Recent Advances in Bovine Vaccine Technology

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ABSTRACT

A description of new commercial and experimental vaccines for viral and bacterial diseases of cattle can be broadly divided into those used for both beef and dairy cows and those used predominantly in dairy cattle. For both types of cattle, newer and experimental vaccines are directed against several of the important viral (e.g., bovine herpesvirus 1, bovine viral diarrhea virus, bovine respiratory syncytial virus, parainfluenza type 3, and foot-and-mouth disease virus) and bacterial pathogens (e.g., Pasteurella spp., Haemophilus somnus). The viral vaccines include gene-deleted, modified live, subunit, and peptide antigens. Newer bacterial vaccines, particularly those for Pasteurella spp., are composed of either modified-live vaccines or bacteria supplemented with toxoid or surface antigens. Haemophilus somnus vaccine research has concentrated mainly on defining unique surface antigens. Novel dairy cow vaccines would include the lipopolysaccharide-core (J5) antigen approach, which has been used for successful immunization against coliform mastitis. Core antigen vaccines also have reduced calf mortality from Gram-negative pathogens. Staphylococcal mastitis vaccines that contain capsular antigens, toxoids, or the staphylococcal fibronectin receptor are of active research interest. Vaccines against mastitis induced by Streptococcus agalactiae and Streptococcus uberis also are areas of intensive research. Delivery of multiple subunit antigens with optimal immune response induction has led to the investigation of attenuated heterologous viral and bacterial expression vectors such as bovine herpesvirus 1, vaccinia, and Salmonella spp. This discussion also demonstrates that molecular biology is being used to advance bovine vaccine technology.

(Key words: vaccines, disease, antigens, mastitis)

Abbreviation key: BHV-1 = bovine herpesvirus 1, BRSV = bovine respiratory syncytial virus, BVDV = bovine viral diarrhea virus, CCM = clinical coliform mastitis, CPS = capsular polysaccharide, F = fusion glycoprotein, FMD = foot-and-mouth disease, FMDV = FMD virus, G = attachment glycoprotein, HN = hemagglutinin neuraminidase, HPI3 = human parainfluenza type 3, HRV = human respiratory syncytial virus, IBR = infectious bovine rhinotracheitis, LKT = leukotoxin, LPS = lipopolysaccharide, MLV = modified live vaccine, PI3 = parainfluenza type 3, PMN = polymorphonuclear leukocyte, R = rough (c = blocked in galactose attachment to core LPS; e = blocked in heptose phosphate attachment to core LPS), TK = thymidine kinase (- = negative).

INTRODUCTION

The present discussion focuses on recent advances in bovine vaccine technology and is confined generally to the newer bovine vaccines, those introduced since 1985, and to vaccine prospects for the near future. Also addressed are the role of rDNA technology in vaccine development and the ways in which knowledge about specific virulence attributes of the pathogen and a better understanding of the immune response of cattle to a given disease agent have defined new directions for vaccine technology. The discussion is divided into two broad categories: vaccines intended for use in both beef and dairy cattle and vaccines intended mainly for dairy cattle, namely, mastitis vaccines. In addition, the use of live vaccines.
attenuated bacteria and viruses as multivalent expression vectors for heterologous antigens is discussed. A comprehensive discussion of the vaccines marketed for use in cattle up to 1990 is provided in the review by Hjerpe (64). For recent overviews of “vaccinology”, the reader is encouraged to consult the reviews of Flexner (45) and Schultz and Israel (134).

**BEEF AND DAIRY COW VACCINES**

When bovine vaccines are discussed, particularly those for respiratory disease, it should be emphasized that the practitioner is generally trying to protect the animals from a disease complex induced by more than one virus or bacterial agent. For the newer vaccines to be effective, then, it will be essential that, as in the past, the veterinarian design herd vaccination programs inclusive of those agents to which the herd is likely to be exposed. In this regard, the reader may again find the review of Hjerpe (64) useful.

**VIRAL VACCINES**

A number of viral vaccines have been marketed for use in beef and dairy cows. One such viral vaccine that is widely used in herd health programs is one for bovine herpesvirus 1 (BHV-1), the virus that induces infectious bovine rhinotracheitis (IBR), an upper respiratory tract infection, which also is the agent of abortions, infectious pustular vulvovaginitis, balanoposthitis, meningencephalitis, enteritis, conjunctivitis, and generalized systemic disease in cattle (54, 64). As with other herpesviruses, protective immunity requires humoral and cell-mediated immune responses (69, 131). Although some reports (52) to the contrary exist, inactivated vaccines are generally considered to be safe and effective. However, inactivated vaccines provide shorter and slower developing immunity than modified live vaccines [MLV (64)]. Modified live vaccines are administered either i.n. or i.m., and both types of MLV are effective (64). Nonetheless, safety concerns with MLV exist; vaccines administered i.m. have been associated with abortions in late gestation cows (93), MLV administered i.v. cause ovarian lesions and infertility (97, 98, 154), and i.n. MLV enhance susceptibility to lesions of infectious keratoconjunctivitis induced by *Moraxella bovis* (53). In addition, conventional, commercial MLV strains can be transmitted between animals (6), produce a latent state from which they can be reactivated (120), and have been implicated in vaccine-induced epizootics of IBR (171).

The new generation MLV for BHV-1 will likely employ defined attenuating mutations. One such MLV has been developed by deletion and insertion into the thymidine kinase (TK) gene, resulting in a stable, irreversible mutation in this gene (81). The TK gene is associated with virulence of several herpesviruses, and mutations within this gene are often attenuating. The TK- BHV-1 was shown to be safe in calves (83) and in pregnant cattle (82, 99), although the virus could be reactivated from apparent latency with dexamethasone (170). In a recent study, a TK- BHV-1 vaccine protected calves from clinical signs following challenge with the virulent BHV-1 strain Cooper (80). One of the more exciting aspects of the defined MLV vaccines represented by the TK- BHV-1 mutants is the possibility of using these viruses not only as specific vaccines but also as heterologous antigen expression vectors. This topic is discussed in a later section.

