A SEROLOGIC STUDY OF SMOOTH AND MUCOID COLONIAL VARIANTS OF FOUR *HAEMOPHILUS SOMNUS* ISOLATES

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ISOLATES

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CHAPTER I

INTRODUCTION

Haemophilus somnus Bailie, 1969 is the etiologic agent of several disease manifestations in cattle, including septicemia, meningoencephalitis, reproductive tract infections, arthritis, and respiratory disease. It has also been reported in association with "weak calf syndrome." Consequently, losses to the cattle industry from H. somnus infections have been recognized to be of major economic importance.

Because the organism has been recognized and identified as a causative agent of cattle disease, there has been interest in its immunologic characterization for diagnostic, epidemiologic, and vaccine developmental reasons. Preliminary serologic surveys suggested that H. somnus was a uniform species with little or no antigenic variation among isolates of different geographic and pathologic origins. However, Corstvet (23) found that three sequential isolates from the trachea of an experimentally infected calf exhibited the same growth characteristics on artificial media, but the three isolates produced different serologic reactions when measured by the Ouchterlony gel diffusion technique. These results suggested antigenic heterogeneity among isolates even when the initial infection was experimentally induced. Similar observations have been made with other bacteria (10, 11, 13, 14, 18, 27, 28, 36).

During the life of a bacterial species, successive or alternating stages or phases of development occur, which results in a multiplicity

of colonial forms. These patterns of bacterial variation are referred to as microbic dissociation (40). In an attempt to organize what is known of these organisms, these patterns of bacterial dissociation have been assigned to a four-phase system: smooth, mucoid, intermediate, and rough. It has been shown with other bacteria that culture phases or colonial variants may also accompany changes in antigenic expression (11, 14, 17, 18, 27, 28, 36, 40).

The purpose of the present investigation was to study the expression of antigens by smooth and mucoid colonial variants of H. somnus. Results were analyzed to determine a possible correlation between antigenic heterogeneity and the dissociation form of the organism.

CHAPTER II

LITERATURE REVIEW

In 1960, Kennedy et al. (50) isolated an organism from cattle suffering from septicemic disease; they described the organism and designated it as Haemophilus-like. Subsequently, the name Haemophilus somnus was proposed by Bailie (2), and it has been adopted even though the organism remains incompletely classified (51). Haemophilus somnus has been isolated from cattle throughout the world, including the United States (3, 4, 15, 31, 39, 40, 42, 65, 70), Canada (34, 54, 56, 69, 82, 84), Switzerland (21, 22), Germany (32, 55, 81), Italy (9), and Scotland (66, 67).

Haemophilus somnus is gram-negative and nonmotile. On primary isolation, it is a pleomorphic bacillus; coccobacillary forms predominate in subsequent passages on artificial media (72). Presence of a capsule has been reported by some investigators (59, 89) but not others (37, 38, 50, 62, 72, 77, 87). Primary isolation of H. somnus seems to be optimal at 37 C in the presence of 8 to 10% CO_2 on a medium containing red blood cells (73). Growth of the organism is enhanced by 0.5% yeast hydrolysate; however, accessory growth factors X (hemin) and V (nicotinamide adenine dinucleotide) are not required (2, 38, 50).

The rate of survivability of the organism in the environment is not known but, under experimental conditions, H. somnus has been shown to survive on a blood agar plate at 22 C in ambient air for one week (43,

50, 80). Dewey and Little (29) studied the influence of body secretions and excretions on H. somnus viability at different temperatures. When the organism was mixed with cerebrospinal fluid, blood plasma, whole blood, milk, or vaginal mucus and held at -70 C, survival exceeded 70 days. Similar results were seen when the organism was mixed with either whole blood or nasal mucosa and kept at 23.5 C. Irrespective of temperature, H. somnus remained viable less than 24 hours when mixed with urine.

Colonies that develop on blood agar plates incubated for 48 hours at 37 C in 8 to 10% CO_2 appear convex, glistening, smooth, moist, entire edged, and 1 to 2 mm in diameter. Haemophilus somnus has a characteristic lemon-yellow pigmentation and a butyrous consistency when collected on a bacteriologic loop. Humphrey et al. (45) report variability in the hemolytic activity of cultured H. somnus, ranging from no hemolysis to complete lysis of the blood cells.

Haemophilus somnus produces acid in dextrin, fructose, galactose, glucose, glycogen, maltose, mannitol, mannose, sorbitol, starch, and trehalose. No acid is produced in adonitol, arabinose, cellobiose, dulcitol, erythritol, glycerol, inositol, inulin, lactose, melezitose, melibiose, raffinose, rhaminose, salicin, and sucrose. It is positive for cytochrome oxidase, variable for production of indole, and reduces nitrate to nitrite. It is catalase, citrate, methyl red, and Voges-Proskauer negative, and it does not hydrolyze gelatin (2).

