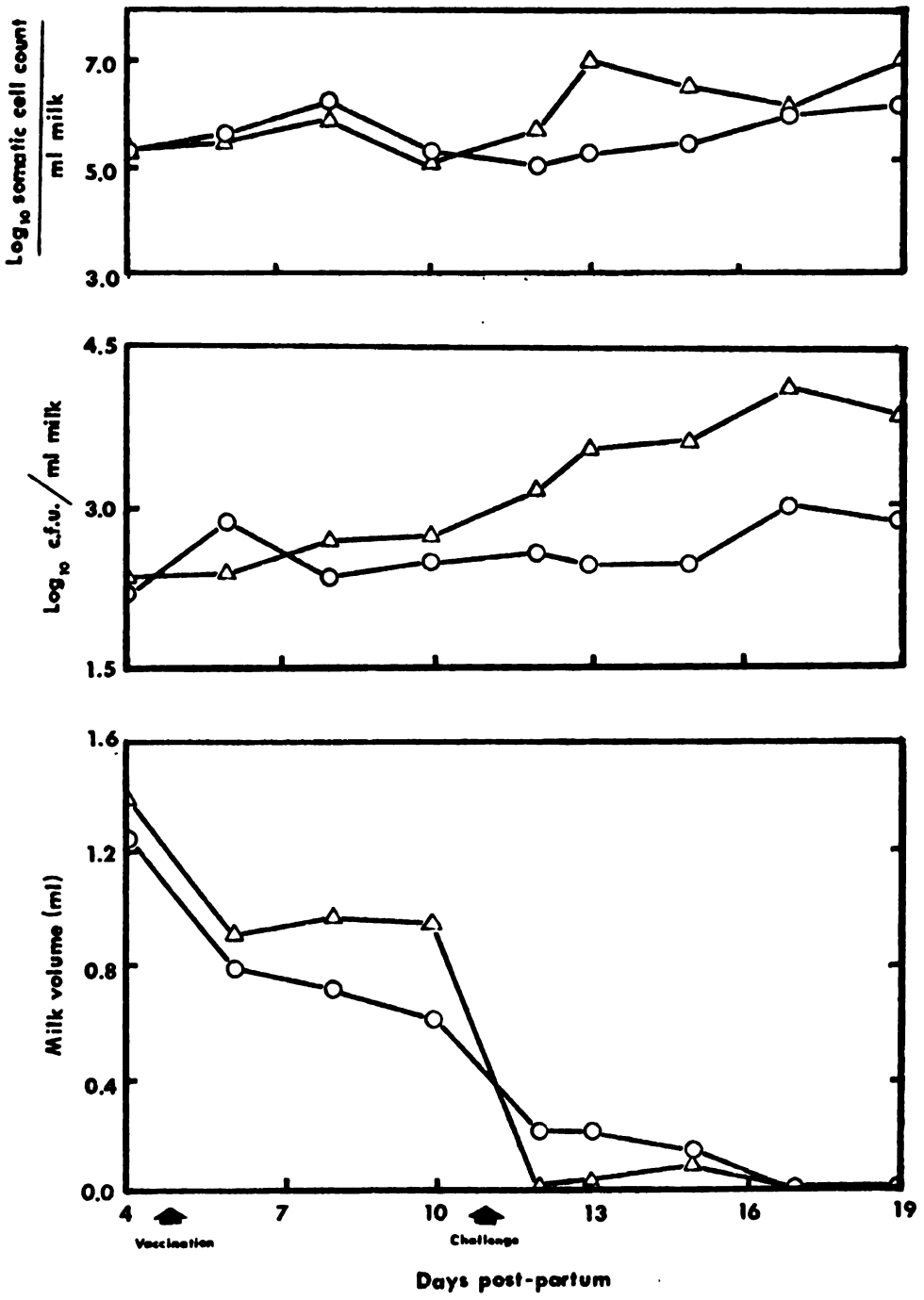

Figure 19.--Subgroup IM-B: Determination of the mean values of total somatic cell count per ml of milk, colony forming units (c.f.u.) per ml of milk, and milk volume of the right (challenged) (-Δ-) and left (non-challenged) (-O-) mammary glands at each collection.