Subunit vaccines for BHV-1 composed of detergent-solubilized virus-infected cells were first shown to be protective in calves by Lupton and Reed (92). Recent attempts to develop subunit vaccines for BHV-1 have focused on the three major envelope glycoproteins of the virus, gI, gIII, and gIV. Babiuk et al. (4), using monoclonal antibody affinity purification, prepared a subunit vaccine composed of one or more of these glycoproteins. Administered i.m. in an avridine adjuvant, this material protected calves from morbidity and mortality in a BHV-1, *Pasteurella haemolytica* aerosol challenge model. Protection was better than with a commercial, killed virus vaccine. Although slightly less active than BHV-1-infected cell lysates, additional studies (155) suggested that gIV was the most protective of the three purified glycoproteins using the aerosol challenge model. As little as 3.1 μg of gIV induced a neutralizing antibody response, and 6.5 μg significantly reduced viral replication in the nasal passages and protected against morbidity and mortality in calves. When 2 μg of immunopurified gIV in avridine adjuvant were ad-
administered concurrently with multiple treatments of the cytokine interleukin-2, serum-neutralizing antibody and cytotoxic T-cell responses specific to BHV-1 were elicited in calves (69). Adjuvants other than avridine were inactive or less effective in inducing a primary antibody response. Another group (149) found that 25 to 50 μg of BHV-1 viral subunit proteins (administered i.m.) presented as immunostimulating complexes [ISCOM; (102)] were more protective than a commercial MLV in a viral challenge model administered i.n. These results imply that a subunit approach to BHV-1 vaccination is feasible and also that recombinant cytokines can function as adjuvants for rDNA-produced subunit antigens (60).

The effectiveness of subunit glycoproteins as vaccines in the aforementioned studies were in contrast to the results of Israel et al. (72), who used intradermally administered, immun-affinity-purified glycoproteins gI, gII, and gIV attached by monoclonal antibody to Sepharose beads (in Freund's incomplete adjuvant). Although these antigens induced serum-neutralizing antibody titers in calves, they did not protect the calves from i.n. challenge with virulent BHV-1. Calves immunized with a commercially obtained, inactivated vaccine had a milder clinical response than the glycoprotein-vaccinated calves. Reasons for this discrepancy are not immediately evident, although the latter group used different preparative methods, a different adjuvant, and a different immunization route than the other groups.

Although several of the BHV-1 glycoproteins have been cloned and sequenced (44, 101, 148, 172), protection studies using purified envelope proteins that have been produced by rDNA methods (e.g., in *Escherichia coli*, baculovirus, yeast, or other eukaryotic expression vectors) have not been published.

Effective vaccines for bovine viral diarrhea virus (BVDV) have been problematic but remain an area of active research. The BVDV is a pestivirus that is very prevalent among ruminant herds throughout the world (5). The BVDV and its biotypes produce numerous disease manifestations, ranging from subclinical infection to diarrhea, erosive lesions of the mucosa (mucosal disease), immunosuppres-

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either HRSV-F or HRSV-G were not protective against BRSV challenge in calves (7). Nevertheless, from the work in cotton rats and other models of HRSV-induced disease, F and G appear to be the viral glycoproteins to which immune responses are directed in HRSV and BRSV infections.

Affinity-purified HRSV-F and HRSV-G were protective in cotton rats against HRSV challenge (105, 157). Clinical vaccine trials with an affinity-purified F subunit vaccine were conducted in humans, but the results have not yet been published (7). Also, truncated F expressed, by baculovirus vector was highly protective in the cotton rat model (159). In addition, a glycoprotein composed of an FG chimera of HRSV produced in baculovirus also was effective and protected better than F alone against challenge in the cotton rat model (17, 160). Protection was afforded without induction of the pulmonary inflammation characteristic of formalin-inactivated vaccines (161). Whether affinity-purified or baculovirus-expressed F, G, or FG affords protection in naive human children is as yet untested (M. W. Wathen, 1992, personal communication). That this subunit vaccine approach may be applicable to construction of a better BRSV vaccine for cattle is implied from studies in which the antiserum from a BRSV-infected calf recognized baculovirus-produced BRSV-F (63).

Parainfluenza virus type 3 (PI3) is another paramyxovirus that causes respiratory disease in calves. The major importance of PI3 as a pathogen appears to be its ability to predispose animals to other pathogens. For instance, PI3 has been used as a tool to establish experimental pneumonic pasteurellosis in calves (73). A number of inactivated PI3 vaccines and two types of MLV (i.m. or i.n.) are currently marketed. The i.n. administered MLV are generally perceived to be more effective than i.m. administered MLV or inactivated vaccines because they are more efficient at inducing secretory immunity (64). Vaccines for PI3 are invariably administered to cattle in preparations containing at least an IBR vaccine and are often included in a mixture containing four or more vaccines. As with BRSV, the lessons of the research with human PI3 (HP13) may be applicable to new directions for vaccines directed against the bovine virus because PI3 and HP13 are quite similar. The bovine PI3 provided protection in squirrel monkeys from challenge with HP13 (24). Challenge studies in the cotton rat model with HP13 have shown that, of the viral envelope glycoproteins, immunity to both the F and hemagglutinin neuraminidase (HN) glycoproteins is necessary for protection from viral infection and replication. These conclusions were deduced from studies with baculovirus-produced subunit preparations and from antigen expression systems vaccinia virus-vectoried (23, 141). In the latter system, vaccinia virus expressing the F glycoprotein was more protective against HP13 challenge of cotton rats than was a vaccinia-HN construct (141). Recently, baculovirus strains expressing an F-HN chimera were constructed (87). This subunit antigen was more protective in cotton rats than F, HN, or mixtures of F and HN (16, 87). Approaches similar to those used for BRSV and for the HP13 may be applicable to bovine PI3.

