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## Current and Future Applications of Monoclonal Antibodies against Bacteria in Veterinary Medicine

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### I. INTRODUCTION

Since Kohler and Milstein first reported the development of hybridoma technology in 1975, a revolution has taken place in biomedical research and clinical medicine. In the area of infectious diseases, monoclonal antibodies have been used as powerful probes for defining the antigenic structure, virulence mechanisms, and host responses to a broad range of pathogens including bacteria (72), viruses (164), and parasites (89). Numerous advances in immunodiagnosis, pro-

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phylaxis, and therapy have resulted, and future applications are forthcoming, at an ever-accelerating rate (26).

A general introduction to hybridoma technology and potential applications of monoclonal antibodies in veterinary medicine first appeared in the veterinary literature in 1982 (5). As is often the case, new knowledge leads to increased specialization, as evidenced by this present volume focusing on monoclonal antibodies solely against bacteria. This particular chapter is further specialized, and deals specifically with a discussion of current and future applications for monoclonal antibodies against bacteria in clinical veterinary medicine.

## II. BACKGROUND

Researchers and clinicians in veterinary medicine are cognizant of the tremendous impact that monoclonal antibodies are having in the areas of biomedical research and clinical application in human medicine, and reports of research involving monoclonal antibodies have begun to appear in the veterinary literature. Relatively little research activity however, is directed toward the study of bacteria. The cumulative Index Veterinarius for 1982 had no entries under monoclonal antibodies, while the 1983 edition had 69. Most entries dealt with viral antigens and identification of eukaryotic cell surface markers. Only three papers dealt specifically with bacterial antigens. In the 1984 edition, 7 of 71 monoclonal antibody citations involved bacteria, while in 1985, 22 of 102 entries dealt specifically with bacteria. In view of the scarcity of published material, the emphasis in this chapter on current and future applications of monoclonal antibodies in veterinary medicine will out of necessity deal largely with future uses.

Understanding and controlling infectious disease continues to be a major challenge in veterinary medicine, and bacterial pathogens are still a major concern. In 1984, \$6 million in research funds were available for veterinary research from the United States Department of Agriculture, Science and Education Administration, on a competitive basis to study all animal diseases (31). Sixty-six percent of these funds were awarded to researchers studying infectious disease problems, and 44% of this portion went specifically to the study of bacterial diseases. It is reasonable to assume that an increasing number of bacterial disease investigations will include the use of monoclonal antibodies in the future.

Four major uses for monoclonal antibodies against bacteria in veterinary medicine are readily identifiable: characterization of bacterial antigens and virulence factors, elucidation of the pathogenesis of bacterial infections, improvements in immunodiagnosis, and clinical applications in treatment and prophylaxis. The focus of this presentation will be primarily on diagnostic and clinical applications, acknowledging from the outset that advances in these areas depend critically on advances in basic research on the structure and function of bacterial

pathogens. The recent development of a monoclonal antibody to the K99 pilus of enteropathogenic *E. coli* for passive immunization of calves against enteric colibacillosis (127) was only possible because the development of hybridoma technology was preceded by years of basic research by numerous workers on the identification of adherence factors on enteric bacteria and their role in the pathogenesis of diarrhea (34). The antigenic structures, virulence attributes, and pathogenic mechanisms of many important bacterial pathogens in veterinary medicine are still uncharacterized. Now that hybridoma technology is readily available as a research tool, monoclonal antibodies will undoubtedly aid in first defining these unknowns and then providing new diagnostic and clinical applications.

There are certain limitations in the practice of veterinary medicine which make the potential diagnostic and clinical uses of monoclonal antibodies very attractive. The food animal practitioner must often carry out a thorough clinical examination and diagnostic workup in the isolation of a barn or feedlot, arrive at a specific diagnosis, and institute a therapeutic plan, all in a single farm visit. When an entire herd or flock is threatened with the spread of a rapidly disseminated infectious disease, this process takes on some urgency and the accuracy of the diagnosis becomes extremely important. Submission of samples to a distant diagnostic laboratory can be impractical. The development of rapid, reliable immunodiagnostic test kits for use on the farm is a recognized need. Certain immunodiagnostic procedures, such as enzyme-linked immunosorbent assays (ELISA), are adaptable for on-farm or in-practice use, and the inclusion of monoclonal antibodies as reagents in these kits will greatly enhance their practicality. The specificity of monoclonal antibodies would allow diagnostic tests to be performed directly on crude specimens such as feces, pus, mastitic milk, and tracheal lavage fluid without the intermediate step of bacterial culture (26). Furthermore, these monospecific reagents virtually eliminate the problems of cross-reactivity which can occur with conventionally derived polyclonal reagents. Specific diseases in which such diagnostic capability would be advantageous to the practitioner will be discussed.

A clinical application with tremendous potential in veterinary medicine is disease prevention by passive immunization, particularly when a newly initiated infection threatens to spread rapidly through a susceptible herd or flock. In many situations, numerous extrinsic factors play a role in the initiation and spread of bacterial disease. These factors include weather conditions, concurrent viral infections, sanitation, nutritional status, stress factors, stocking rates, ventilation, and recent transport (12). Disease outbreaks are often explosive and unpredictable. In many cases, active immunization with vaccines has not been undertaken because disease outbreaks were unexpected, efficacious vaccines are not available, or the cost-benefit ratio of prophylaxis was not considered. In situations such as these, the practitioner currently must intervene with antibiotic

therapy in an effort to reduce morbidity and mortality. However, the use of antibiotic therapy in food-producing animals is coming under increasing scrutiny due to concern about transfer of drug resistance from animal to human pathogens (55). Intervention with parenteral administration of monoclonal antibodies directed against specific virulence factors of the pathogens involved in order to reduce morbidity offers an attractive alternative to antibiotic therapy. As the knowledge of virulence factors in bacterial pathogens increases, more disease outbreaks will undoubtedly be handled in this way. Specific disease entities where this approach is currently being employed as well as diseases in which such intervention is likely to be successful will be discussed in detail.

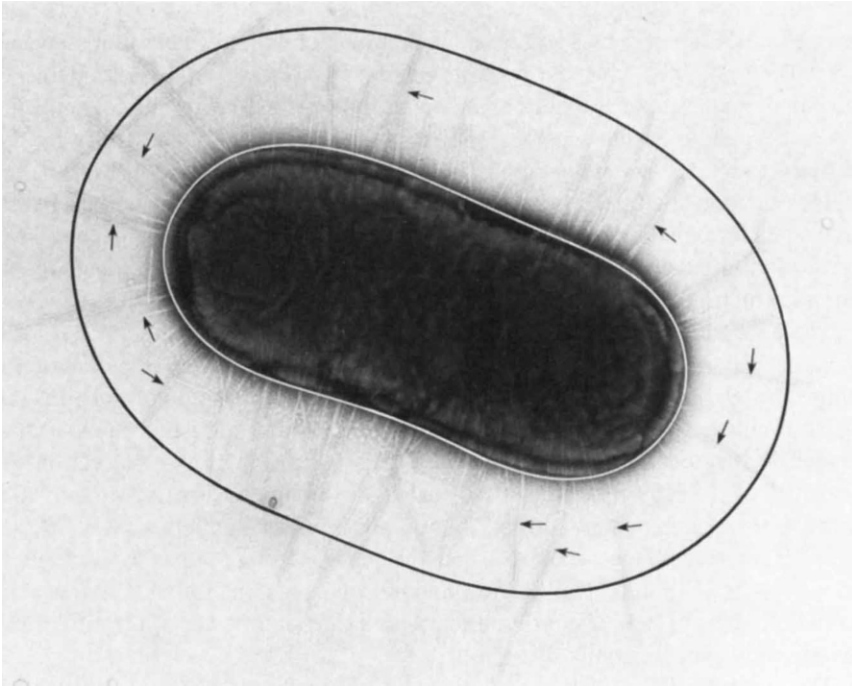
### III. RESULTS AND DISCUSSION

Practical applications of monoclonal antibodies in clinical veterinary medicine must be preceded by a firm basic knowledge of the structural character, virulence factors, and pathogenic mechanisms of disease-producing bacteria. The structure of this chapter is based on the structure of bacteria. It should become readily apparent that as more is known about the role of a particular structural component in the causation of disease, the more firmly based is speculation about the applications of monoclonal antibodies against that bacterial component.

The limitations of space prohibit extensive discussions of the disease conditions presented in this chapter. Although an attempt is made to provide sufficient background material on each disease, readers not familiar with clinical veterinary medicine are referred to a standard textbook of veterinary medicine for further clarification (12).

#### A. Bacterial Pilus Antigens

Pili are proteinaceous, filamentous appendages on the cell surface of certain bacteria (34) (Fig. 1). They are also known as fimbriae, adhesins, adherence factors, or colonizing factors. The role of pili in enabling certain pathogens to colonize epithelial surfaces successfully is well established. In human medicine, pili have been recognized as virulence factors in infections with *Neisseria gonorrhoeae* (107), enteropathogenic *Escherichia coli* (EPEC) (27), and *Pseudomonas aeruginosa* (161), among others. In veterinary medicine, the importance of pilus-mediated adherence has also been identified in EPEC infections causing neonatal enteric colibacillosis of calves, lambs, and pigs (12,34), *Moraxella bovis* infections causing infectious bovine keratoconjunctivitis (IBK; pinkeye) (113), and *Bacteroides nodosus* infections causing foot rot of sheep (145). A role for pilus-mediated adherence is being investigated for a number of other important veterinary pathogens including various *Salmonella* (88), *Pasteurella* (40),



**Fig. 1.** Example of a heavily piliated *Escherichia coli*. This is *E. coli* O78 isolated from a turkey. (Photograph courtesy of Dr. L. H. Arp, Department of Veterinary Pathology, Iowa State University, Ames, Iowa.)

and *Bordetella* (41) species. This knowledge has led to the development of pilus-enriched vaccines for active immunization against some of these pathogens (2,61,91,94,99,100,121,145,160). In the case of the enteropathogenic *E. coli*, monoclonal antibodies have been developed for diagnostic use as well as for passive immunization of newborn calves and pigs (122,127,159). These developments as well as potential applications of monoclonal antibodies for control of pinkeye, foot rot, and other pilus-mediated infections in veterinary medicine will be discussed.

### **1. Pili of Enteropathogenic *Escherichia coli***

#### **a. The K99 Pilus**

*i. Passive Immunization.* The development of a monoclonal antibody against the K99 pilus of EPEC for passive protection of newborn calves against fatal enteric colibacillosis (127) is illustrative of how firm knowledge of the pathogenesis and epidemiology of a specific disease can lead to innovative methods of

disease control. Strains of noninvasive *E. coli* capable of producing diarrhea in neonates must possess two virulence factors, both of which are plasmid mediated (44,93). First, they must be enterotoxigenic, possessing either heat-stable or heat-labile enterotoxin capable of inducing hypersecretion by villous epithelial cells. Second, they must possess pili to colonize the gut successfully by adherence to the villous epithelium. The physical proximity of the bacteria to the villous surface, facilitated by pilus adherence, maximizes the deleterious effects of the enterotoxin. Nonpiliated enterotoxigenic strains and piliated nonenterotoxigenic strains cannot produce disease of equal magnitude to the enteropathogenic strains which possess both pilus and toxin.

