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Common Antigenic Determinant in a Rumen Organism and in Salmonellae Containing the Antigen O4

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Nineteen of 28 strains of rumen organisms isolated from a cow on a high roughage diet and identified morphologically as butyrivibrios, reacted to a low agglutinin titer with salmonella antisera, forming five groups. However only one strain reacted with polyvalent O salmonella antiserum. This strain reacted with O4 factor serum and with antisera to *Salmonella* strains containing the antigen O4, and agglutinin absorption tests showed the presence of an antigen identical to O4. When 16 further strains of butyrivibrio-like rumen organisms isolated from three cows and one steer were examined, one possessed an antigen similar to but not identical with the antigen O9, and two strains reacted with specific O6,7 factor serum but were not examined further. These four strains were presumptively identified by physiological tests as butyrivibrios. The possible site of antigenic stimulation by such organisms is discussed.

Natural antibodies against *Enterobacteriaceae*, including a number of *Salmonella* strains, occur widely in the sera of animal species including ruminants (3, 7). Sharpe, Latham, and Reiter (11) have shown that in the sera of ruminants relatively high titers of agglutinating antibodies occurred against strains of anaerobic bacteria isolated from the bovine rumen. This suggested that the reactions against *Enterobacteriaceae* might be due to antibodies against cross-reacting antigens in these organisms and in rumen strains. Antibodies against motile, curved, gram-negative chain-forming rods (butyrivibrio-like organisms) were particularly prevalent in the sera of the ruminants. This work describes the reaction of some of these organisms to antisera against some *Salmonella* serotypes.

MATERIALS AND METHODS

Organisms from the bovine rumen. Samples of well mixed rumen contents were removed from fistulated bovines, and organisms were isolated by the anaerobic techniques of Hungate (4), with the media and methods of cultivation described by Sharpe et al. (11). Strains of anaerobic, gram-negative, curved, motile rods in chains were identified microscopically.

Presumptive identification of rumen strains. Four serologically reacting isolates were further examined, using medium M10 as basal medium for tests, for growth in relation to oxygen tension, sulfide production, minimum pH in glucose broth, gas production in glucose agar, starch hydrolysis, cellulose digestion (filter paper strip), and production of lactic acid (1, 5, 8).

Salmonellae. Strains were obtained from W. Sojka, Central Veterinary Laboratory, Weybridge, and from the National Collection of Type Cultures. They were grown aerobically in nutrient broth at 37 C. Strains used for preparing antisera were checked at the Salmonella Reference Laboratory, Colindale, to confirm their antigenic patterns.

Preparation of antisera: rabbits. Vaccines consisted of 18-hr cultures of washed cells, either suspended in 0.15 M NaCl + 0.2% (v/v) formaldehyde, or in 0.15 M NaCl and heated at 100 C for 60 min, optical density being adjusted to 0.55 absorbancy at 580 nm (1-cm light path). Rabbits were inoculated intravenously with 1 ml of vaccine every 3 to 4 days for a course of five to eight inoculations.

Goats. Two milliliters of the heat-killed vaccines as above was given intravenously for a course of four to six inoculations.

Commercial antisera. Polyvalent and factor-specific antisera were obtained from Wellcome Reagents Ltd., Beckenham, Kent.

Agglutination tests. Suspensions for agglutination were either (i) suspended in 0.15 M NaCl + 0.2% (v/v) formaldehyde; (ii) suspended in 0.15 M NaCl and heated at 100 C for 60 min; or (iii) (later in the work) heated to 50 C for 30 min while suspended in ethanol, centrifuged, and resuspended in 0.15 M NaCl.

Agglutination tests were read after overnight incubation at 50 C.

Agglutination absorption tests. Antisera were absorbed at a dilution of 1:5 or 1:10 with ethanol-treated packed cells, two parts cells to 10 parts diluted antiserum, for 2 hr at 37 C. Two absorptions were often necessary.

RESULTS

Isolates. Isolations were made from a sample of the rumen contents of cow N13 on a high roughage diet, which promotes the predominance of butyrivibrios (8). Twenty-eight isolates were microscopically identified as motile, gram-negative, curved rods in chains.