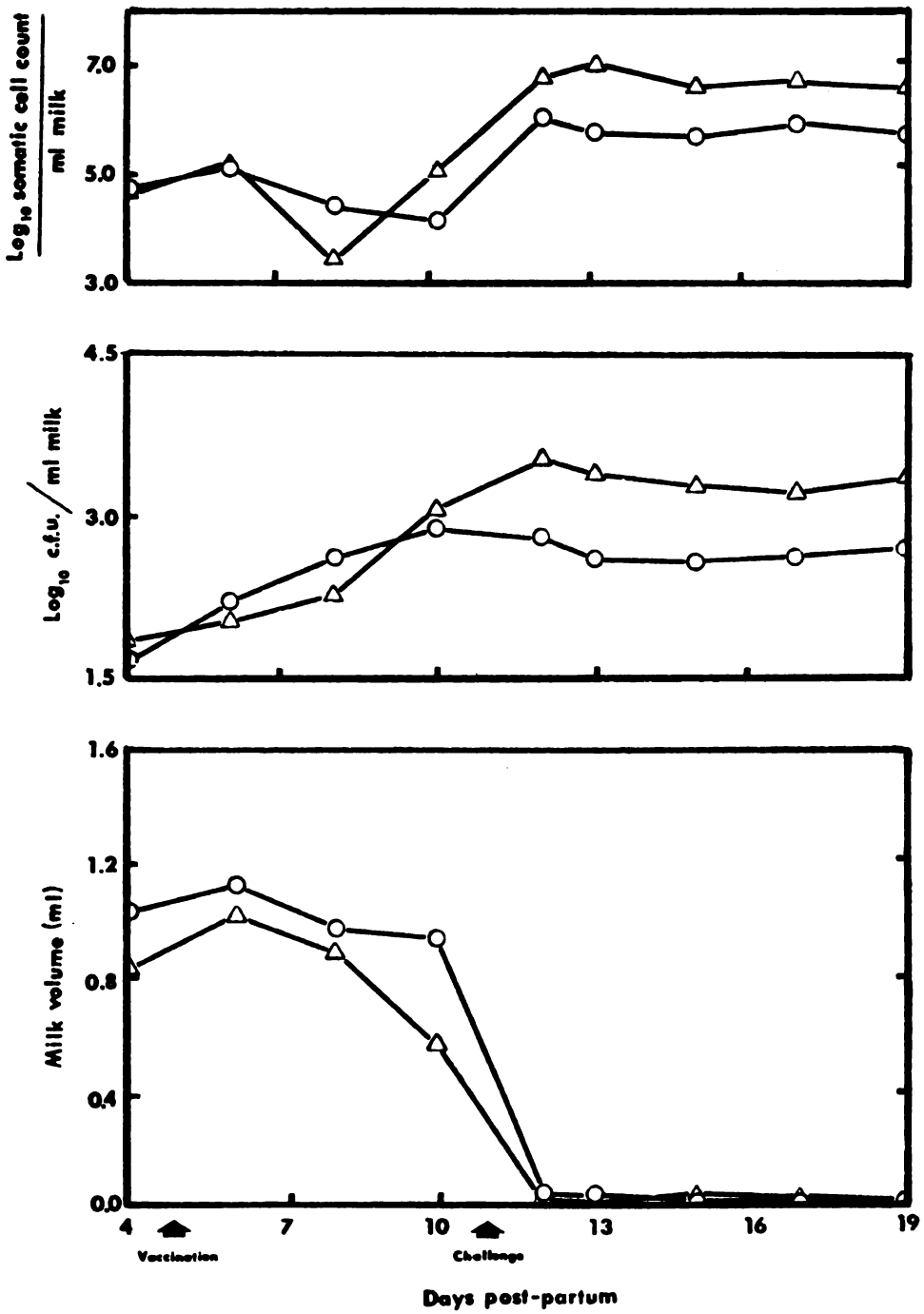

Figure 20.--Subgroup IM-C: Determination of the mean values of total somatic cell count per ml of milk, colony forming units (c.f.u.) per ml of milk, and milk volume of the right (challenged) (-Δ-) and left (non-challenged) (-O-) mammary glands at each collection.