The disease manifestations produced by H. somnus vary. Septicemic disease, which occurs most commonly in yearling beef cattle in feedlots, may be separated into three syndromes which involve the central nervous system, respiratory system, and joints (3, 31, 39, 42, 57, 65, 88). The

peracute syndrome is characterized by fever and rapidly progressive neurologic signs--stiffness, ataxia, weakness of fetlocks, opisthotonus, blindness, and paresis followed by death. Death can occur as early as two to eight hours or occasionally as late as two weeks after onset of symptoms. The acute syndrome primarily affects the respiratory system. Panciera et al. (65) found that 5 to 20% of animals that developed the acute respiratory form suffered neurologic localization that resulted in a rapidly fatal syndrome. The subacute or chronic form of disease involves the joints and produces lameness and stiffness several days to two weeks after commencement of an outbreak. Lameness may persist for weeks or months.

The organism has also been associated with abortions (19, 34, 48, 58, 69, 83), metritis and orchitis (21, 48, 49, 58, 64), and the "weak calf syndrome" (8, 75, 85). Haemophilus somnus has also been isolated from the semen of bulls (21, 47, 58, 85) and from the reproductive tracts of both clinically normal cattle (45, 47, 85) and those with reproductive dysfunction (21, 35, 48, 49, 64). Lesions in the urinary bladder have been observed (39, 62), and the organism has been isolated from urine by several investigators (22, 45, 69, 79, 89).

Little is known concerning the pathogenesis and the epidemiology of H. somnus infections. However, serologic surveys indicate that a high percentage of healthy cattle have antibodies to H. somnus. It appears that natural exposure occurs commonly and causes inapparent infection. Cattle with inapparent infections may harbor H. somnus in the reproductive, respiratory, urinary, and digestive systems (45, 46, 62, 68, 79, 80). Massive shedding of the organism in excreta from these animals could explain outbreaks in cattle that occur on pasture land (39, 65,

74). Data collected in a bacteriologic survey of the tracheae of feedlot cattle indicated that H. somnus is a part of the transient if not indigenous flora (25). Miller (58) has proposed that venereal transmission of H. somnus could interfere with embryo implantation.

Stress factors such as dietary changes, inclement weather, and transport may predispose animals to infection (1, 5, 41, 44, 62, 65, 80). Crandell et al. (26) found that when young calves that were carriers of H. somnus were stressed by introduction of infectious bovine rhinotracheitis virus, a common respiratory virus, horizontal transmission of H. somnus to virus-stressed noncarrier calves occurred.

Serologic studies of apparently normal, healthy cattle show a considerable percent to have agglutinins to H. somnus. Hoerlein et al. (44) used the tube agglutination method to test sera of 1,238 cattle from 25 sources. Twenty-four percent had titers of 25 or greater. Stephens et al. (79) found that up to 91.2% of clinically normal animals had titers of four or greater when a more sensitive bacterial agglutination test that utilized boiled antigen was used.

Serologic surveys on herds of cattle in which the septicemic form of disease occurred show a variable percent of cattle with antibodies to the organism (6, 30, 50). Dierks et al. (30) demonstrated with the complement fixation test (CFT) that nearly 100% of cattle from infected herds were serologically positive and had titers ranging from 29 to 640. The mortality in these herds was in the range of 1 to 2%, and only a small number of cattle were clinically ill. An additional 2,000 serum samples from 284 herds were tested. Complement fixation titers of eight or greater were present in 50% of these herds. In one half the positive herds, the number of serologically positive cattle approached 100%.

Kennedy et al. (50) found that 83% of 83 steers exposed in a natural outbreak of disease three months prior to sampling were serologically positive to H. somnus by the plate agglutination test. Despite the serologic evidence, the relationship between naturally acquired serum antibody and susceptibility to naturally occurring H. somnus disease is not known (46). The serum antibody levels may not be indicative of protection against disease (78). Therefore, the significance of serum antibody titers is unknown.

Serologic cross-reactions between H. somnus and other related or nonrelated bacteria have been observed by several investigators (30, 37, 59, 72, 76). Miller et al. (59) utilized five antigen preparations (whole cell, sonicated, crude polysaccharide, purified polysaccharide, and protein) and found a close antigenic relationship between H. somnus and H. agni with the CFT and agglutination tests. Less consistent and weaker cross-reactions were observed with Actinobacillus actinoides, A. lignieresi, Bordetella bronchiseptica, H. aphrophilus, and Moraxella bovis. A close relationship was observed among H. somnus, H. agni, and Histophilus ovis by Stephens et al. (76) with the gel diffusion technique.

Studies utilizing other techniques have also shown a relationship between H. somnus and other bacteria. Deoxyribonucleic acid hybridization studies have shown a close relationship among H. somnus, H. agni, and Histophilus ovis. Walker et al. (86) suggested that these organisms belong to a single species. The cell envelope proteins of H. somnus, H. agni, and Histophilus ovis were examined by Stephens et al. (76). Results of the polyacrylamide gel electrophoresis tests further demonstrate a close phenotypic relationship among these organisms.