The K99 pilus is found on EPEC infecting calves, lambs, and pigs (92,104). It was first reported in 1972 and was identified at that time as Kco, the common K antigen (139). In 1975 it was designated as K99 (104). Isolation of the pilus can be accomplished with either a salt extraction procedure, ultrasonication, or mechanically, by heating and shaking of a cell suspension. Early reports on the structure of the K99 pilus suggested that it was composed of two protein subunits, a major component of MW 22,500 and a minor subunit of MW 29,500 (59). More recent work indicates that the pilus is composed of only a single subunit of MW 18,400 (19). Electron microscopic examination of K99 reveals it to be a fimbria of helical configuration with a diameter of 4.8 nm. Purified pili are strongly immunogenic.

The susceptibility of calves and lambs to colonization by K99-positive EPEC is age dependent. These species become resistant to experimental challenge after 2 days of age, as has been demonstrated both *in vivo* and *in vitro* (117,136). Although the nature of this natural resistance is not well understood, the clinical significance of this observation was profound. It led to the hypothesis that if susceptibility to enteric colibacillosis occurs only in the first few days of life, the presence of K99-specific antibody in the gut lumen during that time might successfully block intestinal adherence by K99-positive EPEC, thus inhibiting colonization until such time that the calf possessed natural resistance to infection (2). In livestock species, maternal antibodies are derived from colostral ingestion rather than by transplacental transfer (83). Experiments were undertaken to demonstrate whether cows vaccinated prepartum with killed bacterins containing either K99-positive strains of *E. coli* or purified K99 pili would produce and concentrate K99-specific antibodies in their colostrum. Calves ingesting this colostrum at birth would then be protected from enteric colibacillosis. This hypothesis was proven repeatedly to be accurate in experimental challenge studies (2,47,99). As a result, numerous vaccines have become commercially available for active immunization of the cow for passive protection of the calf. The history of vaccine development in enteric colibacillosis of calves has recently been reviewed (46).

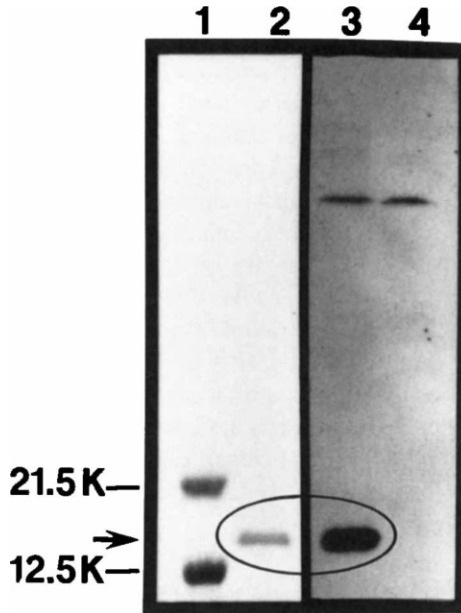
Although these vaccines have been used with apparent success, some practical

disadvantages to maternal vaccination have been recognized. Pregnant cows must be handled for vaccination twice during the first year and once during each subsequent year of the vaccination program (48). Some livestock owners are reluctant to accept the cost and inconvenience of preventative vaccination unless they have recently experienced an outbreak of enteric colibacillosis in their herds. Such outbreaks are difficult to predict, since the epizootiology of neonatal calf diarrhea includes a variety of management and environmental factors. When outbreaks do occur suddenly in unvaccinated herds, it is too late to immunize actively those pregnant cows that are close to parturition. These concerns prompted investigation of an alternative method of passive immunization of newborn calves, namely, the oral administration of K99-specific hybridoma-derived monoclonal antibody shortly after birth. The development of this K99-specific monoclonal antibody and its efficacy in protecting calves against fatal enteric colibacillosis in challenge trials has been reported (127). It represents the first known application of a monoclonal antibody for passive immunization against an important bacterial disease. The details of this work will be briefly reviewed.

The source of antigen was the K99 pilus of strain B41 EPEC, isolated and purified according to the method of Isaacson (59). The antigen, in complete Freund's adjuvant was injected into BALB/c mice. Spleens were removed after a series of three antigen injections and hybridomas were produced by polyethylene glycol-mediated fusion of mouse spleen cells with mouse plasmacytoma cell line P3-NS-1-Ag 4/1. Resulting hybridomas were grown in selective medium containing hypoxanthine, aminopterin, and thymidine in 24-well tissue culture plates. Supernatants from wells with hybridoma clones were screened for K99-reactive antibody using an ELISA technique with purified K99 as the antigen. Hybridoma monoclones producing K99-specific monoclonal antibody were isolated by limiting dilution. A single clone (2BD4E4) was selected and injected intraperitoneally into specific pathogen-free BALB/c mice, preconditioned with pristane. Mice were killed 10–60 days later and ascitic fluid aspirated. The fluid was clarified by centrifugation and stored at  $-20^{\circ}\text{C}$ .

Characterization of the K99-specific monoclonal antibody was accomplished using several techniques including ELISA, bacterial agglutination, and immunoprecipitation of radiolabeled EPEC strain B44. The monoclonal antibody from clone 2BD4E4 was shown to be IgG<sub>1</sub> by ELISA with rabbit anti-mouse IgG<sub>1</sub> antibody. A pool of ascitic fluid from this clone exhibited a titer of  $10^{-5}$  in the K99–ELISA system. Immunoglobulin constituted 45–50% of the protein in the ascites fluid as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 2). The antibody was found to be reactive only with K99 antigen in radioimmunoprecipitation of L-[<sup>35</sup>S]methionine-labeled *E. coli* lysates followed by SDS–PAGE. This immunoglobulin at an initial concentration of 10 mg/ml agglutinated EPEC strains B44 and B41 grown at  $37^{\circ}\text{C}$





**Fig. 2.** Immunoprecipitation of an L-[<sup>35</sup>S]methionine-labeled lysate of EPEC strain B44 with the K99-specific monoclonal antibody orally administered to calves. Immunoprecipitates were subjected to electrophoresis in a 12.5% acrylamide slab gel. Proteins in lanes 1 and 2 were detected by Coomassie Blue staining prior to salicylation of the gel. Molecular weight markers (in thousands, K) are indicated in lane 1. The K99 antigen used subsequently for production of the K99-specific monoclonal antibody is indicated by the arrow in lane 2. Lane 3 represents the radioactive antigen precipitated from EPEC strain B44 by the K99-specific monoclonal antibody. Lane 4 represents a similar precipitate by fibronectin-specific monoclonal antibody. Reprinted from reference 127 by permission of the American Society of Microbiology, Copyright © 1983, from *Infection and Immunity*, 1983, **42**, 653-658.

(K99 pilus expressed) to a dilution of  $10^{-4}$ , but did not agglutinate cells grown at  $18^{\circ}\text{C}$  (K99 pilus not expressed), even when used at  $10^{-1}$ .

Three separate challenge trials were conducted to evaluate the efficacy of orally administered K99-specific monoclonal antibody in protecting neonatal calves against enteric colibacillosis. In the first trial newborn calves were fed colostrum at birth and challenged orally with  $2.6 \times 10^{10}$  organisms of EPEC strain B44 (O9:K30;K99;F41:H-) at 12 hr of age. At 10 hours of age, treated calves received 1 ml of clarified mouse ascites fluid containing K99-specific monoclonal antibody with a titer of 1:12,000 as determined by passive hemagglutination. Control calves received no placebo. In the second and third trials, newborn calves were colostrum deprived at birth. These calves were challenged with an average of  $5 \times 10^9$  organisms of EPEC strain B44 at 12 hr of age. Treated calves received the same oral dose of mouse ascites fluid at 10 hr of age.

Control calves received a placebo of 1 ml mouse ascites fluid containing monoclonal antibody specific for fibronectin. The only difference between trials 2 and 3 was that in trial 2, calves receiving treatment were known to the investigator while trial 3 was performed as a double blind. In all trials calves were examined every 12 hr and their clinical status was scored based on an assessment of state of dehydration, fecal consistency, and mental attitude, up to 72 hr postchallenge.

Treatment with the K99-specific monoclonal antibody did not affect the incidence of diarrhea after challenge with EPEC strain B44. There was no difference in the proportion of treated and control calves that developed diarrhea in any of the three trials (Table I), nor was there any significant difference in the average score of fecal consistency between treated and control groups in any of the three trials (Table II). As calculated in trial 1 only, there was also no significant difference in the time of onset of diarrhea after challenge between the control and treated calves.

In contrast to the above results, there was a significant difference in the incidence of severe dehydration as measured by estimated loss of skin elasticity between control and treated calves (Table I). The proportion of calves that became severely dehydrated after challenge was significantly lower in calves receiving the K99-specific monoclonal antibody as compared with control calves in all three trials. This reduction was highly significant ( $p < 0.001$ ) when the results of the three trials were combined. There was also a significant reduction

TABLE I

Clinical Response of Calves after Challenge with EPEC Strain B44<sup>a</sup>

Trial	K99-Specific monoclonal antibody-treated calves			Controls		
	Diarrhea <sup>b</sup>	Clinical dehydration <sup>c</sup>	Death	Diarrhea	Clinical dehydration	Death
1	4/7	1/7 <sup>d</sup>	1/7 <sup>d</sup>	5/7	5/7	5/7
2	5/5	1/5 <sup>d</sup>	1/5 <sup>d</sup>	4/4	4/4	4/4
3	12/16	6/16 <sup>d</sup>	6/16 <sup>d</sup>	5/6	5/6	5/6
TOTAL	21/28 (75%)	8/28 <sup>e</sup> (29%)	8/28 <sup>e</sup> (29%)	14/17 (82%)	14/17 (82%)	14/17 (82%)

<sup>a</sup> Adapted and reprinted from reference 127 by permission of the American Society of Microbiology, Copyright © 1983, from *Infection and Immunity*, 1983, **42**, 653-658.

<sup>b</sup> Expressed as the proportion of calves in the group that developed a fecal consistency score of 3 after challenge. A score of 3 represented severe, watery diarrhea.

<sup>c</sup> Expressed as the proportion of calves in the group that developed a clinical dehydration score of 2, representing severe dehydration.

<sup>d</sup>  $p < .05$  versus control by one-tailed  $\chi^2$  test.

<sup>e</sup>  $p < .001$  versus control by one-tailed  $\chi^2$  test.

TABLE II

Mean Clinical Scores of Calves after Challenge with EPEC Strain B44<sup>a,b</sup>

Trial	Calves	MCS (0-8)	DEP (0-3)	DEHY (0-2)	FC (0-3)	Weight loss (kg)	Duration of diarrhea (hr)	Onset of diarrhea after challenge (hr)
1	T	2.9 ± 2.7 (<.05)	0.4 ± 1.1 (<.025)	0.4 ± 0.8 (<.0125)	2.0 ± 1.4 (NS)	ND	22.5 ± 3.0 (<.025)	31.0 ± 24.9 (NS)
	C	6.1 ± 3.3	2.1 ± 1.5	1.6 ± 0.8	2.4 ± 1.1	ND	58.8 ± 42.5	14.8 ± 3.7
2	T	4.2 ± 2.2 (<.005)	0.8 ± 1.3 (<.005)	0.4 ± 0.9 (<.005)	3.0 ± 0 (NS)	3.2 ± 2.8 (<.025)	ND	ND
	C	8.0 ± 0	3.0 ± 0	2.0 ± 0	3.0 ± 0	7.6 ± 2.3	ND	ND
3	T	4.7 ± 2.7 (<.0125)	1.3 ± 1.2 (<.0025)	0.9 ± 1.0 (<.0025)	2.6 ± 0.9 (NS)	3.1 ± 3.0 (<.05)	ND	ND
	C	7.3 ± 1.6	2.7 ± 0.8	1.8 ± 0.4	2.8 ± 0.4	5.9 ± 3.3	ND	ND
Combined	T	4.1 ± 2.4 (<.001)	1.0 ± 1.2 (<.001)	0.7 ± 0.9 (<.001)	2.5 ± 1.0 (NS)	3.1 ± 2.9 (<.0025)	ND	ND
	C	7.0 ± 3.8	2.5 ± 1.1	1.8 ± 0.6	2.7 ± 0.8	6.6 ± 3.0	ND	ND

<sup>a</sup> Adapted and reprinted from reference 127 by permission of the American Society of Microbiology, Copyright © 1983, *Infection and Immunity*, 1983, 42, 653-658.

