The Occurrence of Natural Antibodies to Rumen Bacteria

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SUMMARY

The sera of 11 ruminants and one horse contained relatively high titres of agglutinating antibodies against strains of anaerobic bacteria isolated from the bovine rumen. They were not detected in the sera of non-ruminants, i.e. pig, rabbit, guinea pig, rat and man. A close relationship existed between motility of rumen organisms and their ability to evoke detectable antibodies, although antibodies to both flagellar and somatic antigens were produced. The antibodies to the rumen organisms were highly specific and of the IgM type. They occurred in bovine colostrum at the same level as in the blood serum, and were transferred to the calf via the colostrum. After 1 month they had disappeared from the calf serum but between 5 and 8 months later antibodies of a similar activity were detected. In addition, antibodies to the non-motile *Bifidobacterium bifidus* were found in the sera of the young calf.

INTRODUCTION

In the absence of infection or overt stimulation with exogenous antigens, antibodies have been demonstrated in the sera of animals and man which are regarded as natural antibodies (Boyden, 1966). The intestinal flora is considered to be the most important source of antigenic stimulation for the production of such antibodies, providing a small but unremitting source of antigens.

Attempts have been made to define patterns of natural antibodies against Enterobacteriaceae (Gibson, 1930; Landy & Weidanz, 1964) amongst the sera of different animal species. Although it was not possible to make clear-cut distinctions between the animals, there were indications that each species might have its own pattern. It has been suggested (Reiter & Oram, 1967) that in ruminants conditions are particularly favourable for the formation of natural antibodies from rumen organisms. The specialization and high concentrations of organisms within the rumen might give rise to a greater specificity and to a higher titre of antibodies in the sera of these animals.

This paper is concerned with agglutination titres in bovine sera against anaerobic rumen bacteria, the patterns of antibody reactions of different animal sera against these organisms, and the appearance of such antibodies in the bovine colostrum and their transfer to the calf serum.

METHODS

Samples of well-mixed rumen contents were removed from a fistulated cow (A), and organisms isolated by the anaerobic techniques of Hungate (1950). Primary

isolation was made in roll tubes using a slightly modified medium 10 of Caldwell & Bryant (1966). Two stock mineral solutions were prepared (1) K_2HPO_4 0.6 % w/v; (2) NaCl 1.2 %, $(NH_4)_2SO_4$ 1.2 %, KH_2PO_4 0.6 %, $CaCl_2$ 0.12 %, $MgSO_4 \cdot 7H_2O$ 0.25 %, and a stock mixture of volatile fatty acids: acetic 17 ml., propionic 6 ml., *n*-butyric 4 ml., isobutyric 1 ml., DL- α -methyl-*n*-butyric 1 ml., isovaleric 1 ml., *n*-valeric, 1 ml. The composition of the medium was as follows (% w/v final concentration): glucose, 0.05; cellobiose, 0.05; soluble starch, 0.05; maltose, 0.05; yeast extract, 0.05; trypticase, 0.2; resazurin, 0.0001; haemin, 0.0001; agar, 2.0; cysteine-HCl and sodium sulphide each, 0.015; sodium carbonate, 0.4; volatile fatty acid mixture, 0.45 (v/v); mineral solutions 1 and 2, 7.5 (v/v) each; adjusted to pH 6.7 to 6.8 with *N*-NaOH. The cysteine-HCl + sodium sulphide mixture and the sodium carbonate were added separately as sterile solutions to the freshly autoclaved medium. Cleland's reagent, a protective reagent for SH groups (Cleland, 1964), was also added (to a final concentration 0.0001 M) in some instances to help poise the medium at the low Eh value required for growth of some of the rumen organisms. Further cultivation was done on agar slopes and in broth of a similar composition but containing 0.2 %, w/v, each of glucose, cellobiose and maltose and without starch (medium M10).

All media distribution and cultural work was done in an atmosphere of oxygen-free CO_2 .

Isolations and counts of total viable organisms were made from 3 rumen samples taken before, and 1 and 14 days after, a change of diet from 100 % hay to 100 % flaked maize.