Although foot-and-mouth disease (FMD) is not generally found in the US, research on vaccines for FMD illustrate how molecular biological approaches are being utilized in vaccine discovery programs. Worldwide, FMD is one of the most economically important diseases, and more vaccine is used for FMD than for almost any other disease (96). Furthermore, FMD is claimed to be the most contagious disease of cloven-hoofed animals (8). Although mortality from FMD rarely exceeds 5% in adult cattle, mortality rates may exceed 50% in calves (96). The symptoms of FMD, salivation and lameness, are due to the formation of vesicles and blisters on the mouth and feet. The major impact of the disease is in economic loss from reduced production and restriction of importation of agricultural products from areas in which FMD is endemic.

The FMD virus (FMDV) is an aphthovirus with seven serotypes and multiple subtypes (more than 60) within certain of the serotypes (8). Serotype-specific immunity is based on the presence of neutralizing antibody to one of the viral capsid proteins, VP1. Vaccination for FMD in countries in which the disease is endemic is currently conducted with inactivated "current" strains of FMDV. These inactivated vaccines, although quite effective when properly used, are thermally unstable. In addition, incomplete inactivation of vaccine has sometimes resulted in disease outbreaks (8, 79).
As mentioned, neutralizing antibody to VP1 has been correlated with immunity to FMD. Nearly 20 yr ago, virion-extracted VP1 was shown to protect cattle from challenge with FMDV (122), and, accordingly, recombinant and synthetic vaccine approaches have concentrated on this antigen. Cloned VP1 expressed as a fusion protein in E. coli was protective in calves against challenge exposure with the homologous serotype and subtype strain (96, 122). In addition, the predominant immunogenic epitopes of VP1 have been identified as (approximately) AA residues 138 to 160 and 200 to 213 (145). These epitopes, when cloned as fusion proteins with various bacterial proteins including β-galactosidase, Pho E, or LE or with hepatitis B core antigen and produced in several expression systems including E. coli, yeast, or vaccinia virus, were immunogenic, eliciting neutralizing antibody and protection in animal models and even cattle (8, 10, 51, 103). The hepatitis B core antigen may be useful as a general carrier for peptide vaccines because this protein was a potent inducer of helper T cells and a promoter of T-cell-independent antigen responses (51). Synthetic peptides of AA 141 to 160 and AA 200 to 213, when chemically coupled to keyhole limpet hemocyanin, induced neutralizing antibody and gave protection in the guinea pig model and in cattle (13, 50). Even uncoupled synthetic peptides induced peptide- and virus-specific neutralizing antibody in guinea pigs (50) and cattle (39); however, full protective activity was not obtained even with relatively high doses of uncoupled peptide (39).

Kit et al. (80) used a TK- BHV-1 MLV expressing FMDV VP1 epitopes. The VP1 AA 141 to 158 linked through Pro-Pro-Ser to VP1 AA 200 to 213 was constructed as a fusion protein with BHV-1 gIII. This recombinant virus expressed the FMDV epitopes on the surface of infected tissue-culture cells and on the surface of virus particles. The construct induced virus-neutralizing antibody to both BHV-1 and FMDV. Although no data were provided, Kit et al. (80) stated that calves were protected from challenge with both virulent BHV-1 and FMDV.

Although most of these studies were encouraging and although protection in cattle sometimes approached that of inactivated vaccines (96, 103), none of these preparations provided a commercially viable vaccine. A major drawback of the defined peptide approach for FMDV also occurs with other inactivated vaccines. The serologic heterogeneity of FMDV and, possibly, rapid evolution to new antigenic subtypes (8) likely require the vaccine constructs to be multivalent.

**BACTERIAL VACCINES**

Of the bacterial vaccines, those for respiratory disease induced by *P. haemolytica* have been used often because commercial bacterins for *P. haemolytica* provided little or negative efficacy (12, 64), even though efficacy could be demonstrated with oil-adjuvanted, experimental bacterins (27). However, since the introduction of MLV and subunit vaccines that are enriched for the phagocyte-lysing leukotoxin (LKT) of *P. haemolytica*, vaccine efficacy has been greatly enhanced, and the utility of these vaccines has increased.

Immunity to pneumonic pasteurellosis appears to be correlated with activity of alveolar macrophages and neutrophils, the presence of LKT-neutralizing antibody, and the presence of antibody directed toward carbohydrate surface components (26, 104, 130, 142). Although good correlation does not exist between antibody to whole cells and immunity to this disease, correlation is good between antibody titers to LKT and susceptibility to experimental challenge (142).

Although *P. haemolytica* MLV are apparently better than the older bacterins at eliciting a protective immune response, intradermally administered MLV provided protection in some studies (64) but not in others (125). The MLV should not be used within several days of antibacterial therapy or in stressed calves (64). One of the MLV vaccines has been chemically "altered" by passage in the presence of acriflavin·HCl (84, 85). Another recently licensed MLV is a vaccine containing a streptomycin-dependent strain of *P. haemolytica* that was obtained by mutagenesis with nitrosoguanidine (14, 22, 77). Although not proved, the MLV are assumed to provide better immunity than bacterins because they not only present important immunogenic somatic antigens but many of them also produce LKT during their somewhat brief survival in vivo.

As noted previously, LKT-neutralizing antibody has been correlated with immunity to
pneumonic pasteurellosis. This correlation is the basis for the LKT-enriched vaccines. The commercial version of this vaccine most often cited in the literature contains an LKT-rich, cell-free culture supernatant from a log-phase culture of a toxigenic strain of \textit{P. haemolytica.}

The LKT-rich vaccine has provided protection in challenge models and in the feedlot (74, 109, 137). In a recent study, Conlon et al. (28) found that, although LKT produced by rDNA enhanced the efficacy of commercial LKT-rich vaccine, recombinant LKT alone provided no significant protection over that of phosphate-buffered saline. Although the LKT-neutralizing titers were not reported by Conlon et al. (28), those results suggest that, although neutralizing antibody to LKT may be necessary for protection, it is not sufficient for full protection from pneumonic pasteurellosis, and other (soluble?) antigens are likely necessary for full immunity. Based on those results, however, supplementation of the commercially available cell-supernatant vaccine with additional recombinant LKT may be necessary.