<sup>b</sup> All data are expressed as mean ± SD; numbers within parentheses are *p* values versus control, using the one-tailed *t*-test. Abbreviations: T, treated group; C, control group; MCS, maximum clinical score; DEP, degree of depression; DEHY, degree of dehydration; FC, fecal consistency; NS, not significant; ND, not done.

in the severity of systemic illness between treatment and control groups in all three trials as measured by comparison of mean scores earned for degree of clinical dehydration, degree of clinical depression, and maximum clinical score (Table II). In addition, a significant difference was noted in the duration of diarrhea after challenge when measured in trial 1, and a significant difference was observed in the degree of weight loss between treated and control calves when measured in trials 2 and 3 (Table II).

Most importantly, treatment with the K99-specific monoclonal antibody significantly reduced mortality in challenged calves in all three trials (Table I). When the results of all three trials were considered together, the mortality rate in the calves receiving the K99-specific monoclonal antibody was only 29% as compared with 82% in the control calves. This difference was highly significant ( $p < 0.001$ ).

It was concluded that treatment with 1 ml of mouse ascitic fluid containing K99-specific monoclonal antibody reduced the severity of diarrhea and the mortality rate after challenge with EPEC strain B44. This finding supported the earlier work (2) which indicated that immunization of cows with purified K99 antigen before calving stimulated their production of K99-specific antibodies which were passively transferred to their calves and which prevented fatal diarrhea. In this study, antibody directed only against the K99 pilus of EPEC significantly reduced the severity of diarrhea caused by strain B44, which carries two adherence pili (K99 and F41), as well as the K30 capsular antigen, which is also believed to mediate adherence (98). This suggests that the degree of colonization and, hence, the clinical severity of disease, was reduced by the K99-specific monoclonal antibody. The fact that diarrhea was not eliminated completely was attributed to the possibility that either performed enterotoxin was present in the challenge inoculum, that the dose of monoclonal antibody was not sufficient to block adherence by K99 pili completely, or that F41-mediated adherence with some colonization of the gut may have occurred. The fact that 29% of treated calves died was attributed to several factors, including the deprivation of colostrum in trials 2 and 3, and the presence of concurrent infections. Postmortem examination in some treated calves revealed the presence of concurrent rotavirus and/or coronavirus in the intestine.

This work has led to the development and use of a commercially available K99-specific monoclonal antibody preparation for oral administration in newborn calves to prevent fatal enteric colibacillosis.<sup>2</sup> The application of hybridoma technology for the development of a K99-specific antibody which could be prepared by conventional means has been perceived by some as a case of technological overkill. In fact, a conventionally raised K99-specific antibody product for oral administration directly to calves has been developed and marketed in

<sup>2</sup>Genecol™99, Molecular Genetics, Inc., Minnetonka, Minnesota.

Israel (151). However, hybridoma technology has advanced so dramatically since the first published report in 1975, that the cost of producing hybridoma-derived monoclonal antibody compares favorably to the production of conventional antisera (C. C. Muscoplat, Molecular Genetics, Inc., Minnetonka, Minnesota, personal communication). The superiority to conventionally produced antisera is obvious when the specificity, concentration, and uniformity of hybridoma-derived antibody is considered.

*ii. Immunodiagnosis.* The development of a monoclonal antibody to the K99 pilus should have a considerable impact on the diagnosis of enteric colibacillosis. Rapid diagnosis of enteropathogenic *E. coli* as a cause of neonatal diarrhea is important for several reasons. The disease tends to spread rapidly among newborn calves, particularly when cattle are maintained in close quarters (12). Numerous etiologic agents may produce diarrhea in young calves. Although EPEC account for the majority of these infections, rotavirus, coronavirus, *Salmonella* sp., *Clostridium perfringens*, cryptosporidia, and other less common pathogens may also occur (12,16,152). A specific diagnosis of K99-positive EPEC allows the practitioner to choose an appropriate therapy, initiate a preventative program, and offer a more accurate prognosis.

The conventional diagnostic procedure has been the collection of a fecal swab from the live calf or an ileal swab from necropsy, with subsequent culture on bacteriologic medium favoring pilus expression such as Minca-Is (45). After overnight incubation, several colonies are selected and slide agglutination tests are performed using conventionally raised antisera to K99. Alternately, colonies are smeared on a slide and examined using fluorescent-antibody techniques with K99-specific antisera conjugated to fluorescein. Several limitations exist with these methods. With both techniques, overnight culture is required for isolation of EPEC before a diagnosis can be made. Practitioners may not be equipped to perform either test. Since many nonpathogenic *E. coli* may be present in fecal swabs, inappropriate selection of colonies from the initial culture may lead to false-negative results.

In 1979, Ellens *et al.* (24) reported on the development of an ELISA technique which allowed detection of the K99 antigen directly from calf feces. In a comparison with the conventional culture and agglutination technique, the diagnostic sensitivity of the ELISA was equivalent to the standard test. However, overnight culture was eliminated and test results were available in 6 hr. The ELISA employed a rabbit-derived K99-specific antisera purified by adsorption of the hyperimmune serum with a K99-negative variant of the parent strain from which the pilus was first derived. To perform the ELISA, extensive processing of the fecal specimen was first required. Samples were diluted in phosphate-buffered saline, homogenized by ultrasonication, and then centrifuged for clarification. Some cross-reactivity occurred when bovine coronavirus was present in the fecal specimens and required blocking with bovine anticoronavirus serum.

Mills and Tietze (86) have reported on the use of K99-specific monoclonal

antibody (2BD4E4) in a sandwich ELISA for detection of K99 pilus on *E. coli* isolates after bacterial culture of fecal samples. More recently, a sandwich ELISA technique utilizing a K99-specific monoclonal antibody for detection of the K99 antigen directly from fecal samples has been developed (D. E. Reed, Molecular Genetics, Inc., Minnetonka, Minnesota, personal communication). The technique requires no preparation of the fecal sample, yields results in less than an hour, and is designed as a test kit for use in the veterinary practice. The source of the monoclonal antibody is again clone 2BD4E4. A fecal swab or a 0.2-g sample of feces is suspended in a tube of diluent buffer by shaking. The K99-specific monoclonal antibody linked to horseradish peroxidase is added to the tube. A polystyrene wand coated with the K99-specific monoclonal antibody is then placed in the sample tube and incubated for 15 min. The wand is then washed in cold water and placed in a second tube containing the enzyme substrate. After 15 min, the color change is visually observed. A positive control is run with each sample as a color standard. Results are based on the concentration of pilus antigen in the sample and are read as negative ( $< 0.1 \mu\text{g}$  of K99 antigen), low positive ( $0.1\text{--}1.0 \mu\text{g}$ ), or high positive ( $< 1 \mu\text{g}$  of K99 antigen). Due to the consistent concentration and avidity of the monoclonal reagents, the sensitivity of the test can be easily titrated to a desired level of sensitivity.

The specificity of this test system has been demonstrated by testing fecal samples from gnotobiotic calves infected with various enteric viruses as well as by testing bacterial cultures of numerous species and strains of enteric bacteria known to be K99 negative. Two variations of this test system using monoclonal antibody reagents have been reported: a competitive ELISA test and a three-step sandwich ELISA system (159). The latter has since been marketed commercially to practicing veterinarians.<sup>3</sup>

**b. The K88 Pilus.** The K88 pilus is found only on EPEC isolated from porcine enteric colibacillosis. Presence of the pilus is plasmid mediated, and there appears to be a strong association with certain O serotypes of EPEC, most notably O8, O45, O138, O141, O147, O149, and O157 (34). Strains carrying the K88 pilus may produce either heat-stable or heat-labile enterotoxin, or both (135). Pili on K88-positive strains are visible with electron microscopy and have a diameter of 2.1 nm (34). The molecular weight range is 23,500–26,000 (90). Three immunologically distinct K88 pilus structures are recognized (K88ab, K88ac, and K88ad), with the “a” fraction representing a common antigenic determinant (43,106). Partial amino acid sequences of the three K88 antigens have been characterized (34). The plasmid genes responsible for their production have been isolated by molecular cloning (34).

The progress in understanding the role of the K88 adhesin in porcine enteric

<sup>3</sup>Coli-Tect™99, *E. coli* K-99 Antigen Test Kit, Molecular Genetics, Inc., Minnetonka, Minnesota.

colibacillosis, and the development of vaccines to prevent clinical disease is analogous to that described above for the K99 pilus. The K88 antigen was first described in 1961 as a serologically distinct antigen associated with *E. coli* strains isolated from pigs with edema disease and neonatal enteritis (105). It was later demonstrated that the K88 antigen was an adherence factor which facilitated colonization of the small intestine (6,65,158). This was confirmed by several *in vivo* and *in vitro* studies wherein adherence of K88-positive EPEC to intestinal villous epithelium was blocked by antisera against the K88 antigen (65,134,138,158). Based on these findings, vaccine trials were carried out and demonstrated that sows vaccinated with K88-positive EPEC would produce high levels of colostral K88-specific antibody which would passively protect newborn suckling pigs from enteric disease produced by K88-positive EPEC (119,120). Numerous commercial vaccines are now available for immunization of sows to control K88-positive EPEC-induced enteritis in their offspring (157).

As was described in the case of enteric colibacillosis of calves due to K99-positive EPEC, numerous epidemiologic factors contribute to the development of clinical disease, and in swine herds managed in confinement systems, neonatal colibacillosis can be unpredictable in onset and severe in nature (3). Analogous to the situation in calves, in swine herds where active immunization of sows has not been carried out prior to an outbreak of piglet enteritis, morbidity and mortality might be reduced through passive immunization of newborn pigs by direct oral administration of K88-specific antibody. P. L. Sadowski (Molecular Genetics, Inc., Minnetonka, Minnesota, personal communication) has produced K88-specific monoclonal antibodies using techniques similar to those described earlier for the production of the K99-specific monoclonal antibody. Three distinct monoclonal antibodies have been produced, showing specificity for the a, b, and c antigenic determinants of the K88 antigen. The protective effects of both the a and c antibodies when administered orally to newborn pigs have been demonstrated in challenge trials using a K88ac-positive EPEC strain (122).

It is interesting to note that in Europe, where active immunization of sows using vaccines containing K88ab and K88ac-positive strains of EPEC has been carried out for a number of years, EPEC isolates obtained from the intestines of pigs dying of colibacillosis in field outbreaks are increasingly of the K88ad variety and less frequently of the K88ab variety (43). It appears that vaccination is exerting some selection pressure on EPEC, and that the organisms are responding with variation in their adherence structures (34). Passive immunization of piglets with monoclonal antibodies could also potentially contribute to this selection pressure. Alternatively, the avidity of a K88a-specific monoclonal antibody might be capable of blocking adherence by all K88-positive EPEC, despite variations in their secondary structure.