Reaction of isolates to salmonella antisera. Formol-killed and heat-killed suspensions of these organisms were tested by tube agglutination with antisera (diluted 1:20) prepared against Formol-killed vaccines of *S. typhimurium*, *S. dublin*, *S. bovis morbificans*, *S. farmsen*, and *S. give*. Formol-treated suspensions were also tested against 1:20 dilutions of polyvalent H antisera and heated suspensions against 1:20 polyvalent O antiserum. Formol- and heat-treated suspensions both reacted similarly to the antisera against single salmonella strains. As the formolized suspensions did not react with the polyvalent H antisera, no further work was done with these suspensions. As these butyrivibrios possess only a single polar flagellum (1), the use of formolized or heat-treated suspensions, respectively, to determine the types of H or O antigens present, as with the salmonellae, is unlikely to be useful.

Table 1 shows the reactions of suspensions of heat-treated cells of isolates at 1:20 dilution of antiserum. With these antisera it was possible to group 19 of 28 of these isolates into five groups. However, the reactions occurred at the low titre of 1:20 and could not be considered specific. Only one strain, Nor 37, reacted strongly with the polyvalent O antiserum. On testing representative strains of the rumen isolates against eight different specific O factor sera, only Nor 37 reacted, giving a specific reaction with O4 antiserum. This strain was chosen for further work.

TABLE 1. *Agglutination reactions of heat-treated rumen organisms to salmonella antisera*

Serum	O antigens present	No. of strains reacting[a]
Antiserum against:		
S. typhimurium	4,5,12	1
S. dublin	9,12	1
S. farmsen	13,23	14
S. give	3,10	2
S. bovis morbificans	6,8	1
Wellcome polyvalent Factor O	Groups A–G	1
Serum from Cow N13		28

[a] Number of strains of rumen organisms giving a positive reaction with salmonella antisera and with other sera, all diluted 1:20. Rumen organisms were all anaerobic, gram-negative, motile, curved rods in chains isolated from cow N13. Twenty-eight strains were tested.

Reaction of two rumen strains and salmonella serotypes to antisera to Nor 37 and salmonellae. Using heat-treated cells for vaccines, antisera were prepared against Nor 37 and also against *S. abortus equi* and *S. altendorf* (each of which possesses the O4 antigen). These antisera were tested against alcohol-treated suspensions of eleven *Salmonella* serotypes, Nor 37, and a rumen strain, 1L6-31, isolated from another cow. Table 2 shows the titers obtained and also titers against the serum from cow N13. Serum from this cow contained a low titer of antibodies to many of the salmonella strains. The preinoculation goat serum also showed the presence of antibodies to many of the salmonellae. However in the two rabbits' sera, antibodies to salmonellae were absent before inoculation. After inoculation with strain Nor 37 the antibodies raised in rabbit 417 reacted not only with the homologous strain but also specifically with strains of salmonella containing O antigen 4. In the goat a significant rise in titer occurred against these same organisms. Inoculation of rabbit 427 with *S. altendorf* resulted in the presence of antibodies reacting with Nor 37, salmonella serotypes containing the O antigen 4, and also serotypes containing O antigen 12. An antiserum against *S. typhimurium* also reacted in a similar pattern. The Wellcome O4 factor-specific antiserum agglutinated only Nor 37 and salmonellae containing the O antigen 4.

Figure 1 shows the rise and fall of titers of agglutinating antibodies to Nor 37 and to three salmonella strains in a rabbit given five inoculations of alcohol-treated cells of Nor 37. The higher titers observed with the homologous organism may be due to the presence of another specific antigen in this organism but not in the other strains, or to the other O antigens present in the salmonella strains partially obscuring the O4 antigen.

Absorption of antisera. The effect of absorption of antisera by whole cells on agglutinating titers is shown in Table 3. When antiserum to Nor 37 was absorbed with *S. altendorf* all reactions to salmonella strains

TABLE 2. *Agglutination tests on alcohol-treated rumen and salmonella strains with different animal sera and antisera*

Strain	O antigens	Titers against sera								
		Cow N13	Rabbit 417		Goat 293		Rabbit 427		Rabbit 393 a/s *S. typh.*[d]	Wellcome O4 factor
			PI[a]	a/s Nor 37[b]	PI	a/s Nor 37	PI	a/s *S. alt.*[c]		
Nor 37	?	80	—[e]	2,560	80	2,560	—	2,560	160	320
S. abortis equi	4,12	80	—	640	80	1,280	—	2,560	160	320
S. altendorf	4,12	40	—	160	40	640	—	1,280	1,280	80
S. typhimurium	4,5,12	20	—	80	40	160	—	640	2,560	40
S. california	4,5,12	20	—	80	40	320	—	1,280	1,280	80
S. tinda	1,4,12,27	40	—	640	80	640	—	1,280	2,560	160
S. abortus bovis	1,4,12,27	80	—	640	160	1,280	—	2,560	640	320
S. dublin	9,12	20	—	—	—	—	—	320	20	—
S. enteritidis	1,9,12	—	—	—	20	20	—	640	2,560	—
S. bovis morbificans	6,8	—	—	—	—	80	—	w 40[f]	—	—
S. farmsen	13,23	—	—	—	—	—	—	w 40	—	—
S. give	3,10	40	—	—	80	80	—	—	—	—
1L6-31	?	160	—	—	320	320	—	—	—	—