Antigenic variation among isolates of H. somnus has been observed by some (6, 7, 20, 21, 23, 30, 45) but not all (37, 72) investigators. Brown et al. (6) employed the CFT, utilizing homologous and heterologous antisera, and found none or only minor antigenic variation among five Iowa isolates. Similar results were reported by Dierks (30). Garcia-Delgado et al. (37) found no antigenic differences among 68 strains of H. somnus from Canada and the United States when they were tested against the antisera to eight strains using agglutination, complement fixation, immunodiffusion, and counterimmunoelectrophoresis techniques. Shigadi and Hoerlein (72) found three California isolates to be antigenically homologous by utilizing the agglutination test. Corboz and Nicolet (21) demonstrated, by the microtube agglutination test, a close antigenic relationship among five isolates from the United States and Switzerland. However, Humphrey et al. (45) found antigenic variation between survey and reference strains of H. somnus with the immunodiffusion technique. Canto and Biberstein (7) tested 46 strains of Swiss and American origins by the tube agglutination and agglutinin adsorption procedures. Test results suggested the existence of at least three sets of antigens and four agglutination groups. Antigenic variation was observed between both the American and Swiss isolates. Antigenic variability was also demonstrated by the Ouchterlony gel diffusion test on three serial isolates of H. somnus from an experimentally infected calf by Corstvet (23).

The relationship between colonial morphology and antigenic heterogeneity of H. influenzae has been studied (17, 18, 63). Chandler et al. (18) described mucoid colonies to be large, smooth, opaque, and fluorescent in transmitted light. Smooth colonies were observed to be small,

translucent, bluish, and nonfluorescent in transmitted light. They also observed a type-specific soluble substance with the mucoid colonies that was not present in smooth colonies. Morphologic features, structure, and adherence to bovine turbinate cells of three colonial variants of H. somnus from experimentally infected embryonated chicken eggs was studied by Ward et al. (87). The three variants were translucent, small opaque, and large opaque. The translucent variant contained thin-walled and pleomorphic bacillus-shaped organisms. The organisms in the small opaque variant had thicker cell walls and were not pleomorphic. The large opaque variant contained the bacillus-shaped organisms which were very thick walled. Organisms from the translucent and small opaque variants were significantly more adherent to the turbinate cells than were cells from the large opaque colonial variant.

CHAPTER III

MATERIALS AND METHODS

Haemophilus somnus Cultures

Four *Haemophilus somnus* isolates numbered 6396, 6402, 6406 (Dr. R. E. Corstvet, Oklahoma State University) and M677 (Dr. A. B. Hoerlein, Colorado State University) were used in this study. The Oklahoma cultures were serial isolates collected at weekly intervals from the trachea of an experimentally infected calf. Isolate M677 was cultured from the brain of a calf which had died of *H. somnus* septicemia. Primary *H. somnus* isolates were inoculated into the yolk sacs of embryonated chicken eggs. The infected yolks were harvested and lyophilized and stored at -70 C until used.

Culture Media

Each isolate of *H. somnus* was propagated in three different kinds of media. The medium for routine culture consisted of brain heart infusion agar (Difco Laboratories, Detroit, MI), 10% horse serum (Pel-Freez, Rogers, AR), 5% citrated bovine blood, and 1% filter-sterilized yeast hydrolysate (ICN-Pharmaceuticals, Inc., Life Science Group, Cleveland, OH), pH 5.9 (BHIB). For the determination of colonial morphology, the organism was propagated on a medium containing brain heart infusion agar, 10% horse serum, and 1% filter-sterilized yeast hydrolysate, pH 5.9 medium (BHI). A medium composed of Worfel-Ferguson broth

(Difco Laboratories, Detroit, MI), 10% horse serum, 1% yeast hydrolysate, and 2% agar (Difco Laboratories, Detroit, MI) was used for the propagation of H. somnus for the "capsule swelling" reaction (16, 60).

Determination of Colonial Dissociation

Lyophilized cultures of each isolate of H. somnus were rehydrated with 1.0 ml of sterile distilled water and streaked onto BHIB and BHI plates so that isolated colonies could be obtained. The plates were incubated at 37 C in 10% CO_2 and examined at three- to five-hour intervals for 10 to 48 hours. When growth had occurred on the BHI plates, the colonies were examined for colonial morphology. If there was no growth on BHI plates, isolated colonies were picked at random from the BHIB plates, transferred to BHI, and streaked to obtain isolated colonies. The colonial morphology and coloration were examined by oblique light transmitted at an angle of 45^{o} from beneath the colonies with the aid of a dissecting microscope (magnification 45X). Mucoid colonies appeared opaque and large with undulated margins and a yellow-orange coloration, whereas smooth colonies were translucent and small with a regular margin and a grey-blue to grey-green coloration. Colonies selected with respect to the dissociation form were then transferred with a bacteriological loop to BHI and the inoculum streaked so that isolated colonies could be obtained. After incubating as described above, the colonial morphology was again observed by obliquely transmitted light. Colonies with designated morphology and coloration were picked and deposited in the center of a glass microscope slide. To confirm the colonial morphology, one to two drops of 1:10,000 acriflavine solution (National Aniline Division, Allied Chemical and Dye

Corp., Morristown, NJ) were added to the deposited bacterial cells and mixed thoroughly with the inoculating loop. Further mixing was accomplished by rocking and tilting the slide. The precipitation reaction was noted shortly after mixing.