One potential limitation of passive immunization by oral administration of K88-specific monoclonal antibody arises from the fact that, unlike K99-positive

EPEC infections in the calf, which are restricted to the first few days of life, K88-positive EPEC infections in pigs can occur throughout the suckling and weaning periods (157). Continuous administration of monoclonal antibody would be impractical, and passive immunization would have to be employed only in the face of outbreaks, or at known peak periods of susceptibility, namely during the first few days of life and again, just after weaning.

The conventional diagnostic procedures for confirming K88-positive EPEC infection in pigs are similar to those described above for K99-positive EPEC infection. A sandwich ELISA has been described for identification of the K88 pilus from bacterial suspensions of *E. coli* cultured from clinical specimens (87). It is reasonable to assume that K88-specific monoclonal antibody will facilitate the development of ELISA test kits which allow rapid detection of the K88 antigen directly from fecal specimens or swabs.

**c. The 987P Pilus.** The 987P pilus is also restricted to porcine isolates of EPEC (34). It is most often associated with EPEC strains O9, O20, and O141, and always is found with heat-stable enterotoxin only (34). Structurally, 987P is a glycoprotein of MW 20,000 (60). It is a rigid fimbria with a diameter of 7 nm. Unlike the other host-specific adhesins described, 987P does not demonstrate mannose-resistant hemagglutination of erythrocytes, and is coded on the bacterial chromosome rather than by a plasmid (137).

The role of 987P as an adherence factor facilitating colonization of the porcine intestine has been well established. Purified 987P pilus and Fab fragments of antibody specific for 987P have been shown to block adherence of 987P-positive EPEC strains to porcine small intestine epithelial cells *in vitro* (62). Numerous challenge trials have demonstrated that antibody to the 987P pilus will protect piglets from enteric colibacillosis with 987P-positive EPEC. Vaccination of sows with 987P-positive EPEC will increase 987P-specific antibody levels in colostrum and protect suckling pigs from clinical disease (61,91,94).

P. L. Sadowski (Molecular Genetics, Inc., Minnetonka, Minnesota, personal communication) has produced a monoclonal antibody to the 987P pilus using techniques similar to those described above for the production of the K99-specific monoclonal antibody, and protection studies have been carried out. Orally administered 987P-specific monoclonal antibody was shown to protect colostrum-deprived newborn piglets from challenge with EPEC strain 987. Significant reductions in the clinical severity of disease and in mortality rates were demonstrated as compared to challenged control pigs not receiving the monoclonal antibody.

Outbreaks of enteric colibacillosis in young pigs may be due to either K99-positive, K88-positive, or 987P-positive strains of EPEC (28). Although there is a tendency for K88-positive strains to produce diarrhea in pigs over 2 weeks of age and K99-positive and 987P-positive strains to occur more frequently in



younger pigs, there is much overlap in these infections (33). This observation suggests that for prevention of enteric colibacillosis in piglets by oral administration of monoclonal antibody, the specificity of the monoclonal antibody is actually a handicap. The practical solution to this problem would be the administration of a trivalent mixture of monoclonal antibodies to the three known porcine EPEC adherence pili. A commercial product containing monoclonal antibodies against the K99, K88, and 987P adhesins is currently being evaluated for use in controlling outbreaks of enteric colibacillosis in young pigs (C. C. Muscoplat, Molecular Genetics, Inc., Minnetonka, Minnesota, personal communication).

**d. The F41 Pilus.** The F41 pilus is the most recently characterized adhesin of EPEC important in veterinary medicine (20). At present, F41 has been identified only in bovine strains of EPEC and can be found in conjunction with the K99 pilus in certain EPEC strains, most notably B41 (20,95). The presence of F41 is plasmid mediated. It occurs in the O9 and O101 serogroups of EPEC, and is associated exclusively with heat-stable enterotoxin (34). It has a fimbrial structure with a diameter of 3.2 nm (20). Chemical characterization reveals the F41 pilus to be a protein of MW 29,500 whose amino acid sequence has been identified (20). The role of the F41 pilus in the production of bovine enteric colibacillosis is not yet as firmly established as is the role of the K99 pilus. However, there are several observations which suggest that the F41 pilus is a virulence factor. A K99-negative, F41-positive, mutant strain of B41 (B41M) was shown to adhere to calf enterocytes *in vitro* and to produce diarrhea in newborn germfree piglets (95).

No vaccine trials have been reported to date. It is reasonable to assume, however, that once the role of F41 is clarified in bovine neonatal diarrhea, active immunization of the dam to produce F41 specific colostral antibody will be practiced. In turn, the development of an F41-specific monoclonal antibody for direct, oral passive immunization of the calf, is likely to follow.

## 2. Pili of Other Veterinary Bacterial Pathogens

**a. *Moraxella bovis*.** Infectious bovine keratoconjunctivitis (IBK) is a common cattle disease of major economic significance. A comprehensive review of IBK has recently been published (113). It is generally accepted that *Moraxella bovis* is the primary initiator and mediator of this disease, although a role for numerous other pathogens, including viruses, mycoplasma, and chlamydia, have been suggested. Infection of the conjunctiva with *M. bovis* produces severe inflammation resulting in lacrimation, blepharospasm, photophobia, corneal edema, temporary blindness, and residual scarring. Ocular pain and impaired vision lead to reduced feed intake and decreased milk production with substantial economic loss to both beef and dairy cattle producers.

Numerous factors appear to contribute to the initiation of clinical disease. Outbreaks of IBK occur most commonly in the summer and autumn. This is attributed to the increased population of face flies acting as mechanical vectors for *M. bovis* and to the seasonal increase in solar radiation which predisposes the cornea to infection. This has been substantiated in experimental infection where controlled exposure to ultraviolet light prior to challenge with *M. bovis* increases the rate and severity of infection. Lack of skin pigmentation in the eyelids of certain cattle breeds also predisposes to infection. Other corneal irritants such as dust can also increase susceptibility. When multiple predisposing factors are present, severe outbreaks of IBK can occur suddenly and spread rapidly, with the morbidity rate reported as high as 80%.

Although *M. bovis* has been linked to IBK since 1945 (10), considerable doubt existed concerning its role as a primary pathogen, largely due to the difficulties encountered in consistently reproducing experimental infection. In 1972, Pedersen *et al.* (108) demonstrated that only piliated strains of *M. bovis* were capable of colonizing the conjunctiva of calves and reproducing the disease. The presence of pili in virulent strains was associated with a distinctive flat, agar-corroding, colony morphology, and was confirmed by electron microscopy. Since that time, virtually all investigations into the pathogenesis of IBK have utilized piliated strains of *M. bovis*, and, as discussed below, advances in disease control are based on manipulating the immunological response of the host to the pilus. Pili, however, are not the only recognized virulence factors of *M. bovis*. It has been reported that production of hemolysin is also a characteristic of all strains capable of producing ocular disease (110). A role for several other toxins and proteases has also been suggested (113). However, as with the enteropathogenic *E. coli*, the pilus is of major importance as the prime mediator of colonization. If colonization is blocked, the deleterious effects of additional virulence factors may be minimized.

Several lines of investigation suggest that a systemic humoral immune response may prevent infection with *M. bovis*. In field outbreaks of IBK, young cattle are more susceptible than adults, suggesting the development of a natural acquired immunity (57). Pugh *et al.* (112) have demonstrated that calves receiving colostrum from cows vaccinated with *M. bovis* are more resistant to experimental challenge than calves receiving colostrum from unvaccinated dams, when challenged with a homologous strain. It has also been demonstrated by Kopecky *et al.* (68) that calves experimentally infected in one eye subsequently develop less severe clinical disease when challenged in the other eye 21 days later. The conclusion drawn was that systemic rather than local immune responses were responsible for limiting disease. Several investigators have measured antibody responses in the serum and lacrimal secretions of infected calves. Killinger *et al.* (66) reported that in lacrimal secretion the highest and most persistent antibody titers specific for *M. bovis* were of the IgG class despite the fact that, in normal

calves, IgA is the predominant antibody class in lacrimal secretion. Weech and Renshaw (156) demonstrated that the *M. bovis*-specific antibody in lacrimal secretions was directed primarily against protein antigens, including pilus, rather than carbohydrate antigens. These investigators, however, were unable to detect a serum antibody response to infection. Bishop *et al.* (11) identified an *M. bovis*-specific IgG response in the serum of challenged calves but primarily an IgA response in lacrimal secretion. Despite the somewhat contradictory findings of these investigators, it is likely that a systemic humoral immune response is an important defense against infection with *M. bovis*. The apparent efficacy of parenteral vaccination supports this conclusion.

Pugh *et al.* (111) demonstrated the importance of including pilus antigen in parenteral vaccines against *M. bovis* in 1977. Protection was demonstrated against homologous challenge strains. Recently a pilated *M. bovis* vaccine has become commercially available.<sup>4</sup> This vaccine is a formalin-killed, aluminum hydroxide-adjuvanted, whole-cell bacterin containing two highly pilated strains of *M. bovis*, EPP63 and FLA64. In experimental challenge trials (160) the vaccine given subcutaneously at a 28-day interval produced high circulating antibody titers to the pili of both strains, as measured by ELISA, and substantially reduced the rate of infection in vaccinated calves as compared to challenged, unvaccinated controls. This vaccine is now being used widely in the field with apparent success, although the vaccine's ability to induce protection against heterologous field strains of *M. bovis* has not been reported.

Interestingly, little published data exist regarding the characterization of *M. bovis* pilus in terms of chemical and physical structure, genetic derivation, and antigenic homology. Since IBK occurs worldwide and a variety of *M. bovis* strains are isolated from field outbreaks in different geographic areas (39), vaccine failures can be expected to occur unless vaccines are designed to induce strong antibody responses to widely shared pilus antigens. Identification of common epitopes should be considered an important prerequisite for development of efficacious vaccines. Current investigations directed toward development of a pilus vaccine for gonorrhea are instructive.

Schoolnik *et al.* (124) have examined the chemical structure and antigenic diversity of gonococcal pili. Cyanogen bromide fragments of pili from different gonococcal strains were prepared and evaluated. One fragment, designated as CNBr-2 was found to encompass a highly conserved antigenic region that mediates receptor cell site binding function and is immunorecessive. Fragment CNBr-3 was identified as immunodominant and to include a variable antigenic region that confers type specificity to the pilus but is functionally inert. Virji *et al.* (153) have utilized monoclonal antibodies to gonococcal pili to demonstrate a common antigenic region and a type-specific region, both of which contain more than one epitope.

<sup>4</sup>Piliguard™ Pinkeye, Schering Corporation, Kenilworth, New Jersey.

It has been reported that pili from the genera *Neisseria*, *Pseudomonas*, and *Moraxella* show homologous N-terminal amino acid sequences, suggesting that these proteins may be derived from a common ancestral gene (124). This region may show little structural variation because of the necessity of conserving receptor cell site binding specificity for successful colonization of the host. Preliminary work by G. K. Schoolnik (Stanford University, School of Medicine, Stanford, California, personal communication) has begun to clarify the pilus structure of *M. bovis*. Two distinct pili have been identified on *M. bovis* strain EPP63. They are provisionally designated as heavy and light with molecular weights of 18,000 and 17,000, respectively. The amino acid sequence of both proteins has been determined. The coding for these pili is chromosomal and not plasmid mediated, and the DNA sequence coding for the light pilus has been defined. Both the heavy and light pili share a common antigenic region of approximately 40 amino acids at the N-terminus. Serologic studies show this antigenic region to be immunorecessive. The remainders of both pili are antigenically heterologous, and this variable region is immunodominant. Several field isolates of *M. bovis* have also been demonstrated to possess pili of 18,000 and 17,000 MW, but identification of a common epitope has not yet been carried out.