[a] PI, Preinoculation.
[b] a/s Nor 37, Nor 37 antiserum.
[c] a/s *S. alt.*, *Salmonella altendorf* antiserum.
[d] a/s *S. typh.*, *Salmonella typhimurium* antiserum.
[e] —, Titer < 20.
[f] w, Weak reaction.

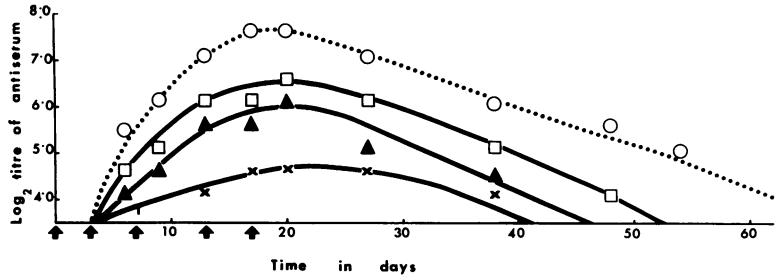

FIG. 1. *Titers of agglutinating antibodies to butyrivibrio Nor 37 and to salmonellae of group B during course of inoculating a rabbit with butyrivibrio NOR 37. Symbols:* ⬆, *Intravenous inoculation given;* ○, *reaction of butyrivibrio NOR 37;* □, *reaction of S. abortus equi;* ▲, *reaction of S. altendorf;* ×, *S. typhimurium.*

were removed, but some of the antibodies to Nor 37 remained, indicating that, in addition to antigen O4, another more specific antigen was indeed present in Nor 37 but not in the other strains. Absorption with nonreacting strains of *S. dublin* or 1L6-31 did not remove the reactions. When antiserum to *S. altendorf* was absorbed with Nor 37, titers were considerably lowered, but reactions, presumably to their common antigen 12, remained for the salmonella strains. Both Nor 37 and *S. altendorf* removed all the reacting antibodies from factor O4 antiserum.

There is therefore a common antigenic factor in the rumen organism Nor 37 and in salmonella of group B containing the O4 factor.

Reaction of further rumen strains to salmonella antisera. Sixteen more strains of anaerobic, motile, curved, gram-negative rods in chains isolated from three other cows and from a steer on different experiments were tested against the Wellcome polyvalent O antiserum and against antisera to *S. typhimurium*, *S. dublin*, and *S. bovis morbificans*, all diluted 1:20. One strain, 1L6-31, reacted with the polyvalent O antiserum and also with antiserum to *S. dublin*. On further testing with factor-specific sera, 1L6-31 reacted with factor O9 to a titer of 160. Antisera against this strain raised in a goat and in a rabbit reacted to titers of only 80 to 160 with strains of *S. strasbourg* (O9, 46), *S. zega* (O9, 12), *S. enteritidis*, and *S.*

TABLE 3. *Effect of absorptions on titers of antisera*

Absorption of sera, and strains tested	Titer of antiserum		
	Nor 37	*S. altendorf*	BW O4
Unabsorbed versus strain:			
NOR 37	2,560	2,560	320
S. abortus equi	640	2,560	320
S. altendorf	160	1,280	80
S. typhimurium	80	640	40
S. bredeney	640	1,280	160
Absorbed with *S. altendorf* versus strain:			
Nor 37	640	—[a]	—
S. abortus equi	—	—	—
S. altendorf	—	—	—
S. typhimurium	—	—	—
S. bredeney	—	—	—
Absorbed with Nor 37 versus strain:			
Nor 37	—	—	—
S. abortus equi	—	160	40
S. altendorf	—	320	—
S. typhimurium	—	320	—
S. bredeney	—	320	—
Absorbed with *S. dublin* versus strain:			
Nor 37	1,280	1,280	ND[b]
S. abortus equi	640	1,280	ND
S. altendorf	80	640	ND
S. typhimurium	80	320	ND
S. bredeney	640	640	ND
Absorbed with 1L6-31 versus strain:			
Nor 37	2,560	1,280	ND
S. abortus equi	640	1,280	ND
S. altendorf	160	640	ND
S. typhimurium	40	640	ND
S. bredeney	640	1,280	ND