Another bacterial pathogen that is a cause of respiratory disease in calves is \textit{Haemophilus somnus}. In addition, \textit{H. somnus} is also a cause of thromboembolic meningoencephalitis, myocardial abscessation, mastitis, arthritis, and reproductive problems (29, 70, 86). This organism is an important pathogen and may also be carried on the nasal, vaginal, or preputial mucosae (70). \textit{Haemophilus somnus} bacteria have been protective in experimentally induced thromboembolic meningoencephalitis and in the feedlot (128, 143), but their use in feedlot cattle is not usually recommended, in part because their efficacy is still somewhat in doubt (64). This lack of consistent efficacy may be related to the recently reported phase variation in the lipooligosaccharide of the bacterium in vivo (71). In addition, none of the \textit{H. somnus} bacteria have demonstrated activity against reproductive problems produced by this organism (64). In the way of subunit vaccines, an anionic, but not the cationic, saline extract was protective in a thromboembolic meningoencephalitis model (142). In addition, Corbeil et al. (30) and Gogolewski et al. (55, 56) proposed that a 40-kDa and, possibly, a 78-kDa outer membrane protein antigen may be excellent candidates for subunit vaccines or diagnostic reagents because these antigens have demonstrated efficacy in pneumonia and abortion models. Furthermore, these antigens appear to be common to all strains of \textit{H. somnus} and were originally reported as not being shared by \textit{P. haemolytica}, \textit{Pasteurella multocida}, \textit{Actinobacillus equuli}, or \textit{E. coli} (30). More recent work revealed that the originally described 40-kDa antigen actually was composed of two antigens, a “protective” 40-kDa antigen, antibody to which cross-reacted with other Gram-negative bacteria, especially members of the Pasteurellaceae, and a 39-kDa antigen, antibody to which cross-reacted only with \textit{Haemophilus suis} (31). Whether only the former or both of these antigens will prove to be a useful vaccine candidate remains to be determined.

The preceding discussion has stressed specific vaccine approaches for a specific pathogen. Another approach that has been intensively pursued recently has been development of a generic, “magic-bullet” vaccine for Gram-negative bacterial pathogens. This approach is best exemplified by the recent work using the common core antigen of Gram-negative bacteria. These vaccines are bacterins composed generally of either \textit{E. coli} or \textit{Salmonella} spp., which have lesions in the ability to synthesize complete lipopolysaccharide (LPS), resulting in colonial rough (R) mutants (33, 152). The most studied of these mutants, \textit{E. coli} J5, a genetically stable, uridine diphosphate, galactose-4-epimerase deficient, \textit{Rc} mutant of \textit{E. coli} O111:B4, and \textit{Salmonella typhimurium} or \textit{Salmonella minnesota} \textit{Rc} mutants have lesions in the early steps in LPS synthesis, and, thus, have exposed LPS core regions. [See reference (152) for a more complete discussion of R mutant classification.] Antibody induced to these exposed core regions are crossreactive with most Gram-negative bacteria, especially during periods of active growth (150, 153). These crossreactive antibodies are likely protective by promoting clearance of LPS by opsonization of the bacteria promoting their killing by phagocytes, or by neutralization of the pharmacologic activity of the toxic portion of LPS, the lipid A region (33, 152). Crossprotection against Gram-positive, viral, or parasitic diseases is not elicited by the common core antigen. Although O-specific immunity probably provides more solid and long-lasting immunity to disease in-
duced by a homologous Gram-negative pathogen, core antigen vaccines have been clinically useful when multiple Gram-negative pathogens or Gram-negative pathogens of unknown specific identity are involved, as in diseases such as coliform mastitis and calf respiratory disease and mortality. Coliform mastitis is discussed later in this review.

Regardless of the pathogens encountered in various cattle-rearing operations, the successful use of core antigen vaccines requires good management practices. Daigneault et al. (35) reported that, in well-managed dairy calf-rearing units, E. coli J5 antigen vaccination was associated with a 2.15-fold reduction in risk of death ($P = .042$) and a 1.34-fold reduction in risk of respiratory disease ($P = .053$). However, in that same study, vaccination of calves in a poorly managed unit was associated with an increased risk of death (35). At the well-managed facility in which bacteriologic isolations were made, 91% of the isolates were Gram-negative bacteria. The primary cause of death in the well-managed unit were bronchopneumonia, salmonellosis, and E. coli septicaemia. In the poorly managed unit, however, emaciation of various etiologies, including BVDV enterocolitis, was the primary cause of death. The apparent adverse reactions associated with E. coli J5 vaccination in the poorly managed unit may have been due to the failure of those calves to mount an Ig response as a result of their poor nutritional status or immunosuppression because of BVDV infection. In a more recent study by the Cullor group (1993, unpublished data), vaccination of calves in a large calf-rearing facility in central California with J5 bacterin was associated with a significantly lower mortality rate in calves 1 to 2 mo of age. The J5 bacterin was significantly effective in reducing mortality in the calves after 2 to 4 wk of age. In that study, which included 250 calves per group, calves were vaccinated at d 3 and 10 of age. Among calves >1 mo of age, 1 death occurred in the J5 vaccines compared with 12 deaths in the placebo group (1993, unpublished data).

Core antigen vaccines have also been protective in feedlot cattle (100). Feedlot calves (approximately 50 calves per group) were administered a commercial bacterin composed of an $R_0$ mutant of Sal. typhimurium in addition to their normal virus-vaccine regimen or not administered antigen other than the normal vaccine regimen. The group receiving the core antigen vaccine had significantly fewer “pulls and repulls” than with the group of unvaccinated calves. In addition, a reduction in treatment cost per head was noted with a decrease from $5.05 to $1.80 for the core antigen-vaccinated calves (100).