Preparation of Antigens

Two types of antigens were prepared from H. somnus cultures. The preparation methods will be discussed separately.

Boiled Antigen

Cultures with designated colonial morphology, as determined by the use of obliquely transmitted light and the acriflavine test, were transferred to BHIB plates by swabbing for confluent bacterial growth. After 18 to 20 hours incubation at 37 C in 10% CO_2, the plates were examined for growth, and the colonial morphology was confirmed by the acriflavine test. The bacterial cells were removed from the culture plates with a glass stick and suspended in precooled (4 C) phosphate-buffered saline (PBS) (0.85% NaCl, 0.01 M phosphate buffer, pH 8.0). Twenty-ml quantities of bacterial suspension (four plates of confluent growth) were centrifuged at 12,062 x g for 10 minutes. The resultant pellet was resuspended in 1.0 ml PBS, placed in a boiling water bath for one hour, and then centrifuged as described previously. The supernatant fluid was removed and stored at either -18 or 4 C. Protein determination of the antigen was by the method of Lowry et al (53).

Whole Cell Antigen

Haemophilus somnus cultures with designated morphology were propa-

gated as described above. The following procedure was used for antisera production and the slide agglutination test. Bacterial growth from five plates was suspended in 2.0 ml precooled (4 C) PBS. For intravenous inoculation, the suspension was diluted to MacFarland No. 3 (10^{-7} to 10^{-8} bacteria/ml) and for the slide agglutination test to MacFarland No. 2 (10^{-5} to 10^{-6} bacteria/ml). Whole cell antigen for the serum adsorption test was propagated as described above. One plate of a 24-hour culture that had confluent growth was used to adsorb 1.0 ml of antiserum (diluted 1:2). Organisms for the "capsule swelling" reaction were cultured on BHI and modified Worfel-Ferguson agar and incubated for 7.5 to 12 hours at 37 C in 10% CO_2.

Preparation of Antisera

Five- to six-week-old, male Cornish cross chickens, negative for antibodies to H. somnus as measured by the Ouchterlony gel diffusion technique, were injected on day 0 with 1.0 ml of antigen and Freund's incomplete adjuvant (Difco Laboratories, Detroit, MI) (equal parts antigen, boiled or whole cell, and Freund's incomplete adjuvant) subcutaneously and 0.5 ml antigen in PBS intravenously. On day 25, the chickens were test-bled and administered 1.0 ml antigen in PBS subcutaneously. Eight days later, the chickens were exsanguinated and the sera collected and stored at either -18 or 4 C without addition of a preservative. Antisera to isolates 6396, 6402, and 6406 were also obtained from Dr. R. E. Corstvet.

Serological Techniques

Ouchterlony Gel Diffusion Precipitin Test

The agar gel consisted of 0.9% Noble agar (Difco Laboratories, Detroit, MI), 8.5% NaCl, and 0.001% Trypan blue (Sigma Chemical Company, Saint Louis, MO). The melted agar was dispensed in 7.0-ml aliquots into 5-cm diameter petri dishes. Seven wells, 6 mm in diameter and 11 mm center to center, were cut in each plate. The wells were filled with appropriate antigens or antisera, incubated in a humidified chamber at 37 C, and read daily for seven days.

Slide Agglutination Test

One drop of whole cell antigen prepared as described above and one drop of antiserum were placed on a glass slide. The slide was tilted back and forth for one minute to mix the antigen and antiserum, and then the mixture was observed macroscopically for agglutination.

Serum Adsorption Test

Serum was adsorbed by the method of Nicolet (61). One petri plate of confluent bacterial growth was suspended in 5.0 ml PBS and centrifuged at 12,062 x g for 10 minutes. One ml of serum, diluted 1:2 with 0.85% NaCl, was added to the resultant pellet, mixed well, and incubated at 25 C for two hours. The suspension was then centrifuged as described above and the supernatant fluid removed for further testing.

Capsular Swelling Test

Whole cell antigen suspension was prepared in 1.0 ml sterile dis-

tilled water as described previously. One bacteriologic loop each of bacterial suspension, antiserum, and methylene blue (Sigma Chemical Company, Saint Louis, MO) solution (1:10,000) was placed on a glass microscope slide and mixed with a loop. The mixture was coverslipped, incubated at 25 C for five minutes, and then observed microscopically (1,500X) for "capsule swelling." Klebsiella pneumoniae type 68 and its homologous antiserum were used as a positive control.

CHAPTER IV

RESULTS

Based upon the colonial morphology, Haemophilus somnus isolates 6396, 6402, 6406, and M677 were assigned to four groups: mucoid, smooth, intermediate, and rough. The classification scheme of the colonial dissociation forms and antigenic variants is shown in Table I.