Clearly monoclonal antibodies could be instrumental in identifying common epitopes on *M. bovis* pili. Furthermore, monoclonal antibodies directed against common pilus epitopes might be used effectively for passive immunization of unvaccinated cattle at risk in outbreaks of IBK. The immunologic studies of host response to infection cited above suggest that parenteral injection of pilus-specific monoclonal antibody may protect against colonization of the conjunctiva, as serum IgG appears to reach the lacrimal fluid. Alternatively, monoclonal antibody could be instilled directly into the eye, injected into the subconjunctival space as is currently done with antibiotic therapy (113), or delivered over time by a sustained-release biodegradable ocular insert which has recently been developed (146). Passive immunization could provide the veterinarian with great flexibility in managing IBK, particularly in situations such as feedlots where cattle are assembled from multiple sources and may develop IBK before active immunization has had adequate time to induce a protective antibody response.

**b. *Bacteroides nodosus*.** Infectious foot rot of sheep is an inflammation of the skin and horny tissues of the foot. When infection progresses to the soft tissue underneath the hoof wall, severe lameness occurs. The pain and impaired mobility, which results in affected pastured sheep, leads to decreased feed intake, reduced weight gains, decreased wool production, increased susceptibility to predation, and, occasionally, starvation. It is a recognized problem in all nonarid intensive sheep-producing areas of the world. Warm temperatures, wet weather, and lush pastures are important triggers of clinical disease, and sudden outbreaks of high morbidity result when climatic conditions are optimal (12).

*Bacteroides nodosus* is the primary etiologic agent of infectious foot rot of

sheep. It is an obligate parasite of the hoof epidermis in sheep and cattle, and is spread during clinical outbreaks by contamination of pasture by purulent discharges from infected feet (13). The pathogenic mechanisms of *B. nodosus* are not clearly defined. Virulent strains are characterized by a distinctive papillate or beaded-colony morphology (B type), a high level of proteolytic elastase activity, and abundant surface pili (29,133). It is widely held that the pilus is an important protective immunogen and has served as the basis for much vaccine research (144,149,155). However, a recent report on antibody responses of naturally infected sheep suggests that pilus may not be the predominant immunogen. Using an electroblot radioimmunoassay, antibody responses to 10 to 15 antigens were identified, with the greatest response being directed against an unidentified nonpilus antigen of 75,000 MW (102). In a comparative electron microscopic study of virulent and nonvirulent strains, Every and Skerman (30) confirmed the presence of abundant pili in virulent strains but also identified the presence of an additional outer membrane layer and aggregates of diffuse polar material which might contribute to virulence.

Beginning around 1970, reports of vaccine efficacy using pilated strains of *B. nodosus* in oil adjuvants were reported from Australia (22), New Zealand (132), and England (116). A protective effect was observed, and the level of protection roughly correlated to serum pilus antibody titers. In virtually all these studies, protection was demonstrated primarily against homologous challenge strains. However, two vaccine trials carried out in the United States using an Australian-produced vaccine demonstrated poor protection (82,140). These failures prompted investigation into serotypic heterogeneity of virulent field strains (123). It appears that pilus (K) antigens of *B. nodosus* are extremely variable. Several studies of K antigen diversity have been reported, and the number of identifiable serogroups has ranged from 3 in Australia (21) to 14 in the United States (123). Using pilus antiserum produced in rabbits, strong agglutination titers to homologous pili are consistently produced, and minor cross-reactions among heterologous pili are widespread (123).

To date, the minor cross-reactions observed in agglutination tests have not been carefully scrutinized. It is conceivable that these cross-reactions represent weak immunologic responses to common epitopes on *B. nodosus* pili but that these epitopes are immunorecessive, a situation analogous to that described for *Neisseria gonorrhoeae* and possibly *Moraxella bovis*, as previously discussed. Clearly, hybridoma-derived monoclonal antibodies to pilus fragments would be far superior to conventionally prepared rabbit antibodies for the purpose of establishing the existence of common epitopes. Identification of a common immunogenic region is critical for the further development of a broadly efficacious pilus vaccine. In addition, the specificity of monoclonal antibodies could facilitate definition of the immunogenic and pathogenic role of other *B. nodosus* surface structures implicated as virulence factors. Once common immunogens

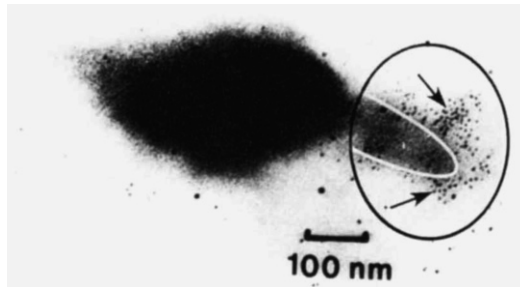
are defined on virulent strains, passive immunization by parenteral injection of monoclonal antibodies might be useful in reducing morbidity in sporadic outbreaks of infectious foot rot in unvaccinated sheep.

**c. Additional Pili of Importance.** As evidenced by the above discussions, attachment to epithelial cell surfaces is a critical first step in the pathogenesis of bacterial infection of mucosal surfaces, particularly when mechanical defenses such as peristalsis or ciliary activity resist colonization. Recognition of this fact has led researchers to a more careful evaluation of both fimbrial and nonfimbrial adherence factors as mediators of infectious disease in both human and veterinary medicine. This is particularly true in the study of respiratory pathogens. Respiratory tract disease continues to be one of the costliest disease entities encountered in veterinary practice. Significant economic loss occurs in the cattle, swine, and poultry industries due to respiratory infections, and trends toward confinement livestock rearing in all of these industry sectors exacerbate the spread of respiratory pathogens (9,109,166).

Studies on the nature of bacterial adherence in respiratory disease have been reported with regard to *Pasteurella multocida* (40), *Bordetella bronchiseptica* (49), *Haemophilus somnus* (143), and *Mycoplasma pneumoniae* (32) infections in various species. The usefulness of monoclonal antibodies in defining the mechanism and structures of adherence by respiratory pathogens has been nicely illustrated by Feldner *et al.* (32) in their work with *Mycoplasma pneumoniae*, a human respiratory pathogen. This study is instructive to veterinary researchers.

What was known about *M. pneumoniae* was its small size, its possession of a specialized tip structure, its mobility, and the ability to adhere to animal cells and inert surfaces. Adherence was considered to be a prerequisite for survival in the host organism, but the location and structure of the mycoplasmal adhesin was unknown. To answer these questions, mice were immunized with whole-cell suspensions of *M. pneumoniae*. Splenic lymphocytes were harvested and hybridomas produced. One hundred twenty hybridoma clones were screened for the production of adherence-inhibiting antibody using a hemadsorption assay. Through limiting dilution techniques, a single clone was isolated producing the desired antibody. Utilizing this monoclonal antibody in an indirect-immunofluorescence test on whole *M. pneumoniae* cells, a localized area of fluorescence was identified on one pole of the cell. Electron microscopic examination using ferritin-labeled antimouse monoclonal antibody confirmed that the monoclonal antibody was bound specifically to the unipolar tip structure of the organism (Fig. 3).

Pretreatment of *M. pneumoniae* cells with the tip-specific monoclonal antibody prevented adherence to sheep, rabbit, guinea pig, and human erythrocytes, demonstrating that adhesive ability was localized to the tip structure. The monoclonal antibody was then radiolabeled by addition of [<sup>35</sup>S]methionine to the



**Fig. 3.** Electron microscopic view of a *Mycoplasma pneumoniae* cell after incubation with adherence-inhibiting monoclonal antibody labeled with ferritin. Ferritin molecules are seen predominantly at the tip structure, confirming that the adhesive property of *M. pneumoniae* is localized to the tip structure. Adapted and reprinted from reference 32 by permission from *Nature (London)*, 1982, 298, 765–767, Copyright © 1982, Macmillan Journals Limited.

hybridoma cell culture. *Mycoplasma pneumoniae* cultures were solubilized in SDS and the proteins separated by electrophoresis in SDS–polyacrylamide gel. The gel was then treated with the labeled monoclonal antibody. Autoradiography revealed antibody bound to a single protein in the molecular weight range 160,000–190,000. Subsequent coating of a second gel with sheep erythrocytes resulted in binding of red cells only on the same protein, indicating that the antigenic protein and the functional binding protein were identical.

Clearly hybridoma technology provides a powerful investigative tool for defining the structure and function of bacterial adhesins. The use of monoclonal antibodies in the study of veterinary respiratory pathogens will undoubtedly lead to greater understanding of host–parasite interactions and novel approaches to immunoprophylaxis by blocking the mechanisms of bacterial adherence.

## B. Bacterial Toxins and Cytoplasmic Proteins

Toxin production is an important virulence mechanism, particularly for non-invasive bacteria which are able to exert profound systemic effects on the host despite localization of the infection. Clostridial infections are the classic example of this virulence mechanism. In human medicine, hybridoma technology has been applied to the study of bacterial toxins to elucidate toxin structure and pathogenic mechanisms, to improve immunodiagnostic capability, and to develop new approaches to immunotherapy. Reports of monoclonal antibodies specific for bacterial toxins include studies of diphtheria toxin (50), *Pseudomonas aeruginosa* exotoxin A (35), *Clostridium botulinum* toxins C<sub>1</sub> (103) and D (96), and *Clostridium tetani* tetanus toxin (154). To illustrate the potential uses of toxin-specific monoclonal antibodies in veterinary medicine, several important toxin-mediated diseases will be discussed.

### 1. *Escherichia coli enterotoxin*

As discussed earlier, enteropathogenic strains of *E. coli* (EPEC) possess two known important virulence factors: adherence pili to implement intestinal colonization in the face of gut peristalsis and a competing resident intestinal flora (118), and enterotoxin which stimulates intestinal epithelial cells to secrete fluid and electrolytes into the gut lumen at an accelerated rate (8). The net results of EPEC infection in the host are dehydration, acidosis, electrolyte imbalance, and death.

Two types of enterotoxin are recognized in EPEC strains isolated from live-stock; heat-stable (ST) and heat-labile (LT) enterotoxins. The former stimulates villous cell hypersecretion through activation of guanylate cyclase in the epithelial cell, while the latter accomplishes the task by activation of adenylate cyclase. Transmission of both enterotoxin types is plasmid mediated. K88-Positive EPEC strains are associated with both LT and ST enterotoxins, whereas K99, F41, and 987P-positive EPEC have been found to produce only ST enterotoxin (34).

Although the value of active immunization of swine (91) and cattle (2) with piliated vaccines has been well established and passive immunization of calves with monoclonal antibody to K99 has been demonstrated to be protective (127), enterotoxin-specific monoclonal antibody could play an important future role in passive immunization of neonates. Observations that K88-positive EPEC isolates from naturally infected swine are less frequently of the K88ab type and more frequently of the K88ad type suggest that the pilus plasmid genome is responsive to selection pressure exerted by vaccination (34). This could potentially impair the long-term efficacy of highly specific monoclonal antibodies directed against variable regions of the pilus. Enterotoxin-specific monoclonal antibody offers a possible alternative for continued passive immunization. A combination of LT- and ST-specific antibodies could conceivably neutralize enterotoxins of all EPEC strains regardless of their current or future pilus specificity. The success of this strategy would depend on the inability of enterotoxin to escape immune recognition through structural variation mediated by the plasmid genome. Furthermore, if colonization is allowed to occur, the question arises of whether or not antibody can successfully bind and neutralize enterotoxins. The physically intimate association of the EPEC organism to the brush border of epithelial cells facilitated by adherence pili may present spatial constraints which limit antibody–enterotoxin interaction.