Sample No.	Cows' diet	Viable organisms/g. rumen contents
1T	100 % hay	2.12×10^9
2T	100 % flaked maize	2.0×10^9
7T	100 % flaked maize	3.5×10^{10}

Colonies were picked from appropriate roll tubes onto slopes of medium M10, were cultured in M10 broth and preserved on slopes of M10 at -60° .

Isolation of Escherichia coli. These organisms were isolated from the rumen contents of two cows B and C on 100 % hay diet, using conventional anaerobic conditions (McIntosh & Fildes jar) and MacConkey media. Counts were estimated by mean probable numbers (m.p.n.), and were positive at 10^{-3} dilutions of rumen contents, but not at 10^{-4} .

Laboratory strains of Enterobacteriaceae. Six human strains of *Escherichia coli* NCTC 8603, 9026, 9703, 9705, 9707, 9855. Ten strains of *Klebsiella aerogenes* isolated previously from bovine mastitis cases and from straw bedding by Dr C. M. Cousins (N.I.R.D.).

Identification of organisms

Anaerobic rumen bacteria. Isolates were presumptively classed in a number of genera and species by morphology, motility, Gram reaction, production of gas from glucose, final pH value in glucose broth, H_2S production and breakdown of cellulose and starch (Hungate, 1966). Lactobacilli were identified by physiological and biochemical tests (Sharpe, Fryer & Smith, 1966), using media containing 0.02 % (w/v) cysteine and anaerobic cultivation by conventional anaerobic methods. The tests described by Shattock (1963) were used to identify *Streptococcus bovis*; *Escherichia coli* strains were identified by morphology and IMVIC tests.

Serological tests

Suspensions for agglutination tests. Anaerobic organisms were grown for 18 hr anaerobically in 100 ml. medium M10, centrifuged, washed and half the organisms suspended in 0.85 % saline + 0.2 % formaldehyde. The other half was suspended in 0.85 % saline and heated to 100° for 1 hr to destroy flagellar antigens. Extinctions were adjusted to a value of 0.55 (1 cm. cell, filter 58) on the Hilger Biochem. Absorptiometer. Lactobacilli were grown anaerobically in medium MRS (de Man, Rogosa & Sharpe, 1960) + 0.02 % (w/v) cysteine; *Escherichia coli* and *Streptococcus bovis* were grown aerobically in yeast glucose broth. All cultures were divided into two portions and suspensions treated in a similar manner to those of the anaerobic bacteria.

Agglutination tests. Results were read after overnight incubation at 50°.

Agglutinin absorption tests. Sera were absorbed with an equal volume of formol treated or heat-treated (100° for 1 hr) bacterial suspensions (extinction 1.4, 1 cm. cell filter 58).

Animal sera. Sera were obtained from: cows A, B, C and D (fistulated experimental cows); cow E from a different part of the country; cows F, G and H from the Institute herd; two goats from the Institute herd, housed and kept quite separately from the cows; one rabbit, one guinea pig, two rats, one pig (all Institute experimental animals); one sheep and one horse from a different part of the country. Sera were preserved with 0.01 % w/v thiomersalate and stored at -20°.

Preparation of antisera in rabbits. Rabbits were inoculated intravenously every 3 to 4 days with an 18 hr culture of washed formaldehyde-treated organisms for a course of eight injections.

Transfer of naturally occurring antibodies from cow to calf. Samples of sera and of the secretion of the non-lactating udders were taken from three cows three weeks before parturition. In addition samples of the first colostrum, of the milk 3 weeks after calving and of the cows' sera 6 weeks after calving were examined. Serum was taken from each calf before the first feed with colostrum and at monthly intervals for 9 months.

Gel filtration. Cow serum and colostrum were filtered on Sephadex G-200, equilibrated with 0.1 M-KCl + 0.01 M-tris buffer + HCl, (pH 7.5).

RESULTS

Agglutination titres of anaerobic rumen bacteria in serum of cow A

Table 1 shows the agglutination reactions of 70 rumen strains isolated from cow A, when titres as high as 320 were sometimes obtained. The close correlation between motility of an organism and its ability to react with the antibodies in the serum can be seen. Agglutinins to flagellar antigens were detected in 37 of the strains and agglutinins to somatic antigens in 17 out of 38 strains examined. With the non-motile strains practically no agglutination reactions were observed. Three strains of non-motile lactobacilli were not agglutinated whereas a relatively high titre was present against the motile strain.