**MASTITIS VACCINES**

A number of problems are uniquely associated with vaccination of dairy cows for mastitis. Although these problems were discussed in earlier reviews on mastitis immunization (3, 25, 126), they bear repeating and are briefly summarized. First, mastitis, an inflammation of the mammary gland, is usually an immune response of the gland to invasive agents; i.e., the disease is equal to the immune response. Therefore, specific enhancement of the immune response may also exacerbate the disease. In addition, because of the large volume of milk in the udder, there is a dilution of the immune components available to fight infection, including Ig, lymphocytes, phagocytes, and complement. Similarly, the enormous surface area of the secretory epithelium greatly complicates immune surveillance of the gland. Also, milk components, particularly fat and casein, greatly reduce the phagocytic and bactericidal activity of professional phagocytes within the milk and gland. This effect is complicated further because milk components are also excellent growth substrates for a number of organisms that produce mastitis. In addition, the organisms that induce mastitis are numerous and heterogenous; Watts (169) estimates that more than 135 agents of mastitis, most of which are bacteria, exist. Finally, the location of the mammary gland itself, only a few inches from mud, manure, and urine, even in the best managed and cleanest facilities, ensures constant exposure of the gland to environmental mastitis pathogens. In addition to these inherent difficulties, the success or failure of a mastitis vaccine often is difficult to define. Should a vaccine reduce severity and frequency of mastitis, prevent new infections, eliminate existing infections, or do all three? Ideally, all three should be accomplished. However, this result is more than is expected of most other successful vaccines because
most vaccines do little more than prevent disease. Difficulties associated with vaccination of the gland notwithstanding, significant progress has been made in the past few years toward development of effective mastitis vaccines.

Commercially available bacterin vaccines for Staphylococcus aureus-induced mastitis are generally of dubious efficacy (64). Pankey et al. (118) found that a commercial bacterin, composed of a lysate of mixed phage types of Staph. aureus, given to cows in commercial dairy herds, reduced the incidence of new IMI by 50% during the second lactation and increased the spontaneous cure rate of these cows by 15%. During the first lactation, however, the incidence of new IMI or the spontaneous cure rates were not different. Pankey et al. (118) also reported that vaccinated cows that were experimentally exposed to Staph. aureus showed no difference from unvaccinated controls in their rate of new IMI. However, the spontaneous cure rates were significantly increased in the vaccinated groups whether the cows were in first or second lactation (118). The differences in spontaneous cure rates as influenced by lactation were not explained in this report (118), nor were the differences in susceptibility to new IMI from natural versus experimental exposure. In the experimental exposure group, however, the fewer cow numbers make these results less reliable. In another study, Pankey et al. (119) evaluated the same commercial bacterin in several hundred commercial dairy cows in three herds in New Zealand. Again, although no difference was observed in new IMI, spontaneous cure rates were increased significantly in the vaccinated groups compared with unvaccinated groups, 62 versus 21%, respectively.

The results of these studies emphasize that, although Staph. aureus bacterins may not be efficacious when IMI is the primary criterion, vaccination may provide some management advantage by increasing spontaneous cure rates, thereby reducing the frequency of subclinical mastitis in the herd.

In our laboratory, we have evaluated a number of commercially available and experimental vaccines using a Staph. aureus-induced mastitis model in lactating mice. The system used was modified from that originated by Chandler (20) and modified by Anderson (2) and was similar to the model that we used for evaluation of antimicrobial compounds (173). Our results with two different lots of the same commercially available bacterin used by Pankey et al. (118, 119) were similar to their results in dairy cows; the bacterin appears to have increased the incidence of spontaneous cures in vaccinated mice compared with unvaccinated or adjuvant-vaccinated mice. These results suggest that the mouse model may be useful for screening vaccine candidates. However, another commercial vaccine composed of inactivated Staph. aureus of three phage types supplemented with toxoids of the α- and β-toxins was tested and was ineffective in this model. The effectiveness of this bacterin in increasing spontaneous cures or in reducing the incidence of mastitis in dairy cows has not been reported.

The rationale for inclusion of staphylococcal exotoxins or toxoids in Staph. aureus vaccine preparations is based on the several studies (1, 15, 48, 91, 121) that have suggested that β-toxin, leucocidin, and, particularly, α-toxin are involved in virulence of Staph. aureus in the mammary gland. In addition, milk and serum antibody titers to α- and β-toxins increased significantly in cows infected with Staph. aureus (90). Although high anti-α-toxin titers protected rabbits from gangrenous mastitis (1) and although one report has suggested that bacterins supplemented with α- and β-toxoids are protective in dairy cows (113), few studies have specifically addressed the importance of the α- and β-toxoids in these preparations (112). Watson (164) reported that toxoid alone did not provide "much protection".

Using a live attenuated strain of Staph. aureus (W 79) administered s.c. as a vaccine, Watson (163) and Watson et al. (165) were able to provide moderate protection in ewes and heifers from challenge with homologous (W 79) and heterologous Staph. aureus stains. Although this work did not progress to a commercially viable product, their study revealed several important characteristics of the immune response in ruminants that confer protection from staphylococcal mastitis (165). Their results suggested that 1) the polymorphonuclear neutrophils (PMN) were the main effector in eliminating Staph. aureus; 2) protective immunization resulted in enhanced
phagocytic capacity of the mammary PMN; 3) elevated opsonic and cytophilic IgG2, but not IgG1 antibody, concentrations were necessary for this enhanced PMN activity (ruminant PMN lack IgG1 receptors); and 4) protective immunity results in an accelerated PMN response to infection. In addition, these and other studies (166) indicated that certain antigens expressed in vivo, but not always under standard in vitro culture conditions, may be important in protection from staphylococcal mastitis. At least one of these antigens expressed in vivo appears to be an antiphagocytic microcapsule or pseudocapsule (47, 110, 162).