Despite these theoretical concerns, Klipstein *et al.* (67) have shown that active immunization with enterotoxin can reduce intestinal hypersecretion. Using a vaccine composed of synthetically produced ST cross-linked to the nontoxic B subunit of LT, rats and rabbits have been protected against challenge with both toxins and heterologous organisms producing these toxins. Protection was measured by a significant decrease in intestinal hypersecretion using a ligated gut



loop assay. The protective effect of the vaccine was attributed to a marked increase in toxin-specific mucosal IgA in the gut.

The feasibility of producing monoclonal antibodies to *E. coli* enterotoxin has already been demonstrated. Hemelhof *et al.* (51) have reported the development of a monoclonal antibody to the heat-stable enterotoxin (STh) of a human EPEC strain. This monoclonal antibody, at a 320-fold dilution, exhibited toxin-neutralizing potency 10 times greater than that shown by conventionally raised antibody to STh. Interestingly, two other monoclonal antibodies to STh have been reported (148,165), but neither demonstrated the ability to neutralize the biologic activity of the enterotoxin. This suggests that immunogenic regions exist outside the region of functional activity in the structure of enterotoxin.

## 2. Cytotoxin of *Pasteurella haemolytica*

Pneumonic pasteurellosis is one of the costliest diseases confronting the cattle industry, particularly in the feedlot sector, where economic considerations dictate cattle management practices which predispose to outbreaks of respiratory disease. Fibrinous pneumonia due to *Pasteurella haemolytica* is considered the terminal event in a multifactorial syndrome known as the bovine respiratory disease (BRD) complex, which occurs with high frequency in North American feedlots (63).

Stress is an important predisposing factor in the development of BRD. Feeder calves entering the feedlot have often been recently weaned, unadapted to grain rations, comingled with strange cattle in sales barns, shipped long distances with inadequate feed or water, and subjected to adverse weather conditions. Comingling with cattle from multiple sources leads to the widespread transmission of respiratory tract viruses, particularly parainfluenza-3, and infectious bovine rhinotracheitis virus (58). Infections with these and other viruses are believed to predispose cattle to the development of *Pasteurella* pneumonia, presumably by impairment of pulmonary clearance and/or immunosuppression. The major cause of mortality in BRD is pneumonic pasteurellosis (64).

In feedlot outbreaks of BRD, morbidity can reach 35% and mortality 10%. In addition to cattle lost, treatment costs can be staggering. Antibiotic therapy is costly and time-consuming, and antibiotic resistance in *Pasteurella* isolates is increasing (4). Efforts to reduce morbidity have traditionally included the practice of vaccinating calves against viral and bacterial pathogens as they enter the feedlot. However, many calves may already be incubating respiratory tract viruses by the time they reach the feedlot, and modified live virus vaccines have been implicated as an added stressor (77). Furthermore, *Pasteurella* bacterins may actually contribute to the severity of respiratory disease, as will be discussed later. In fact, an epidemiologic study of feedlot mortality in Canada identified vaccination of cattle upon arrival at the feedlot as one of the three most important factors contributing to increased mortality rates (78).

Although *Pasteurella multocida* is frequently isolated from outbreaks of BRD, *P. haemolytica* is considered the primary bacterial pathogen. Twelve ruminant serotypes of *P. haemolytica* are recognized, but serotype 1 is most often isolated from pneumonic lungs (115). The virulence factors and pathogenic mechanisms of *P. haemolytica* are not completely understood. While viral impairment of pulmonary clearance may promote increased bacterial entry into the distal lung, bacterial surface adhesins may facilitate continued colonization of the terminal bronchioles and alveoli. After colonization, cell wall endotoxin release is believed by some to be an initiator of the pulmonic lesions which develop (69). However, attention recently has focused on other compounds released by the organism. *Pasteurella haemolytica* has been found to produce a potent cytotoxin capable of killing polymorphonuclear leukocytes and alveolar macrophages, thus disabling the primary immunologic defense of the lung against bacterial pathogens (74,75).

The physicochemical properties of cytotoxin have been studied. Himmel *et al.* (53) have purified and partially characterized cytotoxin as a protein with a molecular weight of approximately 150,000 associated with the bacterial capsule. The protein was isolated from culture supernatant and was shown to be highly immunogenic. In immunodiffusion tests, the antigen was cross-reactive with antisera produced against all 12 serotypes of *P. haemolytica*. No reactivity was demonstrated against antisera to *P. multocida*. Toxicity against bovine alveolar macrophages was demonstrated *in vitro*. Shewen and Wilkie (128) also have reported similar immunogenic and cytotoxic findings with cytotoxin from culture supernatant.

The universal presence of cytotoxin in all serotypes of *P. haemolytica*, its immunogenicity, and its probable role as an important virulence factor make cytotoxin an ideal antigen for active immunization against pneumonic pasteurellosis. Examination of feedlot cattle submitted for necropsy and diagnosed as having respiratory disease had lower levels of serum anticytotoxic neutralizing antibodies than did animals dying of other causes, or than animals bled prior to entry into feedlots (129). Immunization with isolated cytotoxin did confer some protection against experimental challenge, although response to other (surface) antigens appeared to be required for complete protection (130). This may be an analogous situation to the EPEC, where antibodies to both the pilus and the toxin may give the best response.

Recognition of the importance of cytotoxin helps to explain the apparent negative effects of vaccination with killed whole-cell bacterins. Since cytotoxin is produced most abundantly during log-phase growth in culture (36), conventionally prepared bacterins containing bacteria from stationary-phase growth will induce much anticapsular opsonizing antibody but may produce very little cytotoxin-neutralizing antibody. This can lead to increased phagocytic activity but also may result in more efficient killing of phagocytes by cytotoxin released

from ingested bacteria. The net effect of vaccination then may be an impaired immune response.

Although redesigning bacterins to promote a better anticytotoxin response would result in a better vaccine, this does not ensure the development of a better vaccination program. In fact, the realities of feedlot practice argue against the value of active immunization as a technique for controlling BRD. Most outbreaks of BRD occur within 10 to 14 days after arrival in the feedlot, when environmental stresses on the calf are at their greatest. Yet cattle vaccinated upon arrival cannot be expected to develop a strong humoral immune response within the first 2 weeks, if at all. Endogenous steroid release due to stress and concurrent viral infections may impair the animals' ability to mount an effective immune response, even with the best of vaccines. Ideally, calves should be vaccinated before marketing to the feedlot (preconditioning). However, calf raisers in general are unwilling to incur the expense of vaccination to protect the future economic interests of prospective feedlot calf buyers.

Clearly passive immunization could play an important role in controlling BRD, as it fits more logically into the epidemiologic pattern of feedlot disease and feedlot management practices. Antibody administered parenterally to cattle upon arrival at the feedlot could protect them from clinical disease during the critical adaptation period. Hybridoma technology offers a significant advantage over conventionally prepared antisera for passive immunization. It allows for the production of unlimited quantities of high-titer antibodies directed against specific virulence factors. Monoclonal antibody to cytotoxin administered alone or in conjunction with monoclonal antibodies to other antigens of *P. haemolytica* or proteins of known respiratory viral pathogens could dramatically improve current approaches to controlling respiratory disease in cattle.

### 3. Clostridial Toxins

A wide range of clostridial diseases affect farm animals (12), including tetanus (*Cl. tetani*), botulism (*Cl. botulinum*), blackleg (*Cl. chauvoei*), malignant edema (*Cl. septicum*, *Cl. novyi*), necrotic hepatitis (*Cl. novyi*), bacillary hemoglobinuria (*Cl. haemolyticum*), and enterotoxemia (*Cl. perfringens*). All of these diseases are mediated by release of potent clostridial toxins.

In general, these diseases are effectively controlled by vaccination with alum-precipitated toxoids. However, serious outbreaks continue to occur in unvaccinated animals and successful therapy depends on the administration of large doses of specific antitoxins. Commercially available, conventionally prepared antitoxins vary considerably in their cost, availability, and efficacy. The most serious limitation to treatment is the relatively high cost of administering adequate doses of antitoxin. For example, the recommended treatment regimen for an adult horse with tetanus is 300,000 units of tetanus antitoxin administered

three times at 12-hr intervals (12). The current cost of this therapy is approximately \$700.

It has been demonstrated that antibodies to clostridial toxins can be prepared using hybridoma technology. Oguma *et al.* (103) have reported the production of four different monoclonal antibodies against the C<sub>1</sub> toxin of *Cl. botulinum*, only one of which showed neutralizing activity against the biologic activity of the toxin. This suggests that in conventionally prepared antitoxins, a significant proportion of antibody which is produced may not possess neutralizing activity. Volk *et al.* (154) have produced a number of mouse monoclonal antibodies to tetanus toxoid. These antibodies bound to at least 20 different epitopes on the toxoid molecule. Mixtures of these antibodies demonstrated greater neutralizing activity than any of them alone, suggesting that efficient neutralization requires antibody binding at more than one site on the toxoid molecule.

The comparatively high titers of hybridoma-derived antibodies along with their potential for improved specificity could significantly improve the efficacy of antitoxin therapy when monoclonal antibodies are used instead of conventionally prepared antitoxin. Hybridoma technology could conceivably result in lower cost products of higher potency for therapeutic use.

#### **4. Protoplasmic Antigens of *Mycobacterium paratuberculosis***

Paratuberculosis (Johne's disease) is a chronic granulomatous enteric infection of ruminant animals caused by the acid-fast organism *Mycobacterium paratuberculosis*. The disease occurs worldwide and is considered a major problem in the North American cattle industry. In infected herds, clinically ill animals with diarrhea shed the organism in the feces and *M. paratuberculosis* infection can be confirmed by bacterial culture techniques. This is also true of a percentage of infected animals not showing clinical signs. However, many infected animals in a herd are nonshedding carriers and cannot be identified by culture of feces (12).

Efforts at eliminating Johne's disease have been frustrated for decades by the lack of a sensitive and specific immunodiagnostic test for identification of nonshedding carrier animals. A wide range of diagnostic tests has been evaluated to detect both humoral and cellular immune responses to infection. These efforts have been reviewed (84,147). In virtually all cases diagnostic specificity was inadequate as a result of antigenic cross-reactions. *Mycobacterium paratuberculosis* shares common cell wall antigens with a variety of pathogenic and saprophytic bacteria, including other *Mycobacteria* sp., *Corynebacterium* sp., and *Nocardia* sp. (70). These historical difficulties have led to a renewed emphasis on isolation of type-specific antigens from protoplasmic extracts of *M. paratuberculosis* and the utilization of newer, more sensitive diagnostic assays such as the ELISA (85).

Abbas *et al.* (1) have reported on the isolation of an affinity-purified peptide derived from a crude protoplasmic extract of *M. paratuberculosis*. The crude extract is obtained by disruption of whole cells in a Ribi hydraulic cell press, separation of cell wall debris by centrifugation, and lyophilization of the remaining supernatant. Isolation of peptide antigens involved a complicated stepwise procedure. The crude protoplasmic extract was treated by solvent extraction and ammonium sulfate precipitation followed by gel filtration, ion exchange chromatography, and, finally, affinity chromatography. The yield of peptide antigen from crude material applied to the affinity column was 7.8%. Whereas the crude starting material demonstrated cross-reactivity to bovine antisera against *M. avium*, *M. phlei*, *M. fortuitum*, and *Nocardia asteroides* in complement fixation and ELISA tests, the purified peptide reacted only with antisera to *M. paratuberculosis* in complement fixation and ELISA with antisera diluted 1:40.