Microscopically, mucoid colonies appeared opaque, yellow-orange, and noniridescent. This mucoid designate produced a slimy precipitate with clot formation in acriflavine. The smooth colonies appeared grey-blue to grey-green, translucent, and noniridescent. Smooth colonies were divided into two antigenic groups, smooth (S) and smooth-rough (SR), based on their reaction in acriflavine. The S group produced a homogenous suspension in acriflavine, whereas the SR group produced a fine granular precipitate.

The first passage of the reconstituted organisms on artificial media yielded a mixture of colonial forms. It was also observed that single colonies could have two or more zones of different dissociation. The mucoid and smooth forms were not stable. Transformations from mucoid to smooth to intermediate and smooth to mucoid were observed. However, subcultures (18 to 24 hours) of smooth and mucoid colonial forms were predominantly pure cultures of the same variant. Smooth colonial forms appeared to be more stable than the mucoid forms. When two mucoid colonies which appeared identical were transferred, sub-

TABLE I

COLONIAL DISSOCIATION FORMS AND ANTIGENIC VARIANTS OF FOUR *HAEMOPHILUS SOMNUS* ISOLATES

Colonial Morphology[a]	Reaction in Acriflavine[b]	Designation	
		Colonial Form	Antigenic Type
Large, circular, or undulated margin, surface slimy with even circular rings; older cultures often have small, bud-like marginal colonies; yellow-orange, opaque, non-iridescent	Slimy precipitate, often with clot formation	Mucoid	M
Small, circular, regular margin, smooth surface, grey-blue, grey-green, translucent, noniridescent	Homogenous suspension	Smooth	S
	Fine granular precipitate		SR
Large, circular or undulated margin; surface more or less granulated; blue-green to yellow-green, opaque, non-iridescent	Partial granular precipitate	Intermediate	I
	Granular precipitate	Rough	R

[a]When observed with obliquely transmitted light.
[b]1:10,000 dilution in distilled water.

cultures rarely resulted in two mucoid cultures of the same quality. There was a greater degree of variation within this phase.

Results of the "capsule swelling" test were negative for H. somnus isolates; however, a broad halo of "swelling" was observed for the positive control (Klebsiella pneumoniae type 68).

Antisera prepared by Corstvet against isolates 6396, 6402, and 6406 each reacted with antigen preparations of 6396 and 6402, protein concentration 800 ug/ml, and with antigen 6406, protein concentration 400 ug/ml. These results were confirmed by the slide agglutination test. The adsorption of antiserum to 6402 with 6402, 6406, and 6396 cells removed agglutinating activity of the antiserum for all of the isolates.

Table II shows the results of the Ouchterlony gel diffusion and slide agglutination tests of strain 6396 with antisera to the soluble antigens of smooth or mucoid forms or antisera to a mixed culture containing both forms. Both tests were positive with the antiserum to the mixed culture. Unadsorbed antiserum reacted only with homologous antigen except in one instance when smooth antiserum reacted with mucoid antigen. Adsorption of the antiserum against the smooth form with either the smooth or mucoid particulate antigens removed reactivity. However, the activity of the mucoid antiserum was removed only by adsorption of its homologous antigen.

Results of the Ouchterlony gel diffusion and slide agglutination tests of strain 6402 with antisera to the soluble antigens of smooth or mucoid forms or antisera to a culture containing both forms are shown in Table III. These results are the same as those shown for isolate 6396 in Table II.

Results of Ouchterlony gel diffusion and slide agglutination tests

TABLE II

OUCHTERLONY GEL DIFFUSION AND SLIDE AGGLUTINATION TESTS OF ISOLATE 6396, SMOOTH (S) AND MUCOID (M) FORMS IN HOMOLOGOUS ANTISERA PREPARED AGAINST SOLUBLE ANTIGENS OF BOTH FORMS

	Antiserum[a]									
	S+M[b]				S[c]			M[d]		
Test Antigen	uad[e]	ad-S[f]	ad-M[g]	ad-M+S[h]	uad	ad-S	ad-M	uad	ad-S	ad-M
Gel Diffusion:[i]										
S+M[j]	+	+	+	-	+	-	-	+	+	-
S[k]	+	-	+	-	+	-	-	-	-	-
S[j]	ND[l]	ND	ND	ND	+	-	-	-	-	-
M[k]	+	+	-	-	-	-	-	+	+	-
M[j]	ND	ND	ND	ND	+	-	-	+	+	-
Slide Agglutination:[m]										
S+M	+	+	+	-	+	-	-	+	+	-
S	+	-	+	-	+	-	-	-	-	-
M	+	+	-	-	-	-	-	+	+	-

[a]Undiluted for gel diffusion test. Diluted 1:10 in 0.85% NaCl for the slide agglutination test.
[b]Mixed culture containing smooth and mucoid forms.
[c]Smooth form.
[d]Mucoid form.
[e]Unadsorbed.
[f]Adsorbed with the smooth form.
[g]Adsorbed with the mucoid form.
[h]Adsorbed with the mixed culture.
[i]Soluble antigen reaction at seven days.
[j]New antigen from the same lot as k.
[k]Same antigen as used for immunization.
[l]Not done.
[m]Whole cell antigen diluted in PBS, pH 8.0, to MacFarland standard No. 2.