Watson (164) and Watson and Watson (168) found that a vaccine composed of killed Staph. aureus, which had been cultured under conditions to induce a pseudocapsule, when combined with inactivated B-hemolysin (B-toxin) and dextran sulfate as the adjuvant, provided significant protection in ewes from intramammary challenge with homologous and heterologous Staph. aureus strains (164). Purportedly, dextran sulfate selectively enhanced production of antcapsular antibody of the IgG2 isotype. In a more recent study (167) in five commercial dairy herds in Australia involving 582 cows, a blind field trial with a similar vaccine administered i.m. to cows at 8 wk and 4 wk postcalving demonstrated that this capsule-enhanced bacterin could significantly reduce Staph. aureus mastitis in those herds. The incidence of clinical mastitis, the level of subclinical infection, and the level of new subclinical IMI that was due to Staph. aureus were reduced by 50, 18, and 25%, respectively (167). In studies by Nickerson (110, 111) with Watson’s vaccine, vaccinated cows also showed a reduction in new IMI compared with that of controls when the cows were challenged with Staph. aureus. In addition, in a study reported by Sears et al. (135) in which a bacterin composed of capsule-enhanced cells was supplemented with α-toxin in an oil-based adjuvant, the rate of new IMI was reduced in a small number (n = 19) of challenge-exposed cows in first lactation. Although statistical comparisons were not possible because of the small number of cows in the unvaccinated group, if the authors included historical, unvaccinated controls, the risk of new IMI for the unvaccinated cows was 4.6 times greater (P < .001) than for vaccinated cows (135). These studies suggest that a surface antigen, probably the pseudocapsular material, and perhaps the α- or β-toxins are important protective antigenic components for improved Staph. aureus mastitis vaccines.

Most mastitis-inducing strains of Staph. aureus do not produce classic capsules, as visualized with India ink, but can be demonstrated to produce a diffuse polysaccharide slime layer or a pseudocapsule, at least on primary culture (47, 114, 115, 117, 127, 168). Capsular polysaccharide (CPS) could be detected in milk of cows naturally infected with Staph. aureus if the milk was incubated at 38°C for 20 h (146). The CPS is antiphagocytic, and antibodies to the CPS are opsonic (59, 147). Although antibody in the serum and milk directed at the pseudocapsule could be detected with CPS-enhanced bacterins (110, 111, 116, 135, 164), infection by Staph. aureus alone, however, did not result in a detectable antibody response to CPS (90).

The present classification scheme of the CPS is based on immunological specificity for 11 chemically and configurationally different capsular antigens detected on strains of Staph. aureus (47, 49). Approximately 70% of strains isolated from bovine mastitis produce CPS serologically classified as type 5 or 8 (106, 123). Purified types 5 and 8 CPS are not immunogenic, at least for mice (41, 42, 43). This lack of immunogenicity has led to conjugation of types 5 and 8 CPS to carrier proteins such as Pseudomonas aeruginosa exotoxin A or diphtheria toxoid (41, 42, 43). These preparations induced antibody responses to CPS in mice that were high titer and T-cell-dependent. The antibody induced was opsonic, as indicated by enhanced type-specific phagocytosis of Staph. aureus by human PMN. These materials are in phase II clinical trials in humans (43). Although testing of conjugated CPS in cattle has yet to be reported, if CPS is as important an antigen as the work in mice has suggested, then conjugated CPS, combined with an adjuvant to induce opsonic IgG2 antibody in the cow, may well provide protection from Staph. aureus mastitis.

Another recent approach to provide a vaccine for Staph. aureus mastitis has been to use one of the presumed staphylococcal adhesion proteins as a vaccine. Staphylococcus aureus binds to the protein fibronectin in vitro and in
some infection models (36, 47, 106, 124, 133, 156). Also, fibronectin has been proposed to be one of the specific receptors for *Staph. aureus* adhesion and colonization (108, 133). This ubiquitous mammalian glycoprotein, which is found in either a soluble or tissue-bound form, is involved in eucaryotic cell adhesion, migration, and wound healing (36). *Staphylococcus aureus* has a fibronectin-binding protein on its surface that binds to a specific portion of the N-terminal region of fibronectin (108). Although fibronectin has never been conclusively demonstrated to play a role in primary colonization by *Staph. aureus* in the mammary gland, rDNA-produced fibronectin-binding protein was used by Flock (46) and Nelson et al. (108) to immunize against *Staph. aureus*-induced mastitis in the mouse or dairy cow. The rDNA-produced fusion proteins reduced the severity of *Staph. aureus*-induced mastitis in the mouse model (46, 108) and reduced incidence of clinical mastitis in dairy cows (108). In the dairy cow study, the fusion protein was administered as immunostimulating complexes (ISCOM) into the supramammary lymph node 4 wk prior to calving and again at 85 and 193 d later, during the lactation period. Cows were challenged with *Staph. aureus* strain Newbould 305 1 wk after the last vaccination. Three of 5 unvaccinated cows developed clinical mastitis, but only 1 of 5 vaccinated cows developed mastitis. The 1 vaccinated cow that developed mastitis for unexplained reasons did not respond immunologically to the fusion protein (108). Although these studies are interesting, whether a vaccine based on the fibronectin-binding protein or other *Staph. aureus* adhesion will be effective remains to be seen.

Research on vaccines for streptococcal mastitis has not been as prolific as for *Staph. aureus* and *E. coli* mastitis. For *Streptococcus agalactiae*, some studies indicated that previous infection and even hyperimmunization with *Strep. agalactiae* cells treated with formalin were not protective (94). However, progress has been reported more recently. Rainard and Poutrel (126) reported that they were evaluating a vaccine based on a protein that they designated “X”, which is shared by most mastitis strains, but not by human strains of *Strep. agalactiae*. This protein was immunogenic, resulting in opsonic antibody in the cow. Protein X may be similar to the 97,000- to 104,000-kDa protein antigen described by Wanger and Dunny (158); however, not enough information was provided to determine whether these proteins were identical (126). The French researchers’ (126) strategy was to couple protein X to streptococcal group B CPS, which gave opsonic antibodies when coupled to carrier protein. The results of trials with these materials are eagerly awaited.