Although the purification of this antigen and its application in the ELISA system represent major advances in the immunodiagnosis of paratuberculosis, the application of hybridoma technology could eliminate the need for exhaustive purification of protoplasmic antigens. Hewitt *et al.* (52) have reported on the use of monoclonal antibodies in the serodiagnosis of human tuberculosis without the need for purification of antigen. The assay described is a competitive-inhibition assay wherein crude *M. tuberculosis* antigen (pressate) is bound to microtiter plate wells. Test sera are then incubated in coated wells, followed by the addition of  $^{125}\text{I}$ -labeled murine monoclonal antibodies directed against type-specific antigens of *M. tuberculosis*. Plates are then washed and the amount of bound monoclonal antibody is determined by  $\gamma$  counting as an indirect measure of *M. tuberculosis*-specific antibody in test sera. Combinations of different antigen-specific monoclonal antibodies were employed in the assay to improve sensitivity. It was emphasized that mycobacterial infections present a wide range of antigens to the host which may be recognized or ignored to variable degrees during the host immune response. Therefore, the sensitivity of the immunoassay can be improved by utilizing monoclonal antibodies against several known type-specific antigens.

The prevalence of host recognition of the peptide antigen of *M. paratuberculosis* described by Abbas *et al.* (1) is currently unknown, although it was reported in their study that sera from 83% of the known infected animals tested were identified as positive in the ELISA assay. This antigen could be used to produce a mouse monoclonal antibody for use in the competitive-inhibition assay described, with crude protoplasmic extract of *M. paratuberculosis* serving as the test antigen. In addition, monoclonal antibodies could be used as probes to identify other protoplasmic antigens of *M. paratuberculosis* which are not cross-reactive and could therefore be used to improve the sensitivity of a competitive-inhibition test.

Morris and Ivanyi (95a) have recently reported on patterns of cross reactivity

in protoplasmic antigens of various mycobacteria including *M. paratuberculosis* to a panel of ten monoclonal antibodies produced against *M. tuberculosis* and *M. leprae* antigens. A radioimmunoassay was employed to detect binding activity.

### C. Bacterial Capsular Antigens

The bacterial capsule plays an important role in the pathogenesis of some bacterial infections, largely through its ability to inhibit phagocytosis. Two general mechanisms of antiphagocytic activity are recognized (56). First, abundant capsule may cover cell surface antigens such as pili and cell wall lipopolysaccharide (LPS), preventing phagocytic interaction with subcapsular determinants. Second, polysaccharide capsule does not fix complement in the absence of antibody, thus inhibiting complement-mediated opsonization. The polysaccharide capsule of many bacteria is highly immunogenic, however, and immunization with capsule-rich vaccines will promote anticapsular antibody production capable of mediating complement fixation and phagocytosis (7).

In human medicine, an exciting approach to immunotherapy is being explored using monoclonal antibodies to capsular antigens of group B *Streptococcus*, bacteria responsible for potentially fatal septicemias in newborn infants. Since these infections occur during passage through the birth canal and their occurrence is unpredictable, active immunization does not represent a practical approach to management of the problem, a situation analogous to many of the veterinary diseases already discussed. Shigeoka *et al.* (131) have reported on the development of murine monoclonal antibodies of the IgM class prepared against group B streptococcal (GBS) type III polysaccharide antigens. One of these monoclonal antibodies, when used in a rat model of infection, significantly reduced mortality in rats challenged with five different GBS type III strains. Two of these strains normally resist opsonization by human sera containing opsonizing antibody. Mortality in rats was significantly reduced even when monoclonal antibody was administered as late as 24 hr after challenge. This suggests that the antibody could be used for immunotherapy as well as immunoprophylaxis. A similar protective effect has been shown for monoclonal antibodies to capsular antigens of *Haemophilus influenzae* (37).

In veterinary medicine, the role of capsule is not well defined for many bacterial pathogens. Capsular antigens do occur widely among the Enterobacteriaceae and they have been proposed as playing a role in the adherence of enteropathogenic *E. coli* (98), in addition to the aforementioned pili. Capsular K antigens are also involved in the pathogenesis of septicemic *E. coli* infections (163). Among the gram-positive organisms, *Staphylococcus aureus*, an important mastitis pathogen, possesses numerous mechanisms for escaping phagocytosis, among them being capsule (163). Two streptococci, *S. equi* and the group E *Streptococcus* of swine, are recognized as having antiphagocytic properties.

However, this is due to possession of cell wall-associated proteins rather than polysaccharide capsule (18,162).

At present, the diagnostic, therapeutic, and prophylactic applications of monoclonal antibodies against capsular antigens of veterinary pathogens remain largely unexplored. Hybridoma technology can contribute to a better understanding of the structure, function, and importance of capsular antigens. This in turn may lead to clearer ideas for clinical applications of monoclonal antibodies.

#### **D. Bacterial Cell Wall Antigens**

Cell wall structures have received a great deal of attention both as immunogens and as virulence factors. The cell wall "O" LPS of gram-negative bacteria are strongly antigenic and serve as the basis for taxonomic classification of gram-negative organisms (42). The lipid A moiety of gram-negative bacterial LPS has been identified as a potent component of the endotoxin molecule exerting powerful, well-defined, toxic effects in infected hosts (17). The waxy cell walls of acid-fast organisms such as the mycobacteria, are instrumental in facilitating the intracellular survival of phagocytized bacteria, leading to chronic granulomatous infections (163). The peptidoglycan component of gram-positive bacterial cell wall has also been demonstrated to produce endotoxinlike activity and adjuvant activity (126). In addition, some gram-positive organisms possess cell wall-related proteins, such as protein A of *Staphylococcus aureus* and the M protein of group A streptococci, which assist the organism in avoiding phagocytosis and establishing infection (7). In this section, three potential applications for monoclonal antibodies against cell wall components of veterinary pathogens will be discussed.

##### **I. *Brucella abortus***

Brucellosis, due to *Brucella abortus*, is an important zoonotic disease of ruminant animals which can cause undulant fever in humans. In the United States, a federal regulatory disease control program established in 1934, has significantly reduced, but not eliminated brucellosis in cattle. The essential elements of the control program are restriction of cattle movement, serologic identification and destruction of infected animals, and vaccination of calves with a live avirulent *B. abortus* (strain 19) vaccine. Vaccination is an important component of the program. However, the production of antibodies in vaccinated animals as well as the occurrence of other organisms which induce cross-reactive antibodies, confound the accurate serologic diagnosis of naturally occurring brucellosis infection. This is recognized as a serious obstacle to the total elimination of brucellosis from the national herd (12).

Numerous approaches to improving the strain specificity of serologic tests have been explored. Now, hybridoma technology offers the most promising

solution to this long-standing problem. Identification of strain 19-specific cell wall antigens as well as type-specific antigens of *Yersinia enterocolitica*, a common cross-reacting organism, might be expedited by the use of monoclonal antibody probes. Monoclonal antibodies developed against strain-specific antigens could then be used in competitive serologic assays such as the competitive-inhibition assay described earlier for the diagnosis of tuberculosis, to discriminate natural *B. abortus* infections from both strain 19 vaccination and spontaneous infections with cross-reactive organisms. Several reports on the development of monoclonal antibodies against cell wall LPS antigens of *Brucella* strains for serodiagnostic application have already appeared (54,125,125a). The first work by Schurig (125) illustrated the use of monoclonal antibody to *Brucella* in a competitive ELISA test to discriminate antibody against *E. coli* LPS antigen from antibody to *Brucella* LPS antigen in sera from immunized animals. In another study (15), a monoclonal antibody specific for the "O"-chain polysaccharide of *Yersinia enterocolitica* was found to agglutinate several biotypes of *B. abortus*. It was concluded that the "A" antigen of *B. abortus* is identical to the "O"-chain polysaccharide of *Y. enterocolitica*.

## 2. *Streptococcus equi*

*Streptococcus equi* causes a severe lymphadenopathy in horses resulting in abscessation and rupture of lymph nodes primarily in the head and neck region. The condition is commonly known as strangles due to the severe respiratory impairment which can result from swelling of the pharyngeal lymph nodes. In certain management situations, *S. equi* infection can reach epidemic proportions. This is particularly true on breeding farms and in training stables where there is a continual turnover of large numbers of horses. Contamination of the environment with purulent material from draining abscesses promotes the spread of disease, and in some facilities strangles becomes an endemic problem (12). Young foals accompanying their mares to breeding farms are particularly susceptible. A commercial bacterin is available for vaccination against *S. equi*, but the degree of protection afforded by immunization is variable (25). Vaccination of susceptible foals after they have been introduced onto contaminated premises is of little value. This represents another situation in veterinary medicine where an effective product for passive immunization of populations at risk would be helpful in controlling disease.

Numerous *S. equi* antigens have been examined for their potential as protective immunogens (101), including peptidoglycans, murein-teichoic acid complex, group C carbohydrate, Lancefield extracted protein, and a cell wall protein similar to the M protein of group A streptococci. The M-like protein, first characterized by Woolcock in 1974, is of particular interest (162). It has been shown to be a potent immunogen, producing high antibody titers in both horses and rabbits. Srivastava and Barnum (142) have reported that vaccination of pony



foals with purified, alum-precipitated M-like protein protected them from infection via contact exposure to a horse with active strangles. Antibody levels produced by vaccination with M-like protein were equivalent to the levels which occur after natural infection with *S. equi*.

These findings suggest that a monoclonal antibody directed against the M-like protein could be useful for passive immunization of foals against *S. equi* infection, although at this time it is unclear whether circulating serum antibody alone affords adequate protection against disease. Some evidence exists that a cellular immune response as well as a local mucosal immune response in the nasopharynx play a role in host protection (150). Nevertheless, the value of passive immunization with parenterally administered monoclonal antibody merits future evaluation.

### 3. Core LPS of Gram-Negative Bacterial Endotoxin

One of the most exciting potential applications of hybridoma technology in veterinary and human medicine is the development of a monoclonal antibody to the common core LPS fraction of endotoxin for uses in immunotherapy and immunoprophylaxis against a broad range of Gram-negative pathogens involved in a wide variety of specific disease entities. That such a possibility can even be theorized is a credit to the many basic and clinical researchers who have carefully studied the structure, function, and clinical effects of Gram-negative endotoxin.

Variations in "O" antigenicity of Gram-negative bacteria result from the diversity of structure of the oligosaccharide side chains of cell wall LPS. Although these "O" antigens are highly immunogenic, their structural diversity leads to serologically distinct antibody responses, with little or no cross-reactivity occurring between heterologous "O" strains. Given the staggering number of Gram-negative bacterial species and strains capable of producing serious infection, active immunization has never received serious consideration as a means of effectively controlling Gram-negative infections. However, recognition of the problem of antigenic diversity has prompted investigations into the identification of shared cell wall antigens which might serve as common immunogens in a cross-protective vaccine. McCabe *et al.* (80) have reviewed the background and developments in this area of research, and the major advances described will be briefly summarized here.