[a] —, Titer < 20.
[b] ND, Not done.

dublin, whereas titers against the homologous organism were 5,120 and 2,560. However, absorption of factor O9 antiserum with cells of strain 1L6-31 failed to remove the reactions of the four salmonella strains, although some drop in titer occurred, and the reaction against 1L6-31 was completely removed. This suggests that the antigenic determinant present in 1L6-31 which reacted with the O9 factor antiserum was similar but not identical to the O9 antigenic determinant present in salmonella of group D. Two other strains of butyrivibrios, both from a steer, reacted with factor-specific serum O6, 7 to a titer of 1:80. No further tests were done on these strains.

Further identification of rumen strains. Strains Nor 37 and 1L6-31 and the two strains reacting with the O6, 7 serum produced a final *p*H in glucose broth of 5.0 to 5.4, none were cellulolytic, none produced lactic acid, only one produced sulfide, one produced gas from glucose, and two hydrolyzed starch. These characteristics were compatible with the strains being non-cellulolytic butyrivibrios.

Production of antibodies to strain Nor 37 in animals by other routes of administration. Attempts were made to raise serum antibodies against strain Nor 37 in the rabbit and in the goat by oral infusion of large numbers of viable cells of the culture, given at twice-weekly intervals for 5 weeks. In the rabbit no antibodies were detected in the serum after this treatment. In the goat, which had an initial titer of 40, the titer rose to 160, but on further infusion did not rise any higher.

Attempts were also made to raise antibodies to this organism in the ruminating calf by infusing twice weekly very large numbers of viable cells directly into the rumen of fistulated animals or into the duodenum of duodenum-cannulated animals. No increases in the initial low titers (40–80) occurred with any of these calves. Bacteriological and serological examination of isolates from rumen contents indicated, however, that strain Nor 37 did not become established in the rumen flora of the animals inoculated.

DISCUSSION

Common antigenic determinants between unrelated organisms are not uncommon particularly with the O antigens of the *Enterobacteriaceae*, serological cross-reactivity being due to identical chemical structures in the polysaccharide of cross-reacting species (reviewed Kwapinski [6], Wilson and Miles [12]). If the rumen strain Nor 37 has the same O4 antigenic determinant as group B salmonellae it should contain abequosyl-mannosyl-rhamnosyl-galactose repeat units.

It is of interest that the blood serum of an uninoculated cow and of two goats contained O antibodies to a variety of salmonella agglutinating antigens whereas that of rabbits did not. This may be correlated with the presence or absence of antibodies to rumen organisms in the serum of animals observed by Sharpe et al. (11), who showed that the two former species of animals possess such antibodies but the rabbit does not.

Our results indicate that previous reports of the presence of antibodies to a wide range of *Enterobacteriaceae*, including salmonellae, in bovine serum, may be due to cross-reactions with identical or similar polysaccharides present in rumen organisms.

Although only a small number of the butyri-

vibrios tested (4 of 44) reacted initially with the polyvalent O serum (containing factors of groups A through G) and then with factor-specific sera, these strains were isolated from three out of five of the animals examined. Further work is necessary to determine the incidence of such cross-reacting strains.

Failure to raise antibodies against strain Nor 37 with massive doses of live organisms by oral, direct ruminal or duodenal routes, may have been due to two factors: either a constant persistent stimulation is required over a long period of time and our treatment did not continue for long enough, or the organism must become established in the rumen for stimulation to occur.

To elicit an immune response, organisms should penetrate or at least survive on the mucosal epithelia (10). For this, viable bacteria are necessary, as shown by the production of antibodies on ingestion of live but not heat-killed cells of *S. enteritidis* (2).

As the caecum may be colonized by species of bacteria similar to those in the rumen and producing similar volatile fatty acid patterns (9), this could be the site of antigenic stimulation. Histological examination of the cecum wall showed clearly that it contained large numbers of plasma cells and mucosal scrapings were found to contain immunoglobulins (*unpublished data*). These observations may point to the origin of the antibody production in the cecum, as the rumen wall is devoid of plasma cells.

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