Enumeration of Cellulolytic Cocci in Sheep Rumen by Using a Fluorescent Antibody Technique

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SUMMARY

Strains of *Ruminococcus albus* were isolated from sheep fed lucerne chaff once daily. Antisera to these bacteria were prepared in rabbits and labelled with fluorescein isothiocyanate. A specific labelling reagent was produced from these conjugates by chromatography on Sephadex G-25 and two absorptions with mouse liver powder. The Breed smear technique was adapted for counting fluorescent cocci. Background fluorescence in rumen fluid samples was controlled by making suitable dilutions. A sample taken 10 hr after feeding had a mean value of 5.76 (S.E. 1.16) $\times 10^6$ cocci/ml. Samples taken from different parts of the rumen at the same time showed random but significant variation in the number of fluorescent cocci/ml. The natural fluorescence of some particles of digesta was bright enough to obscure the specific fluorescence and attempts were made to release fluorescent cocci by homogenization; this was partially successful, and the average ratio of untreated to homogenized counts was 1:1.17. Diurnal variations in the number of cellulolytic cocci were followed over a 4-day period. Minimum numbers were recorded 6 hr after feeding and maximum numbers 18 hr later. There was a measurable day-to-day variation.

INTRODUCTION

Several cultural methods have been described for the enumeration of cellulolytic cocci in the rumen (Hungate, 1950; Kistner, 1960). These methods usually depend on the use of ball-milled cellulose as the sole substrate in a habitat-simulating medium. After incubation cellulolytic colonies are detected by cleared zones in the opaque medium. Cellulolytic bacteria have also been recovered from high dilutions of rumen liquor by picking colonies from a non-selective medium and observing whether they hydrolyse cellulose in liquid culture (Bryant & Burkey, 1953).

Serological studies of ruminococci (Jarvis, 1967) demonstrated immunological relationships among strains of both *Ruminococcus albus* and *R. flavifaciens* and serologically related strains persisted in the rumen. It was concluded that the fluorescent antibody technique could be adapted to permit direct microscopic examination of rumen contents for cellulolytic cocci. This paper describes the development and use of a simple counting method for *R. albus*, based on the direct fluorescent antibody technique.

METHODS

Sheep. Two fistulated merino sheep, C6 and C18, were housed indoors in separate pens on a slatted floor and fed rations which would maintain them at roughly constant body weight. Each sheep received 800 g. chaffed lucerne hay at 9.00 a.m. daily and had free access to water. Both sheep had been established on their diets more than 6 months before bacterial isolations were made.

Bacterial strains. *Ruminococcus albus* strains LY3 and LY4 were isolated from sheep C6 using a modification of the anaerobic culture technique described by Hungate (1950). The characteristics of these strains were described by Jarvis & Annison (1967). *Escherichia coli* and *Staphylococcus aureus* were obtained from stock cultures in a collection maintained at the School of Rural Science.

Production of antiserum. Antisera to LY3 and LY4 were produced in rabbits using the methods described by Jarvis (1967).

Preparation of fluorescein-labelled antiserum

Labelled antiserum was prepared by the method of Marshall, Eveland & Smith (1958) as described by Hobson, Mann & Smith (1962) with the exception that the proportion of fluorescein isothiocyanate (Baltimore Biological Laboratory, Division of B-D Laboratories, Inc., Baltimore, Maryland, 21204, U.S.A.) to protein used was 0.05 mg./mg. protein.

Estimation of total serum protein. Serum protein was determined spectrophotometrically from the extinction of a 1/100 dilution of serum in a 1 cm. cuvette. Readings were taken at 260 m μ and at 280 m μ and applied to a nomograph based on data given by Warburg & Christian (1941) (prepared by California Corporation for Biochemical Research, Los Angeles 63).

Conjugation of antiserum with fluorescein isothiocyanate. A magnetic stirrer was used to mix 2.5 ml. whole serum, 4.2 ml. 0.15 M-NaCl and 1.7 ml. 0.5 M-carbonate + bicarbonate buffer (pH 9.0) at 2° in a cold room. Cold acetone (1 ml.) containing fluorescein isothiocyanate (FITC) was added dropwise to the stirred solution from a syringe fitted with a 26-gauge needle. Stirring was continued for 18 hr at 2°.

Separation of fluorescein-conjugated protein from unreacted fluorescein. When dialysis was used for this purpose (Hobson *et al.* 1962) the solution of serum proteins in buffered saline (conjugated antiserum) was dialysed against litre volumes of 0.01 M-phosphate buffered saline (pH 7.0) at 2°. The dialysing solution was stirred continually and changed twice daily. After 3 days an ultraviolet lamp was required to detect dialysed fluorescein but a slight amount was present in the saline when dialysis was stopped after 9 days. Chromatography on Sephadex G-25 (Gordon, Edwards & Tompkins, 1962; Peters, 1963) was found to be a rapid and efficient means of separating conjugated antiserum and unreacted FITC.

Estimation of labelling efficiency. After the unconjugated fluorescein had been separated on a Sephadex column the relative fluorescein:protein ratios of different conjugates were compared by reading the extinction at 280 m μ and 495 m μ (Tokumaru, 1962). It was confirmed that FITC was degraded during prolonged storage at 2° (McKinney, Spillane & Pearce, 1964). Use of degraded FITC resulted in the production of conjugate with a low fluorescein:protein ratio. Fluorescence with such conjugates was dull although the titre was high.