TABLE III

OUCHTERLONY GEL DIFFUSION AND SLIDE AGGLUTINATION TESTS OF ISOLATE 6402, SMOOTH (S) AND MUCOID (M) FORMS IN HOMOLOGOUS ANTISERA PREPARED AGAINST SOLUBLE ANTIGENS OF BOTH FORMS

	Antiserum[a]									
	S+M[b]				S[c]			M[d]		
Test Antigen	uad[e]	ad-S[f]	ad-M[g]	ad-M+S[h]	uad	ad-S	ad-M	uad	ad-S	ad-M
Gel Diffusion:[i]										
S+M[j]	+	+	+	-	+	-	-	+	+	-
S[k]	+	-	+	-	+	-	-	-	-	-
S[j]	ND[l]	ND	ND	ND	+	-	-	-	-	-
M[k]	+	+	-	-	-	-	-	+	+	-
M[j]	ND	ND	ND	ND	+	-	-	+	+	-
Slide Agglutination:[m]										
S+M	+	+	+	-	+	-	-	+	+	-
S	+	-	+	-	+	-	-	-	-	-
M	+	+	-	-	-	-	-	+	+	-

[a]Undiluted for gel diffusion test. Diluted 1:10 in 0.85% NaCl for the slide agglutination test.
[b]Mixed culture containing smooth and mucoid forms.
[c]Smooth form.
[d]Mucoid form.
[e]Unadsorbed.
[f]Adsorbed with the smooth form.
[g]Adsorbed with the mucoid form.
[h]Adsorbed with the mixed culture.
[i]Soluble antigen reaction at seven days.
[j]New antigen from the same lot as k.
[k]Same antigen as used for immunization.
[l]Not done.
[m]Whole cell antigen diluted in PBS, pH 8.0, to MacFarland standard No. 2.

of isolates 6406 and M677 (smooth and mucoid forms) in homologous antisera prepared against soluble antigens of both forms are shown in Tables IV and V, respectively. Both tests were positive with antisera to the mixed culture. Unadsorbed antisera reacted with its homologous antigen. Adsorption of the antisera with homologous but not heterologous antigen removed reactivity.

Results of the Ouchterlony gel diffusion test with adsorbed and unadsorbed antisera prepared against the smooth variant of all four isolates are shown in Table VI. Antigens of all four H. somnus isolates failed to react with both homologous and heterologous antisera produced against the smooth form of the isolate when adsorbed with either homologous or heterologous cultures of each isolate. The unadsorbed antisera reacted with both homologous and heterologous antigens.

TABLE IV

OUCHTERLONY GEL DIFFUSION AND SLIDE AGGLUTINATION TESTS OF ISOLATE 6406, SMOOTH (S) AND MUCOID (M) FORMS IN HOMOLOGOUS ANTISERA PREPARED AGAINST SOLUBLE ANTIGENS OF BOTH FORMS

	Antiserum[a]									
	S+M[b]				S[c]			M[d]		
Test Antigen	uad[e]	ad-S[f]	ad-M[g]	ad-M+S[h]	uad	ad-S	ad-M	uad	ad-S	ad-M
Gel Diffusion:[i]										
S+M[j]	+	+	+	-	+	-	+	+	+	-
S[k]	+	-	+	-	+	-	+	-	-	-
S[j]	ND[l]	ND	ND	ND	+	-	+	-	-	-
M[k]	+	+	-	-	-	-	-	+	+	-
M[j]	ND	ND	ND	ND	-	-	-	+	+	-
Slide Agglutination:[m]										
S+M	+	+	+	-	+	-	+	+	+	-
S	+	-	+	-	+	-	+	-	-	-
M	+	+	-	-	-	-	-	+	+	-

[a]Undiluted for gel diffusion test. Diluted 1:10 in 0.85% NaCl for the slide agglutination test.
[b]Mixed culture containing smooth and mucoid forms.
[c]Smooth form.
[d]Mucoid form.
[e]Unadsorbed.
[f]Adsorbed with the smooth form.
[g]Adsorbed with the mucoid form.
[h]Adsorbed with the mixed culture.
[i]Soluble antigen reaction at seven days.
[j]New antigen from the same lot as k.
[k]Same antigen as used for immunization.
[l]Not done.
[m]Whole cell antigen diluted in PBS, pH 8.0, to MacFarland standard No. 2.