Agglutination titres of anaerobic rumen organisms with sera of different animals

Some of the rumen isolates from cow A were then tested against the sera of five other cows, two goats, rabbit, two rats, pig, horse and sheep (Table 2). The sheep, one cow and the horse were from different environments, the other sera came from animals housed at the Institute. The same organisms were agglutinated in a similar pattern and to similar titres against both flagellar and somatic antigens with the sera of the cows, the goats, the sheep and the horse. The sera of the other animals however showed only an occasional weak reaction with rumen organisms.

Table 1. *Agglutination reactions of rumen bacteria isolated from cow A with serum from cow A*

Genus or species	Formol-treated organisms			Heat-treated organisms		
	No. of strains examined	No. of strains in which motility observed	Titres	No. of strains	No. of strains in which motility observed	Titres
<i>Butyrivibrio</i>	15	14	40-320	7	7	40-160
<i>Selenomonas</i>	12	12	40-160	6	6	20-80
<i>Bacteroides</i>	12	0	< 20	7	0	< 20
<i>Streptococcus bovis</i>	10	0	< 20	8	0	< 20
<i>Streptococcus bovis</i>	2	0	20-40	.	.	.
<i>Lactobacillus</i>	3	0	< 20	3	0	< 20
<i>Lactobacillus</i>	1	1	160	1	1	80
<i>Ruminococcus</i>	2	0	< 20	1	0	< 20
<i>Spirillum</i>	1	1	20	.	.	.
<i>Succinivibrio</i>	1	1	80	.	.	.
<i>Lachnospira</i>	1	1	80	1	1	40
<i>Unclassified</i>	8	7	20-160	2	2	20-40
<i>Unclassified</i>	2	0	< 20	2	0	< 20
	70			38		

. = not tested.

Agglutination titres of anaerobic rumen organisms with human sera

Eleven of 12 human sera tested had no agglutinins for selected rumen organisms: the exception agglutinated to a titre $> \frac{1}{20}$ the somatic antigens only, of 2 *Butyrivibrio* strains, 2 *Lachnospira* and one *Lactobacillus* strain.

Agglutination reactions of anaerobic rumen bacteria isolated from a different cow

Organisms isolated from cow D in the same way as from cow A were characterized and motility noted. Of 14 strains examined 12 gave characteristic patterns of agglutination similar to those of isolates from cow A, when tested with sera from three cows, one goat, horse and rabbit. Two motile strains behaved somewhat differently in that they gave no reaction with the goat and cow A sera although they gave a strong reaction with the sera of cows D and F and with horse serum.

Agglutination reactions of Escherichia coli

Sixteen strains isolated from the rumen of cows B and C showed agglutination patterns against cow A serum similar to those of the motile anaerobic rumen bacteria

Table 2. Agglutination reactions of 14 strains of rumen bacteria isolated from cow A, tested against sera from different animals

Strain	Treatment of organisms	Five cows A, D, E*, F, G	Two goats 1, 2	Horse*	Titre of serum			Pig
					Sheep*	Two rats, one rabbit	Guinea pig	
†Butyrivibrio 1T18	F	160-640	160-320	320	160	—	40	—
	T	40-640	80-160	160	160	—	20	—
†Butyrivibrio 1T44	F	80-640	320	160	160	.	.	.
	T	40-160	80	80	80	.	.	.
†Butyrivibrio 2T70	F	80-640	160-320	320	80	.	.	.
	T	40-320	80	160	80	.	.	.
†Selenomonas 1T60	F	80-320	80-160	160	40	—	—	—
	T	40-320	20-40	160	20	—	—	—
†Selenomonas 1T68	F	40-320	20	80	20	—	—	—
	T	40-320	20	160	20	—	—	—
†Lachnospira 1T24	F	80-320	160	160	80	—	—	—
	T	40-160	80	160	40	—	—	—
†Lactobacillus 2T59	F	40-320	80	80	80	—	40	80
	T	40-640	40	320	80	—	20	20
Bacteroides 1T11	F	—	—	—	—	—	—	—
Bacteroides 1T14	F	—	—	—	—	—	—	—
Bacteroides 1T31	F	—	—	—	—	—	—	—
<i>Streptococcus bovis</i> A2/3	F	—	—	—	—	—	—	—
<i>Streptococcus bovis</i> B3/9	F	—	—	—	—	—	—	—
<i>L. acidophilus</i> 7T25	F	—	—	—	—	—	—	—
<i>Bifidobacterium bifidus</i> 7T4	F	—	—	—	—	—	—	—

* , Animal from a completely different environment; †, motility observed; F, formol treated; T, heat treated; —, titre of less than 20; ., not tested.