In 1966, Lüderitz *et al.* (71) reported on the structural similarities of the core portions of Gram-negative cell wall LPS in *Salmonella* sp. and other related *Enterobacteriaceae*. It was observed that, despite variations in the terminal oligosaccharide structures which confer "O" antigenicity, all species examined possessed a common core antigen composed of lipid A attached to ketodeoxyoctonate (KDO) and heptose. This core structure is immunogenic.

Mutant strains of *S. typhimurium* (14), *S. minnesota* (79), and *E. coli* (23) have been identified which fail to synthesize oligosaccharide side chains of cell

wall LPS. Two stable mutants, the Re mutant of *S. minnesota* and the J5 mutant of *E. coli* O111:B<sub>4</sub>, have been used in a number of immunization studies to demonstrate that antibody produced against the core LPS determinants only will significantly protect laboratory animals from challenge infection with a broad range of Gram-negative organisms including other *Salmonella* and *E. coli* species (79,167), *Klebsiella* sp. (79,167), *Pseudomonas* sp. (168), and *Haemophilus influenzae* (76).

These encouraging findings have led to investigations into the role of core LPS antibody in protecting human patients from the effects of Gram-negative septicemia. McCabe *et al.*, (81) measured naturally occurring antibody to core LPS determinants as well as antibody to type-specific antigens in 400 human patients with Gram-negative septicemia. A marked correlation was observed between antibody titer to core LPS and severity of clinical disease. Patients with high antibody titers to core LPS of 1:80 or more as measured by indirect hemagglutination were far less likely to develop shock or die than patients with low antibody titer to core LPS. This phenomenon appeared to be independent of antibody titer to type-specific antigens of infecting organisms. Ziegler *et al.* (169) immunized human volunteers with the J5 mutant of *E. coli* O111:B<sub>4</sub> and produced a human antiserum to core LPS, which was then administered to hospital patients with Gram-negative septicemia in a double-blind clinical trial. Mortality was significantly reduced in patients receiving J5 antiserum in comparison to patients receiving nonimmune serum, even among patients experiencing profound shock. These patients were infected with a broad range of unrelated Gram-negative organisms.

Mutharia *et al.* (97) have recently produced four monoclonal antibodies against the J5 mutant of *E. coli*, all of which react with purified LPS from J5 *E. coli*, the Re 595 mutant of *S. minnesota*, *Agrobacterium tumefaciens*, and *Pseudomonas aeruginosa* in addition to purified lipid A of *P. aeruginosa*. These antibodies also react in ELISA with outer membrane preparations of more than 30 strains of various Gram-negative bacteria while showing no reactivity against Gram-positive species. These results add credence to the concept of widespread conservation of single antigenic sites in the lipid A of diverse gram-negative bacteria and illustrate the power of monoclonal antibodies as investigative tools. A more recent report (87a) has shown that the cross reactivity of monoclonal antibodies against *E. coli* J5 endotoxin was even greater when the antibodies were reacted with whole heterologous organisms rather than the purified LPS extracts.

These findings have raised hopes that a vaccine containing core LPS determinants can be developed for immunization against diverse gram-negative bacterial infections. In addition, antibody against core LPS could be used for passive immunization of patients at risk, such as burn patients or immunocompromised patients on chemotherapy, as well as for immunotherapy in the early stages of

Gram-negative bacterial septicemia. Monoclonal antibody derived by hybridoma technology would be a logical method for producing therapeutic and prophylactic antibody. It obviates the problem of raising conventional antisera in human beings and would yield specific antibody in much higher concentration. The concentration of antibody administered for therapeutic purposes is probably a critical factor because, in overwhelming Gram-negative sepsis, antibody is rapidly consumed.

The potential applications of a monoclonal antibody to core LPS in veterinary medicine are numerous and the economic benefit to be derived from passive immunization and immunotherapy is enormous. Many costly clinical syndromes in livestock are known or thought to involve Gram-negative bacterial septicemia or endotoxemia (12,127a). These include, among others, colisepticemia in newborn calves, shigellosis in foals, agalactia of sows, salmonellosis of cattle and horses, postoperative endotoxemia in horses, and coliform mastitis in cattle. Many of these conditions are characterized by a high mortality rate even in the face of aggressive antibiotic therapy and supportive care. The ability to intervene in these conditions with a monoclonal antibody to core LPS would be of incalculable benefit to clinical veterinary medicine.

#### IV. CONCLUSIONS

Three main areas of application for monoclonal antibodies against bacterial antigens in veterinary medicine have been discussed, namely, passive immunization, improved immunodiagnostics, and immunotherapy. Although many of the proposed applications presented in this chapter are speculative, the authors have attempted to limit discussion to bacterial diseases where existing knowledge of the antigenic structure, virulence mechanisms, and host responses to infection is sufficient to make such speculation realistic. Whenever possible, these potential applications have been supported by discussion of analogous developments in human medical research to lend credence to the speculations in veterinary medicine. Only additional research involving these specific veterinary pathogens will determine whether or not the potential applications we have hypothesized can be successfully implemented in practice. It is hoped that this chapter will encourage veterinary researchers to examine critically some of our proposed applications for monoclonal antibodies. Undoubtedly many additional practical applications for hybridoma technology in veterinary medicine have eluded our imagination, and interested readers are very likely to develop additional ideas of their own. If this occurs, then the chapter has served a useful purpose.

Passive immunization using monoclonal antibodies may dramatically alter traditional approaches to disease control in veterinary medicine. Several aspects of veterinary livestock practice underscore the need for effective passive immu-

nizing agents. Livestock producers resist the idea of active immunization against infectious diseases even when efficacious vaccines are available. Much of this resistance is due to the economic realities of livestock production where profit margins are narrow and the cost of vaccination is weighed against the risk of experiencing disease outbreaks. Unfortunately, in many cases the risks are weighed incorrectly and the veterinarian is then called in to halt the rapid spread of infectious disease through a highly susceptible population of livestock. The value of passive immunization in these situations is unquestionable, as evidenced by the apparent success of the K99-specific monoclonal antibody in controlling outbreaks of fatal diarrhea in neonatal calves due to enteropathogenic *E. coli*. In other situations, the risk of disease is recognized to be high and active immunization is carried out, but management factors preclude the value of vaccination. This is particularly true in feedlots where susceptible populations of cattle are not accessible for vaccination prior to the period of highest disease risk. Infections are established before protective antibody is produced by the host and passive immunization represents the only logical immunologic defense.

Monoclonal antibodies, because of their specificity, unlimited availability, and high titer, represent excellent passive immunizing agents. However, their potential usefulness in preventing infection must be evaluated on a case-by-case basis. When the host naturally responds to infection with a local mucosal immune response or a cellular immune response, parenteral administration of monoclonal antibody may not provide adequate protection against infection. In localized infections of mucosal surfaces, monoclonal antibody may need to be administered to specific sites when circulating antibody does not participate in immunologic control of infection. Whereas this was easily accomplished in enteric colibacillosis by oral administration of K99-specific monoclonal antibody, the logistics of administering monoclonal antibody to the terminal airways in respiratory disease are mechanically complex.

The selection of the appropriate antibody class may also be important depending on whether or not opsonization of bacteria is dependent on complement activation. It appears that in the work with antibody to the core LPS of endotoxin, antibody of the IgM class is more protective than IgG antibody. In other situations, bacteria are capable of producing proteases which degrade secretory IgA (73), and monoclonal antibodies of other classes might be more effective. The half-lives of either heterologous or homologous monoclonal antibodies must also be taken into account, especially if passive immunization or long-term therapy is anticipated.

Perhaps the most significant limitation to widespread application of monoclonal antibodies for passive immunization is the current dependency of hybridoma technology on murine cell lines. Repeated application of murine-derived monoclonal antibodies to livestock species will undoubtedly lead to the development of host antimurine antibodies which would destroy the immunologic ac-

tivity of the administered monoclonal antibody, or perhaps induce allergic sensitivity. This problem requires the development of new cell lines capable of producing species-specific antibody. Recent reports of successful human-murine (38), bovine-murine (114a,141,141a), and porcine-murine (114) fusions capable of producing human, bovine, and porcine immunoglobulins, respectively, offer much hope for the future.

In the area of immunodiagnosics, monoclonal antibodies will be of particular value to veterinary medicine. As a result of their specificity, monoclonal antibodies are ideally suited for identifying specific antigens out of crude antigenic mixtures in unpurified specimens. This has been illustrated by the development of an ELISA test kit using K99-specific monoclonal antibody for detection of the K99 antigen directly from fecal samples. For the veterinarian in livestock practice who works independently and in relative isolation, the development of test kits for field use will dramatically reduce dependency on a central diagnostic laboratory and increase the availability of test results. In addition, monoclonal antibodies will undoubtedly lead to refinements in current methods of serodiagnosis, particularly in those cases where antigenic cross-reactivity leads to decreased specificity. Paratuberculosis and brucellosis are prime examples.

Immunotherapy is becoming increasingly popular in human medicine, and many potential applications exist in veterinary medicine as well. Immunotherapy is particularly attractive in veterinary medicine as an alternative to antibiotic therapy. The use of antibiotics in livestock is strictly regulated and the selection of antibiotics by practitioners often depends as much on their persistence in meat, milk, and eggs as it does on the susceptibility of the infecting organism. For example, the use of aminoglycoside antibiotics in cattle requires a 30-day withholding period before treated cattle can be marketed for meat. This inhibits the use of aminoglycosides even when their therapeutic use is indicated. Even more importantly, societal awareness and concern over adulterants in the food supply and the potential for transfer of drug resistance from animal pathogens to human pathogens, demands that the profession of veterinary medicine identify and implement effective alternatives to antibiotic use. Immunotherapy with monoclonal antibodies represents one likely alternative, and new research activity should be directed toward the development of a broad range of immunotherapeutic agents using hybridoma technology.

Although the scope of this chapter has been limited to applications of monoclonal antibodies against bacteria in veterinary medicine, equally exciting developments are occurring in the areas of veterinary virology, mycology, parasitology, and neoplastic disease. There is no doubt that hybridoma technology will accelerate the pace of basic research in veterinary medicine and lead to a broad range of diagnostic and clinical applications that will change the face of veterinary practice for years to come.

## V. SUMMARY

Current and future applications of monoclonal antibodies against bacteria in veterinary medicine have been discussed. Those aspects of veterinary practice which make developments in hybridoma technology particularly attractive to veterinary medicine have been emphasized.

Existing applications of monoclonal antibodies were detailed, including the use of pilus-specific monoclonal antibodies for passive immunization of calves and piglets against enteropathogenic *E. coli* (EPEC) infections as well as the development of rapid diagnostic test kits for field diagnosis of EPEC infections.

Potential applications of monoclonal antibodies for passive immunization against a variety of veterinary pathogens were presented, including passive immunization against *Moraxella bovis* (pinkeye), *Bacteroides nodosus* (foot rot), EPEC enterotoxin (enteric colibacillosis), *Pasteurella haemolytica* (pneumonic pasteurellosis), and *Streptococcus equi* (strangles).

Potential diagnostic applications for important veterinary pathogens were discussed, including diagnosis of *Mycobacterium paratuberculosis* infection (Johne's disease) and *Brucella abortus* (brucellosis).

Potential applications for immunotherapy using monoclonal antibodies were also discussed, including therapy for clostridial infections using monoclonal antibodies against clostridial toxins, and for gram-negative bacterial infections, using monoclonal antibodies against the common core lipopolysaccharide determinants of gram-negative cell wall endotoxin.

Conclusions were presented concerning the role of hybridoma technology in the future progress of veterinary medicine.

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