With *Streptococcus uberis*, previous exposure does provide protection, at least with the homologous strain. Hill (61) found that previous infection with *Strep. uberis* strain 01401J significantly reduced the incidence of clinical mastitis on subsequent rechallenge of the same or other quarters with this same strain. In a recent study with a bacterin of formalin-killed *Strep. uberis* (without adjuvant), which was infused directly into the udder of dry cows, IgG1 and IgA antibodies specific for *Strep. uberis* could be detected in the milk for up to 2 mo after calving (107). Whether this response was protective from clinical mastitis was not tested. In addition, if an enhanced phagocytic response is necessary for immunity, IgG2 antibody response may need to be induced.

One of the major success stories in recent mastitis research has been the application of the core antigen vaccines, namely, the *E. coli* J5 vaccine, for coliform mastitis (32). As mentioned, J5 vaccination provides antibody responses that recognize heterologous Gram-negative bacteria. Therefore, vaccination with J5 could provide protection against not only the multiple serotypes of *E. coli* involved in coliform infections but also against the multiple genera and species of other Gram-negative bacteria involved in this disease (40). One of the first observations by the Cullor group (151) that indicated the potential usefulness of J5 as a mastitis vaccine was that dairy cows from a large herd in central California, which had serum IgG1 titers of <1:240 in an ELISA specific for J5 core antigen, had 5.33 times the risk of developing clinical coliform mastitis (CCM) than did cows with titers >1:240 (151). Subsequently, a field trial was conducted in two commercial dairies in California (57). The treatment group (246 cows) received three doses of J5 bacterin in Freund’s incomplete

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pyogenes, were unaffected by vaccination. Pro–
consideration when calculating the economic

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In contrast to the results of the field trials, some experimental challenge trials have not been as successful in demonstrating efficacy with J5 vaccines. Hill (62) found that vaccination of cows with either J5 or an Re mutant of E. coli K-12 failed to protect cows against experimental challenge with a virulent strain of E. coli. All cows developed acute clinical mastitis. Hogan et al. (66) also found that a J5 vaccine would not prevent IMI or CCM in an experimental challenge model using a heterologous, smooth strain of E. coli that had been isolated from a cow with CCM. However, the authors (66) did find that the J5-vaccinated cows had decreased severity in clinical signs compared with the unvaccinated control cows. In addition, the vaccinated cows had significantly lower bacterial counts in their milk and significantly better milk production and dry matter intake than unvaccinated controls (66). No obvious reasons explain the difference in results between field and challenge trials other than, perhaps, differences in virulence between field and challenge strains, differences in challenge numbers in natural versus experimental conditions, or potencies of the vaccine preparations.

Although the J5 bacterin is commercially available only in California, several companies are trying to obtain federal licensure. Theoretically, the core antigen vaccine composed of the Re mutant of Sal. typhimurium should have a similar application in reduction of CCM, but, at this writing, no data have been published demonstrating that this material is safe and efficacious for prevention of CCM, nor does the USDA-approved label (US veterinary license 345) stipulate that the Sal. typhimurium Re bacterin can be used for prevention of CCM.

BACTERIAL AND VIRAL HETEROLOGOUS
EXPRESSION VECTORS

The final area of discussion is the use of live attenuated viruses and bacteria as multivalent expression vectors for heterologous antigens. Although other vectors are possible, the live recombinant vectors that induce, or have the greatest potential to induce, a protective...
response in cattle include vaccinia virus, BHV-1, and Salmonella (19, 34, 45, 68).

Vaccinia virus has been the prototype virus for most poxvirus-based vector systems. Vaccinia is a large, DNA-containing virus that replicates in the cytoplasm of susceptible host cells. It has a genome large enough to allow the insertion of approximately 20 kb of additional genetic material and could probably be used to construct polyvalent vaccines for several different pathogens. This virus is unusual among the poxviruses in that it has an especially broad host range. Although originally isolated from cattle, vaccinia virus is infective for humans, rodents, pigs, and other mammals. However, this broad host range can also be a cause for concern over safety of vaccinia virus as an expression vector vaccine. Although a rare event, vaccinia can cause life-threatening complications even among healthy humans (68). Therefore, attenuation of vaccinia by deletion of the TK gene or the use of avian poxviruses that do not cause productive infections in mammals are currently being actively investigated as safer delivery systems for humans (9, 18, 68).

In cattle, vaccinia virus has been used as a heterologous expression vector to vaccinate against vesicular stomatitis virus and rinderpest virus (11, 68, 93, 174). In the former study, cattle were immunized intradermally with vesicular stomatitis virus-vaccinia recombinants. Although lesion development was reduced significantly from that of controls, some cattle immunized with vaccinia recombinants still developed lesions with an experimental challenge (93). With rinderpest virus, cattle immunized with a TK- vaccinia virus strain expressing either the hemagglutinin or the fusion protein of rinderpest virus were fully protected from all clinical manifestations of the disease when they were challenged with a 100% lethal dose (11, 174). These studies indicate that recombinant vaccinia or other poxviruses may have a practical place in control of certain diseases in cattle.

The use of TK- BHV-1 to express the FMDV VP1 epitopes has been discussed. In addition, use of i.n. administered BHV-1 recombinant, with deletion of gIII and insertion of E. coli β-galactosidase gene in its place, attenuated BHV-1 and induced a mucosal antibody response to the heterologous antigen (88). This study demonstrated that mutations other than in the TK gene can attenuate BHV-1 and that mucosal antibody responses to heterologous antigens are possible with BHV-1 vectors. Whether these responses will be protective for cattle has yet to be tested with relevant heterologous antigens using the gIII mutant viruses.