Table 3. *Agglutination titre, against rumen bacteria, found in blood serum, dry period secretion, colostrum and milk of a cow and in the blood serum of its calf*

Organism	Titres in cow H of:						Titres in calf H serum at				
	Serum before calving	Dry period secretion	Colostrum	Milk 3 weeks after calving	Serum 6 weeks after calving	Before colostrum	Five days after colostrum	One month	Four months	Six months	Nine months
Butyrivibrio IT18	F 320 T 80	5120 2560	320 80	80 40	320 160	— —	40 80	— —	20 —	— —	320 40
Butyrivibrio IT35	F 40 T 40	640 320	80 40	— —	20 40	— —	40 80	— —	— —	— —	40 20
Selenomonas IT60	F 160 T 40	1280 1280	80 80	20 20	80 80	— —	80 —	— —	40 —	20 —	160 80
Selenomonas IT68	F 40 T 40	640 320	80 40	— —	20 20	— —	40 20	— —	— —	40 20	40 20
Lachnospira IT24	F 160 T 160 T 160	320 640 640	160 80 80	— — —	320 160 160	— — —	160 — —	— — —	— 40 40	80 40 40	40 80 80
Bacteroides IT11	F —	—	—	—	—	—	—	—	—	—	—
Lactobacillus 2T59	F 160 T 160	640 320	80 40	— —	160 40	— —	80 —	— 20	40 —	160 20	640 40
Lactobacillus 7T25	F — F —	— —	— —	— —	— —	— —	— —	— —	— —	— —	— —
<i>S. bovis</i> A2/3	F . F . F .			— — —	— — —				— — —	— — —	— — —
<i>B. bifidus</i> 2T4	F . T .	. . .	. . .	— —	160 160	. . .	. . .	. . .	— —	— —	— 20
<i>E. coli</i> BC3	T .	. .	. .	. .	160	. .	. .	. .	—	—	—

F, Formol-treated cells; T, heat-treated cells; —, titre of less than 20; ., not done.

except that no agglutination occurred with the sheep serum but it did occur with the pig serum. Four human strains were not agglutinated by the sera, and two were only weakly agglutinated. Ten non-motile *Klebsiella aerogenes* strains were not agglutinated.

Serological relationships between strains of rumen bacteria

Absorption tests showed that the serum of cow A had natural agglutinins specific for many of the organisms, despite some sharing of antigens. Thus there were four different serotypes in five *Butyrivibrio* strains, with variations in both somatic and flagellar antigens; 3 *Selenomonas* strains had different antigens, but 2 *Lachnospira* strains appeared identical. Injection of the organisms into rabbits induced specific somatic and flagella agglutinins.

Retesting serum from cow A

Eleven months later another sample of serum was taken from cow A. During this time the cow had been on several different diets and at the time of sampling was out at pasture. The pattern of agglutination against rumen organisms was similar to that found with the previous sample of serum.

Transfer of naturally occurring antibodies from the cow to the calf

Table 3 shows the agglutination titres found in one of the three experiments with a cow and its calf. The level of antibodies in the secretion of the non-lactating udder was approximately 8-fold that of the blood serum, whereas in the colostrum the levels were the same as the serum. In the milk 3 weeks after parturition only a very low level of antibodies could be detected. In serum, 6 weeks after calving the level was similar to that found three weeks before calving. The concentration of these antibodies in the serum of a pregnant cow was therefore the same as in the lactating cow.

No antibodies were detected in the calf (J) serum before colostrum was given, whilst after feeding colostrum for 5 days agglutination titres were detected. However at 1 month, on a diet of milk and roughage, these passively acquired antibodies had disappeared and even at 3 months the calf had manufactured only traces of its own antibodies against these strains.

