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

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Received for publication 22 June 1972

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 antiserum 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 antiserum 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 antiserum 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 Welcome 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 butyrovibrios (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 antiserum (diluted 1:20) prepared against Formol-killed vaccines of S. typhimurium, S. dublin, S. bovis morficsans, S. farnsen, and S. give. Formol-treated suspensions were also tested against 1:20 dilutions of polyvalent H antiserum and heated suspensions against 1:20 polyvalent O antiserum. Formol- and heat-treated suspensions both reacted similarly to the antiserum against single salmonella strains. As the formalized suspensions did not react with the polyvalent H antiserum, no further work was done with these suspensions. As these butyrovibrios possess only a single polar flagellum (1), the use of formalized 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.

Reaction of two rumen strains and salmonella serotypes to antiseria 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 antiseria 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 293 a/s S. typh. | Wellcome O4 factor |
|                 |            | a/s | a/s | a/s | a/s | PI | a/s Nor 37 | Pl | a/s Nor 37 | Pl | a/s S. alt. | Pl | a/s S. alt. | Pl |
| Nor 37          | ?          | 80 | - | - | - | - | - | 2,560 | 80 | 2,560 | - | 2,560 | 160 | 160 | 320 |
| S. abortis equi | 4,12       | 80 | - | 640 | 80 | 1,280 | - | - | 2,560 | - | 2,560 | 160 | 160 | 320 |
| S. altendorf    | 4,12       | 40 | - | 160 | 40 | 640 | - | - | 1,280 | - | 1,280 | 1,280 | 1,280 | 80 |
| S. typhimurium  | 4,5,12     | 20 | - | 80 | 40 | 160 | - | - | 640 | - | 2,560 | 2,560 | 160 |
| S. california   | 4,5,12     | 20 | - | 80 | 40 | 320 | - | - | 1,280 | - | 1,280 | 1,280 | 1,280 | 80 |
| S. tinda        | 1,4,12,27  | 40 | - | 640 | 80 | 640 | - | - | 1,280 | - | 2,560 | 2,560 | 160 |
| S. abortus bovis| 1,4,12,27  | 80 | - | 640 | 160 | 1,280 | - | - | 2,560 | - | 640 | 640 | 320 |
| S. dublin       | 9,12       | 20 | - | - | - | - | - | 320 | - | 20 | - | - | 320 |
| S. enteritidis  | 1,9,12     | - | - | - | - | 20 | 20 | - | - | 640 | - | - | 640 |
| S. bovis morbillicans | 6,8 | - | - | - | - | - | 80 | - | - | w 40 | - | - | 40 |
| S. farmesen     | 13,23      | - | - | - | - | - | - | - | - | - | - | - | - |
| S. give         | 3,10       | 40 | - | - | 80 | 80 | - | - | - | - | - | - | - | - |
| 1L6-31          | 160        | - | - | 320 | 320 | - | - | - | - | - | - | - | - | - |

* PI, Preinoculation.  
* a/s Nor 37, Nor 37 antiserum.  
* a/s S. alt., Salmonella altendorf antiserum.  
* a/s S. typh., Salmonella typhimurium antiserum.  
* —, Titer < 20.  
* w, Weak reaction.

**Fig. 1.** Titers of agglutinating antibodies to butyrylribio Nor 37 and to salmonellae of group B during course of inoculating a rabbit with butyrylribio N0R 37. Symbols: , Intravenous inoculation given; O, reaction of butyrylribio NOR 37; □, reaction of S. abortus equi; ▲, reaction of S. altendorf; x, 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 morbillicans, 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. Antiseria against this strain raised in a goat and in a rabbit reacted to titers of only 80 to 160 with strains of S. strasbour (O9, 46), S. zega (O9, 12), S. enteritidis, and S.
Table 3. Effect of absorptions on titers of antiserum

| Absorption of sera, and strains tested | Titer of antiserum |  |
|---------------------------------------|--------------------|---|
|                                       | Nor 37             | S. altendorf |
|                                       | S. abortus equi    | S. typhimurium |
|                                       | S. bredeney        | S. bredeney |
| Unabsorbed versus strain:             |                   |  |
| NOR 37                               | 2,560              | 2,560 320 |
| S. abortus equi                      | 640               | 640 320 |
| 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               | — |
| S. abortus equi                      | —                 | — |
| S. altendorf                         | —                 | — |
| S. typhimurium                       | —                 | — |
| S. bredeney                          | —                 | — |
| Absorbed with Nor 37 versus strain:  |                   |  |
| Nor 37                               | —                 | — |
| S. abortus equi                      | —                 | — |
| S. altendorf                         | —                 | — |
| S. typhimurium                       | —                 | — |
| S. bredeney                          | —                 | — |
| Absorbed with S. dublin versus strain: |                   |  |
| Nor 37                               | 1,280             | 1,280 ND* |
| 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                         | 640               | 640 ND |
| S. typhimurium                       | 40                | 640 ND |
| S. bredeney                          | 640               | 1,280 ND |

* ND, Not done.

...unrelated organisms were 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 caecum 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 caecum, as the rumen wall is devoid of plasma cells.

ACKNOWLEDGMENTS

We thank W. Sojka for supplying some of the salmonella strains, G. S. Knaggs for preparation of the antisera raised in the goat, and B. A. Phillips for technical assistance.

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