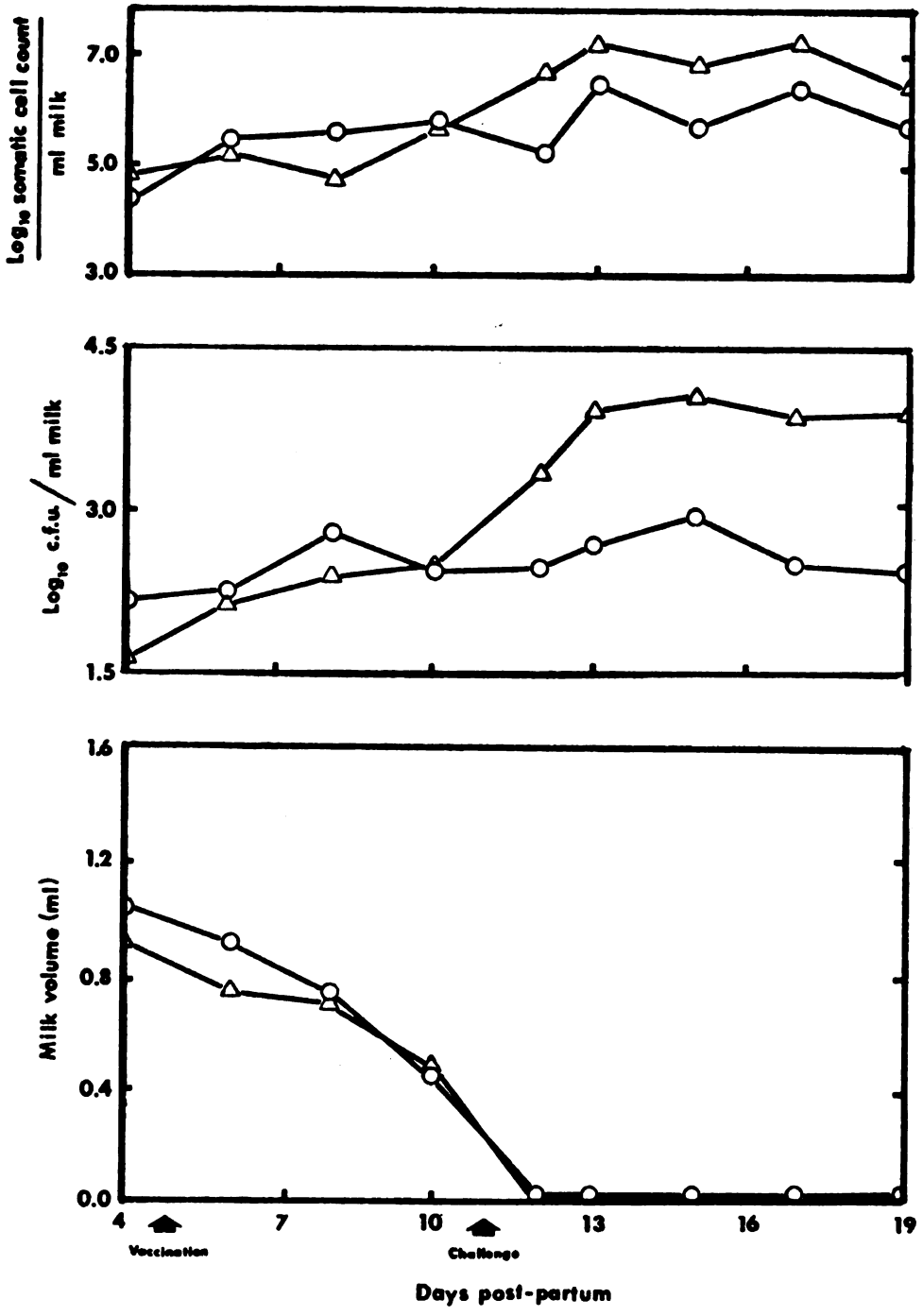

Figure 21.--Subgroup IM-D: Determination of the mean values of total somatic cell count per ml of milk, colony forming units (c.f.u.) per ml of milk, and milk volume of the right (challenged) (-Δ-) and left (non-challenged) (-O-) mammary glands at each collection.