In the two other experiments (Cows F and G) the titres of antibodies in the serum after parturition were similar to the levels in the colostrum. In the milk, levels were again very low. With the two calves K and L the results were similar to calf J except that in one of them (K) the level of absorbed antibodies was much lower than in the other calves although the titres in the mother's serum were as high as in the other two cows. With calf L, which was on a milk diet for 2 months, results were obtained similar to those from the other two on milk and roughage diets.

At 4 months traces of antibodies which agglutinated some of the rumen strains were observed in all three calves. At 6 months the titres had risen, particularly in calf K, where titres against all except one of the normally positive rumen strains varied from 80 to 320. Eventually, after 9 months, agglutinin patterns similar to those in adult ruminants became established.

To investigate whether the calves developed any antibodies against their own rumen organisms, samples of rumen contents were taken by stomach tube from calves K

Table 4. *Agglutinating titres of sera of two calves K and L aged 5 months, against organisms isolated from their own rumens*

Agglutination reactions of strains isolated from calf K with calf serum K					Agglutination reactions of strains isolated from calf L with calf serum L				
Strain	Motility	Presumptive identifications	Titre of serum		Strain	Motility	Presumptive identifications	Titre of serum	
			Formol-treated	Heat-treated				Formol-treated	Heat-treated
A18	+	Selenomonas	40	80	P2	+	Selenomonas	80	<20
A27	+	Selenomonas	80	40	P19	+	?	40	40
A36	+	?	20	<20	P7	+	?	<20	<20
A30	+	Butyrivibrio	<20	<20	P26	+	?	<20	<20
A35	+	Butyrivibrio	<20	<20	P30	+	?	<20	<20
A40	+	Butyrivibrio	<20	<20	P36	+	?	20	<20
A2	—	<i>B. bifidus</i>	80	40	P8	—	<i>B. bifidus</i>	320	20
A12	—	<i>B. bifidus</i>	320	80	P28	—	?	640	160
A45	—	<i>B. bifidus</i>	320	<20	P34	—	<i>B. bifidus</i>	80	80
A1	—	?	<20	<20	7 strains	—	?	<20	<20
A5	—	Bacteroides	<20	<20					
A39	—	Bacteroides	<20	<20					
A46	—	Bacteroides	<20	<20					

<20, Titre less than 20; ?, strain not identified; F, formol-treated cells; T, heat-treated cells.

—, non-motile; +, motile

and L at 5 months of age, organisms were isolated by anaerobic techniques and tested against the calves' own sera. Identification of these organisms was by microscopic appearance and presence or absence of motility only. Table 4 shows that there was not the same correlation between motility and ability to react with antibodies as with the sera of adult ruminants. No agglutinins were observed for some of the motile strains, including some selenomonds, whilst relatively high titres occurred with some non-motile strains, in particular some *Bifidobacterium bifidus* strains. No antibodies to *Bacteroides* were observed in calf K, where these organisms were among the majority flora. It thus appears that motility is not a necessary factor to stimulate the formation of antibodies in the calf against its own rumen strains.

Fractionation of serum and colostrum from cow H

One of the physical criteria of natural antibodies is that they are predominantly present in the IgM fraction of immunoglobulins, apparently because of the very low level, but continuous antigenic stimulation (Michael & Rosen, 1963). Cow serum was chromatographed on Sephadex 200 and the fractions of the first two peaks tested against a *Butyrivibrio* strain and a *Selenomonas* strain. All the agglutinating activity for both flagellar and somatic agglutinins appeared in the fraction which was completely excluded from the gel, being contained in the front peak. No agglutinins were found in the later fractions, indicating that the antibodies were of the IgM type, and confirming the findings of previous workers (Landy & Weidanz, 1964). Similar results were obtained with colostrum, which also contained all the agglutinins in the high molecular fractions of the first peak.