TABLE V

OUCHTERLONY GEL DIFFUSION AND SLIDE AGGLUTINATION TESTS OF ISOLATE M677, SMOOTH (S) AND MUCOID (M) FORMS IN HOMOLOGOUS ANTISERA PREPARED AGAINST SOLUBLE ANTIGENS OF BOTH FORMS

	Antiserum[a]									
	S+M[b]				S[c]			M[d]		
Test Antigen	uad[e]	ad-S[f]	ad-M[g]	ad-M+S[h]	uad	ad-S	ad-M	uad	ad-S	ad-M
Gel Diffusion:[i]										
S+M[j]	+	+	+	-	+	-	+	+	+	-
S[k]	+	-	+	-	+	-	+	-	-	-
S[j]	ND[l]	ND	ND	ND	+	-	+	-	-	-
M[k]	+	+	-	-	-	-	-	+	+	-
M[j]	ND	ND	ND	ND	-	-	-	+	+	-
Slide Agglutination:[m]										
S+M	+	+	+	-	+	-	+	+	+	-
S	+	-	+	-	+	-	+	-	-	-
M	+	+	-	-	-	-	-	+	+	-

[a]Undiluted for gel diffusion test. Diluted 1:10 in 0.85% NaCl for the slide agglutination test.
[b]Mixed culture containing smooth and mucoid forms.
[c]Smooth form.
[d]Mucoid form.
[e]Unadsorbed.
[f]Adsorbed with the smooth form.
[g]Adsorbed with the mucoid form.
[h]Adsorbed with the mixed culture.
[i]Soluble antigen reaction at seven days.
[j]New antigen from the same lot as k.
[k]Same antigen as used for immunization.
[l]Not done.
[m]Whole cell antigen diluted in PBS, pH 8.0, to MacFarland standard No. 2.

TABLE VI

OUCHTERLONY GEL DIFFUSION REACTIONS OF *HAEMOPHILUS SOMNUS* ISOLATES 6396/S, 6402/S, 6406/S, AND M677/S WITH ANTISERA PREPARED AGAINST SOLUBLE SMOOTH ANTIGEN

	Antiserum															
	6396					6402					6406					M677				
	uad[a]	ad[b]				uad	ad				uad	ad				uad	ad			
		A	B	C	D		A	B	C	D		A	B	C	D		A	B	C	D
Antigen:																				
6396	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-
6402	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-
6406	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-
M677	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-

[a]Unadsorbed.
[b]Adsorbed with: A--6396, B--6402, C--6406, D--M677.

CHAPTER V

DISCUSSION

Corstvet previously reported that serial isolates of Haemophilus somnus collected at weekly intervals from the trachea of an experimentally infected calf displayed antigenic heterogeneity. He postulated that "antigenic drift" had occurred either as a result of the acquisition of or loss of a capsule or because of microbial dissociation (23). The present investigation was initiated in an attempt to relate the observed variation in antigenic expression to changes in the physical morphology of cells or colonies of cells.

Microscopic observation of the colonial morphology revealed a considerable variation not only within a subculture of an isolate but also within a single colony of the subculture. Many colonies showed evidence of a variety of secondary growths, including daughter colonies, pie-shaped sections, and localized marginal outgrowths. These patterns of sectioning and marginal transformations have been observed with other bacteria (27, 28, 40). The colonial variants were designated into four groups: mucoid, smooth, intermediate, and rough. These colonial designations followed the pattern used by Carter (11) with Pasteurella multocida and were based upon colonial morphology observed microscopically and with acriflavine testing. The smooth and mucoid forms were chosen for further study because they were easiest to differentiate. Additionally, H. somnus strain M677 in mixed culture and subculture of

its mucoid and smooth forms could produce typical lesions of infectious septicemic thrombotic meningoencephalitis after intravenous or intratracheal inoculation (22).

Corstvet (24) proposed that serologic variation of the smooth and mucoid forms could be due to the presence or absence of a capsule; however, lack of colonial iridescence which is characteristic of capsulated H. influenzae strains (17, 18, 33) and negative "capsule swelling" test seem to indicate that if a capsule were present the capsular material might not be capable of "capsular swelling." Haemophilus influenzae type b has been observed to undergo genetic changes that affect capsule production. These variant bacteria and their progeny are incapable of producing complete capsules; therefore, they are nontypable by "capsular swelling" test but can be typed by immunofluorescence (17, 71). If H. somnus is capable of producing a capsule, the modified Worfel-Ferguson medium used for culture might not enhance the production of capsular material as seen in other bacteria. Another possible explanation for the negative "capsule swelling" test could have been that the antisera produced for use in the test might not have had antibodies specific to the capsular material. Light microscope observations of capsules using the Hiss stain and India ink preparations have been reported (59, 89); however, electron micrographs of thin sections stained with ruthenium red do not show H. somnus to be encapsulated (77, 87). A possible explanation for the discrepancy in reports of capsules could be that the diffraction halo around bacteria, which is a common aberration in light microscopy, might have been interpreted as a capsule.

Carter (11, 12) observed two types of smooth colonial variants of

Pasteurella multocida. The variant S which was iridescent was also encapsulated and produced a homogenous suspension in acriflavine. The second type of smooth variant (SR) was noniridescent and possessed less capsular material; however, in acriflavine, a partial flocculation reaction was observed. Similar results were seen in the present study. Antigenic S cells remained in a homogenous suspension; however, iridescence was not observed. Cells of the SR type produced a fine granular precipitate in acriflavine, a finding similar to that of Carter (11, 12) with P. multocida. Since the colonial morphology and acriflavine reactions were similar to those seen by Carter (11, 12), one might also postulate the presence of a capsule in the S cells of H. somnus.