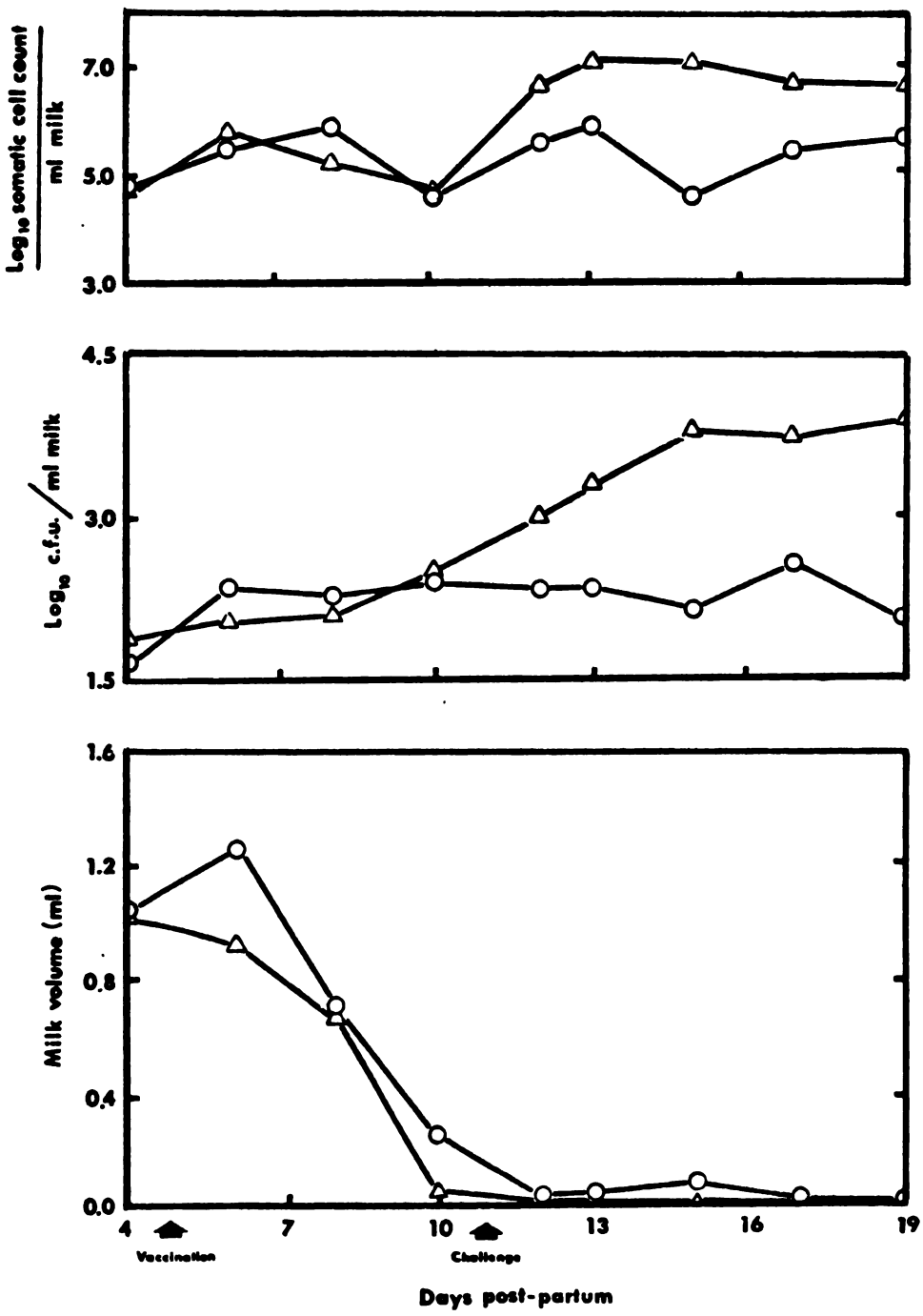

Figure 22.--Subgroup IM-E: Determination of the mean values of total somatic cell count per ml of milk, colony forming units (c.f.u.) per ml of milk, and milk volume of the right (challenged) (-Δ-) and left (non-challenged) (-O-) mammary glands at each collection.