DISCUSSION

The agglutinin titres obtained in bovine serum against rumen bacteria substantiated our suggestion that the high concentration of these organisms might evoke a strong antigenic stimulus. A sensitive bactericidal test (Landy & Weidanz, 1964) has been used to detect the presence of natural antibodies against Gram-negative bacteria, but the relatively high titres obtained with agglutination tests against the rumen bacteria made such tests unnecessary. The high specificity of the natural agglutinating antibodies in ruminants was shown by absorption tests where, with only 10 strains of three different genera, the presence of eight different serotypes was demonstrated. Specificity is further illustrated by the absence of agglutinins against a human *Escherichia coli* strain although they were detected against a rumen *E. coli* strain. The presence of antibodies against the indigenous rumen flora differentiate the ruminants and the horse from other mammals such as the rat, rabbit, guinea pig, pig and man. Horse serum may contain the same antibodies as the ruminant because microbial digestion of plant residues takes place in the caecum which probably contains similar organisms to those in the rumen (Davies, 1964).

The site of antigenic stimulation against these rumen organisms is unknown. Rumen organisms are transferred almost continuously from the rumen to the omasum and abomasum (Smiles & Dobson, 1956) so that only small numbers of whole organisms pass to the small intestine. The low pH of about 2.0 in the abomasum would probably destroy most of the survivors, except lactobacilli and coliforms (Smith, 1965). No information is available on the obligate anaerobic flora of the adult bovine intestine.

The presence of antibodies in the sera against rumen strains of *Escherichia coli*, which were only present in very small numbers in the rumen, suggests that they colonize the intestine where antigenic stimulation could occur. Microbial cells may also be translocated across the gut wall and eventually enter the lymph nodes (Wolochow, Hildebrand & Lamanna, 1966; Grys, 1966). Such translocation might also occur in the rumen wall.

The reason for the selective stimulation in the adult ruminant of antibodies against motile strains only of rumen organisms is not yet clear. Lovell (1932) detected flagellar agglutinins against *Salmonella* in 67% of animals examined (horses, cattle, sheep, pigs) so that flagellar antibodies may be widespread. Flagellins are not destroyed by pepsin and trypsin but are rendered soluble at pH 2.0 (Kobayashi, Rinker & Koffler, 1959; Stocker & Campbell, 1959). Solubilized flagellins are antigenic (Fitch & Winebright, 1962), and could therefore be a source of antigenic stimulation in the intestine. *Butyrivibrio fibrisolvens* (Bryant & Small, 1956*a*) is monotrichous; *Selenomonas ruminantium* (Bryant, 1956) has a tuft of lateral flagella; *Lachnospira multiparvus* (Bryant & Small, 1956*b*) has one lateral flagellum. The single flagellum or laterally placed flagella are unlikely to be fixed over the whole cell surface in formalized cultures, and some of the somatic antigens might also be taking part in agglutination.

In the calf strong agglutinins to the non-motile Gram-positive *Bifidobacterium bifidus* were present. These bifidus organisms are present in much larger numbers in the calf; they were isolated as part of the majority flora in the rumen of all three calves examined aged 3, 4 and 5 months respectively. The calf has therefore been subjected to much greater exposure to bifidus than has the adult animal.

As natural antibodies are reported to be of the IgM type, it was to be expected that the agglutinins specific for the rumen organisms would be IgM. The lactating bovine mammary gland is not very permeable to immune globulins, although low agglutinin titres against streptococci, staphylococci and coliforms have been detected in milk (Reiter & Oram, 1967). During the dry period the gland becomes selective and concentrates the immunoelectrophoretically fast globulins of IgG (Murphy, Aalund, Osebold & Carroll, 1964; Pierce & Feinstein, 1965). However IgM has been detected by immunoelectrophoresis in colostrum and in calf serum after feeding colostrum (Popovici & Jurenkova, 1967; Baglioni & Fioretti, 1967). Dr W. J. Penhale ((Royal Dick) School of Veterinary Studies, Edinburgh, personal communication and 1965) has made quantitative studies of the concentration of IgM in colostrum and its absorption into the calf serum using purified IgM, IgG; he also found that IgM was absorbed through the calf intestines.

The specific agglutinins against rumen organisms were found to be sensitive markers of the transfer of IgM to the secretion and colostrum and then to the calf serum. Our results show that IgM antibodies against rumen organisms were present in the same concentration in the colostrum as in the blood serum, indicating that there was no selective concentration of this type of immunoglobulin in the udder. The increased titres in the non-lactating secretion compared with those in blood serum are due to physical concentration because of the re-absorption which takes place during drying off. Our results also confirm that in the calf IgM is freely taken up through the gut.

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