Because of failure to observe iridescence and "capsular swelling", it was postulated that serologic variation could be due to the presence or absence of specific surface antigens. Corstvet (24) previously reported that H. somnus isolates 6396, 6402, and 6406 reacted in the Ouchterlony gel diffusion test with antiserum to isolate 6406, and no lines of precipitate were formed with antigen 6406 when tested with antisera to 6396 and 6402. These results suggest that antigenicity may vary among sequential isolates.

Preliminary experiments in the present studies, which utilized antisera provided by Corstvet and newly prepared soluble antigens from the same lyophilized cultures, did not confirm these findings. These antisera to isolates 6396, 6402, and 6406 each formed a line of precipitate with antigens 6396 and 6402, protein concentration 800 ug/ml. Similar results were seen with isolate 6406, protein concentration 400 ug/ml, and with antisera to 6396, 6402, and 6406. Antiserum to 6402 when adsorbed with 6396, 6402, or 6406 removed reactivity in the slide

agglutination test.

Antigenic heterogeneity may also be due to the colonial dissociation of the organism, as has been demonstrated with other bacteria (11, 18, 52). With homologous antisera, a serological difference between smooth and mucoid dissociation forms of the same isolate could be demonstrated as seen with 6396, 6402, 6406, and M677 (Tables II, III, IV, and V). However, with isolates 6396 and 6402 (Tables II and III), new lots of soluble M antigens reacted with both the S and M antisera. Results indicate that 6396 and 6402 mucoid soluble antigens were a mixture of mucoid and smooth forms. Furthermore, antisera prepared against both the smooth and mucoid isolates of 6396 and 6402 (Tables II and III) could be adsorbed by the homologous mucoid particulate antigens. These inconsistent test results suggest that the mucoid particulate antigen used for serum adsorption was actually a mixture of both smooth and mucoid forms.

It should be noted that the particulate antigens utilized for adsorptions of smooth and mucoid antisera of isolates 6396, 6402, 6406, and M677 (Tables II, III, IV, and V) are of different lots than the antigen used for adsorption of antisera to the mixed culture forms of all isolates. Particulate antigens used for the slide agglutination and serum adsorption studies were from the same lot.

Serologic differences between smooth and mucoid forms of H. somnus were demonstrated with both the Ouchterlony gel diffusion and slide agglutination tests. Difficulty in obtaining pure smooth and pure mucoid cultures may be due to many factors. The method of selection of the dissociation forms was relatively crude, and the selection of identical colony types was difficult. Furthermore, the appearance of

the colonies may not accurately reflect the nature of the individual bacteria. Genetic variations may affect different cellular properties and yet not influence colonial morphology. During bacterial replication that results in formation of a colony, there is opportunity for genetic change. Consequently, the cells of a given colony may be heterogenous and give rise on subculture to more than one kind of colony (17). Perhaps the mucoid dissociation form of cells is dominant and masks the presence of smooth organisms. When two mucoid colonies presumed to be pure were subcultured, the progeny rarely resulted in two subcultures with the same physical attributes. Similar observations have been made by others. Hadley (40), in his discourse on microbial dissociation, noted that one must make allowances for some degree of variation within each phase. The culture phases are interconvertible, and there is no evidence that any culture phase is characterized by stability in the absolute sense.

Serologic differences of the smooth and mucoid dissociation forms of H. somnus have been demonstrated with the Ouchterlony gel diffusion and slide agglutination tests, and the differences have been shown to be due to the presence or absence of specific surface antigens. Inconsistent test results in the present study were probably due to the purity of the smooth or mucoid cultures. Such variations in purity could result in quantitative or qualitative variations of the surface antigens or antibodies corresponding to the dissociation form. Using cultures of unknown dissociation form, one might expect a line of precipitate in the Ouchterlony gel diffusion test unless the optimal proportions of antigen and antibody were not equivalent. Such an occurrence could explain the descrepancies between the Corstvet study (23) and preliminary experi-

ments in the present study.

In the present study, it was observed that expression of surface antigens varies according to the dissociation form of the organism. The smooth dissociation form of all four isolates reacted similarly with homologous and heterologous antisera (Table VI), and they appear to be serologically identical.

CHAPTER VI

SUMMARY

The colonial dissociation of four isolates of Haemophilus somnus was observed microscopically. Smooth and mucoid colonial variants of each isolate were selected on the basis of microscopic appearance and reaction in acriflavine. "Capsular swelling" was not observed with any of the isolates, irrespective of form. Antisera prepared against a particular dissociation form were specific for that form in the Ouchterlony gel diffusion and slide agglutination tests. The activity could be eliminated by adsorption with cells of the same form used for antiserum production but not by cells of the other form. Results indicate that the dissociation form directly influences the antigenic expression of H. somnus.

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