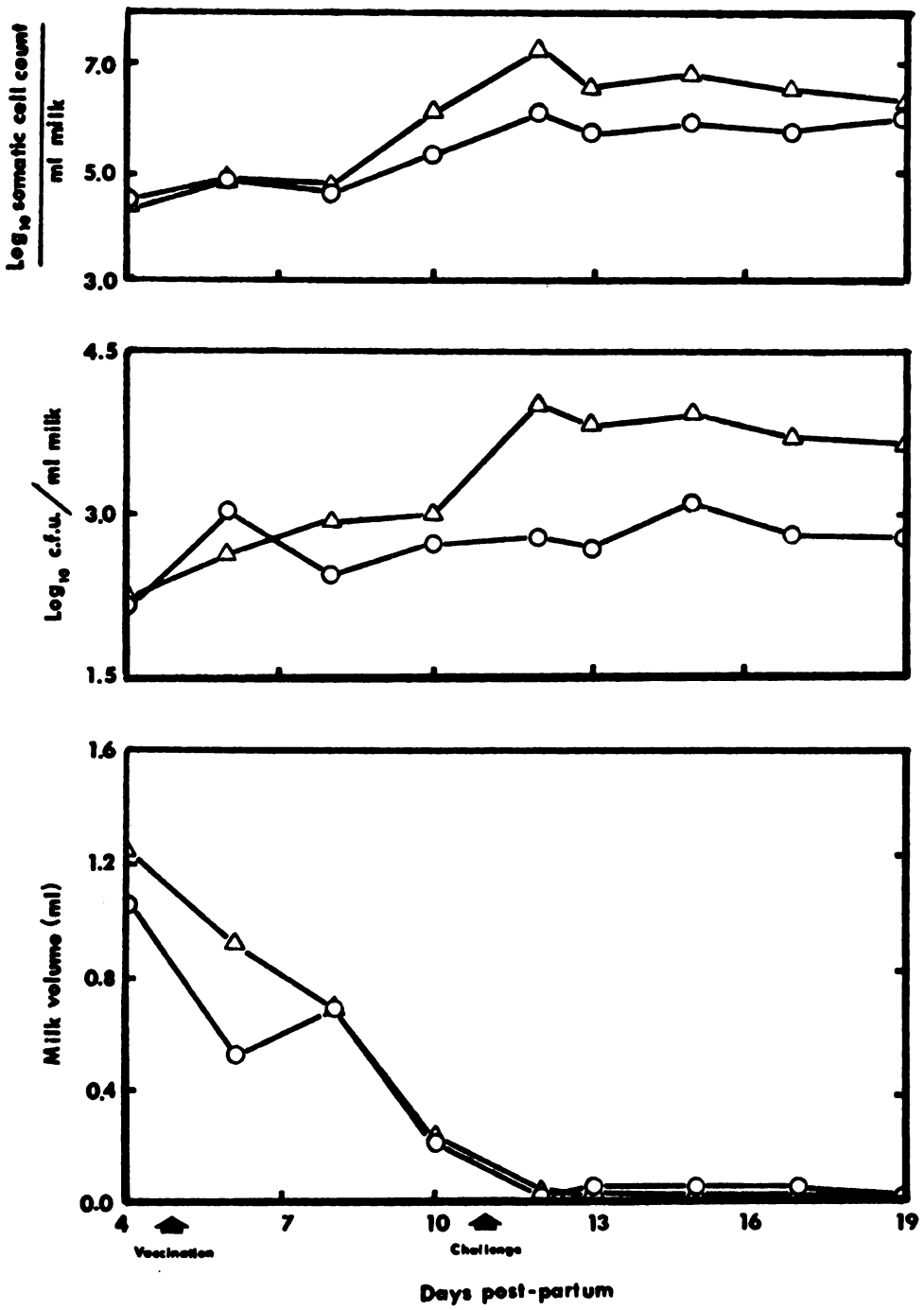

Figure 23.--Subgroup IM-F: Determination of the mean values of total somatic cell count per ml of milk, colony forming units (c.f.u.) per ml of milk, and milk volume of the right (challenged) (-Δ-) and left (non-challenged) (-O-) mammary glands at each collection.