The use of attenuated Salmonella strains as vaccine delivery vectors has been extensively studied in a number of animal species and in humans (19, 21, 34). Attenuated Salmonella allow the possibility of providing an excellent vaccine for the salmonellae themselves (MLV), and properties related to the pathogenesis of the organism make these enteric pathogens uniquely suited as heterologous antigen expression vehicles. The main advantages of the salmonellae are that they 1) stimulate secretory, humoral, and cellular immune responses; 2) can be provided by oral immunization; and 3) may persist, providing long-term immunity to a heterologous antigen. The key disadvantage of Salmonella as a vaccine vector in cattle is the public health perception that Salmonella may be added to the human food chain, even when these strains are attenuated.

Although attenuation strategies for Salmonella spp. have taken several routes, the most studied and defined avenues have involved deletion of one or more of the genes in the aromatic biosynthetic pathways (ΔaroA, C, or D mutations) or deletions in both the genes encoding adenyl cyclase and cyclic AMP receptor protein (ΔcyaΔcrp) (19, 34). The Δaro mutations result in a requirement for the essential aromatic AA, tryptophan, phenylalanine, and tyrosine, which occur in the mammalian host, and a requirement for the essential metabolites 2,3-dihydroxybenzoic acid (precursor of enterochelin, the siderophore) and p-aminobenzoate (precursor to folate), which do not occur in mammalian tissues. The ΔcyaΔcrp mutations in Salmonella result in problems in global gene expression via the catabolite repression system. Cyclic AMP binding to the cyclic AMP receptor protein is required for expression of genes necessary for carbohydrate and AA transport and utilization, glycogen synthesis, type I pilus expression, flagellar synthesis, and expression of certain outer membrane proteins (34). Because these mutations are constructed as deletions, the or-

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organisms cannot revert to wild type. In addition, combination deletions, such as those occurring in the \( \Delta \text{aroA} \Delta \text{aroC} \), \( \Delta \text{aroA} \Delta \text{aroD} \), and \( \Delta \text{ctx} \alpha \Delta \text{crp} \) mutants, further reduce the probability of restoration to wild type as a result of a recombinational event because of the physical separation of these genes on the \textit{Salmonella} chromosome (132).

The use of \textit{Salmonella}-based bacterial vectors with defined, attenuating deletions was stimulated by the construction of \( \Delta \text{aroA} \text{Sal. typhimurium} \) mutants by transposon-mediated mutagenesis (67). Hoiseth and Stocker (67) were able to generate genetically stable \( \Delta \text{aroA} \) mutants that were avirulent for mice. Subsequently, \( \Delta \text{aroA} \) mutants of calf-virulent \textit{Sal. typhimurium} and \textit{Salmonella dublin} were constructed (129, 138, 139, 140). These mutants were attenuated for calves, producing only mild and transient symptoms or no symptoms on either oral or parenteral vaccination. Calves vaccinated by either oral or parenteral route were protected from challenge with lethal doses of virulent \textit{Salmonella} strains. However, \( \Delta \text{aroA} \) mutants of \textit{Sal. typhimurium} varied in their ability to provide solid immunity; of the three \textit{Sal. typhimurium} \( \Delta \text{aroA} \) mutant strains tested by Smith et al. (138), only one strain, a derivative of an originally calf-virulent isolate, was protective. Some of the \( \Delta \text{aroA} \) vaccines were able to protect calves against challenge with a homologous serotype as well as a heterologous serotype (139, 140). Calves vaccinated with either \( \Delta \text{aroA} \text{Sal. typhimurium} \) (group B) or \textit{Sal. dublin} (group D) were protected from challenge with normal lethal doses of the other serotype. At least part of the basis of this heterologous protection appeared to have been due to crossreactive opsonic antibody, perhaps directed at common somatic (both serotypes contain somatic antigens 1 and 12) or core antigens (75).

Recently, a double \( \Delta \text{aroA} \) mutant of \textit{Sal. typhimurium} was found to be safe and effective against experimental challenge with its calf-virulent parent strain (76). Seven-day-old calves were orally immunized with approximately \( 10^{10} \) cfu of a \( \Delta \text{aroA} \Delta \text{aroD} \) strain. Ten of 10 calves excreted the vaccine strain in the feces for \( \leq 7 \) d. Four of the 10 calves experienced a mild, transient diarrhea, and 1 of 10 calves showed a mild pyrexia. On challenge of 8 of the vaccinated and 4 unvaccinated (control) calves with the virulent parent 3 wk later, 7 of the 8 vaccinated calves were resistant to the challenge, exhibiting only transient, mild pyrexia. These calves excreted the challenge strain only for short periods and in low numbers. One of the vaccinated calves and 4 of 4 control calves, however, exhibited severe scouring, pyrexia, and were killed \textit{in extremis} or for humane reasons. The virulent challenge isolate was recovered from most of the tissues sampled from the scouring calves. Although it was not clear why 1 of the 8 vaccinated calves was not protected, the results are highly encouraging because these calves were very young on primary vaccination and received no booster dose (76).

Although no defined attenuation mutants are commercially available as vaccines for salmonellosis in calves currently, recent reports suggest that these may soon be available (38). Also, although no studies have been reported using cattle with \textit{Salmonella} spp. expressing heterologous antigens, recent studies using pigs and other species suggest that live \textit{Salmonella} vectors that provide protection against non-\textit{Salmonella} diseases are a practical possibility for cattle (19, 144).

**CONCLUSIONS**

Advances toward new and improved vaccines for calves, beef, and dairy cows are occurring rapidly. Recombinant DNA technology has had an important part in the recent advances in this area and will play an increasingly important role in the future toward the development of “perfect” vaccines. However, rDNA technology has not proved to be the panacea it was originally touted to be. Recombinant DNA technology provides a very powerful set of tools that, without knowledge about the pathogenesis of disease agents and the immune response of the host, been only crudely applied to some of the important questions still to be addressed in vaccine development.

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