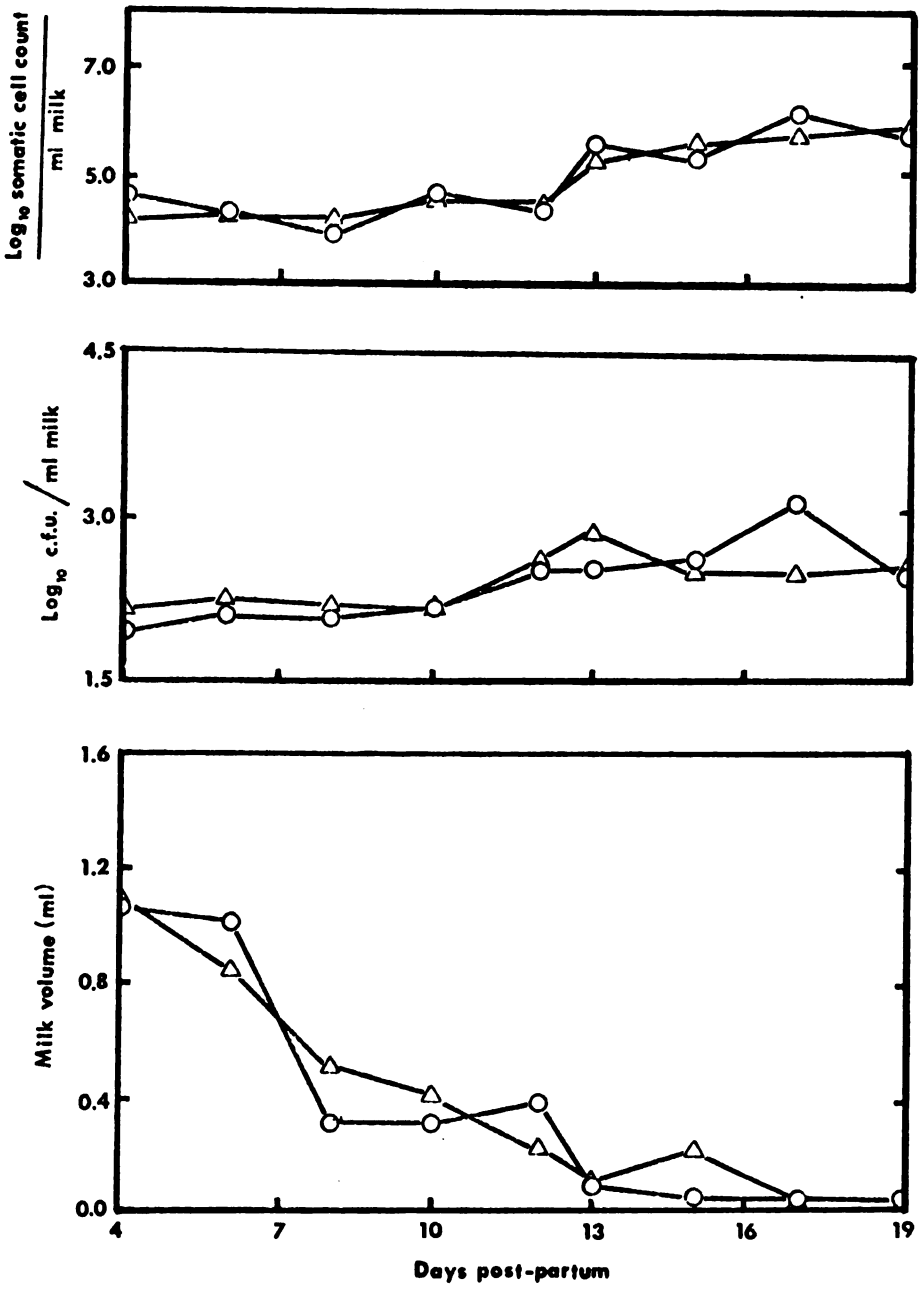

Figure 24.--Control Group: Determination of mean values of total somatic cell count per ml of milk, colony forming units (c.f.u.) per ml of milk, and milk volume of the right (-Δ-) and left (-O-) mammary glands at each collection.