Absorption of conjugated antiserum. Dialysed solutions were cleared by centrifugation at 22,000 g for 35 min. at 2° and then absorbed. Sephadex-treated conjugates were not cleared before adsorption. Acetone-dried mouse liver powder (100 mg./ml. of conjugate) was added to conjugated antiserum and the mixture was shaken for 1 hr at room temperature, then centrifuged at 22,000 g for 20 min. at 0°. The supernatant absorbed conjugated antiserum was recovered, preserved with Thiomersal (British Drug Houses Ltd., England) and deep frozen until required.

Antibody titre of labelled antiserum. The effect of fluorescein labelling treatments on the antibody activity of the antiserum was followed by a tube agglutination test against homologous antigen (Jarvis, 1967).

Preparation of slides from pure cultures

A thin smear of bacteria was spread on an ultraviolet transmitting glass slide (Shandon Scientific Co., Cromwell Place, London) and allowed to dry on a warm surface. The smear was fixed by immersing the slide in acetone for 20 min. The acetone was allowed to evaporate from the slide and it was then immersed in 0.1 M-phosphate buffered saline (pH 7.0) for 1 min. Excess saline was drained from the slide and two drops of absorbed conjugated antiserum were spread over the moist smear with a glass rod. The slide was incubated 30 min. at 37° in a moist chamber. After incubation the slide was rinsed with a phosphate buffered saline and washed by immersion in three lots of buffered saline for 3–5 min. each. Excess saline was drained off and a drop of redistilled glycerol containing 10 % (v/v) buffered saline added to the smear. A cover-slip was applied (Chance No. 1) and the slide blotted to remove excess buffered glycerol. These smears were examined using the methods and apparatus described earlier (Jarvis, 1967).

Counting ruminococci in samples of rumen liquor

A wide-bore glass tube was used to collect samples of rumen content from fistulated sheep. Each sample was transferred to a 25 × 150 mm. test-tube, stoppered and allowed to stand for 20 min. Counts were made on liquid drawn from a point 4 mm. below the surface and diluted 1:1 in 0.01 M-phosphate-buffered saline. After thorough mixing 0.01 ml. of the dilution was pipetted on to a slide and immediately spread over an area of 1 cm.² with the end of a thin glass rod. Smears were dried on a warm horizontal surface (5–10 min.) and then fixed in methanol (10 min.). The methanol was allowed to evaporate, and smears were moistened in phosphate-buffered saline (1–2 min.), drained and covered with conjugated specific antiserum (1–2 drops). After incubation at 37° for 30 min. in a moist chamber the slides were removed, rinsed, washed and mounted as described above.

A × 100 objective and × 10 eyepiece were used for counting. The field diameter was measured with a slide micrometer and a microscope factor was computed (American Public Health Assoc., 1948) allowing for the appropriate dilution 1/1, 1/5 or 1/10. With a dilution of 1/1 the average count per field × 10⁶ represented the count/ml. of rumen fluid. Fields were counted at approximately equal distances along two diameters of the smear at right angles to one another. Individual cocci were counted in the occasional small colonies encountered. Diplococci were considered separate cocci when any clear division between daughters could be distinguished.

RESULTS AND DISCUSSION

Development of a fluorescent antiserum specific for ruminococci

Cherry, Goldman & Carski (1960) discussed the steps necessary to demonstrate the serological specificity of observed immunofluorescence; and the following list is taken from their work. (1) Uninfected tissue or uninoculated culture media should not stain. (2) Conjugates of normal serum should not stain the antigen. (3) Staining should be inhibited by pretreatment of the smear with unlabelled specific antibody or by dilution of the conjugate in unlabelled specific antibody. (4) Staining should be inhibited by adsorption of the antiserum with homologous antigen prior to staining. These criteria were applied to show the specificity of antisera to strains of *Ruminococcus albus* for their homologous antigen.

Unstained antigen suspensions were found to have absolutely no natural fluorescence. Unstrained smears of rumen content contained particles which fluoresced red or yellow (Hobson & Mann, 1957) and also green-fluorescing particles, some of which were small enough to be confusing. These particles could be distinguished from bacteria by their lack of regular shape.

Table 1. *Staining of Escherichia coli and Ruminococcus albus LY3 by conjugated normal serum fractions*

Normal serum	<i>E. coli</i>	<i>R. albus</i> LY3
Liver-absorbed protein fraction	Positive but dull	Negative. No visible cocci by blue-light fluorescence
Liver-absorbed fractions immediately behind the protein peak	Negative. No visible rods	Very faint labelling

Conjugates of normal serum were prepared by the method of Hobson *et al.* (1962), dialysed for 7 days and absorbed with mouse liver powder. When smears of *Escherichia coli* and *Ruminococcus albus* LY3 were treated with this material *R. albus* LY3 fluoresced but *E. coli* did not. The labelled normal serum was passed through a Sephadex G-25 column and this revealed considerable fluorescence which did not move with the protein peak. The extinction of successive fractions was measured at 280 m μ and 495 m μ and the values obtained were corrected for absorption at 280 m μ due to FITC (Tokomaru, 1962). Most of the FITC travelled with the protein peak but the extinction at 280 m μ decreased much more rapidly than that at 495 m μ , indicating a tail of unconjugated FITC. Table 1 records the results obtained when the liver absorbed protein fraction from the Sephadex column was used to label *E. coli* and *R. albus* LY3. It was concluded that dialysis would not remove traces of FITC which were sufficient to cause the observed non-specific staining. When unconjugated FITC was removed labelled normal serum could not cause appreciable fluorescence.

The blocking effect of unlabelled specific antiserum was shown by comparing two treatments: (a) 2 vol. homologous unlabelled antiserum to 1 vol. homologous labelled antiserum; (b) 2 vol. normal serum to 1 vol. homologous labelled antiserum. There was a considerable decrease in fluorescence in (a) as compared with (b) because of the blocking effect of unlabelled homologous antibodies.

Smears of *Ruminococcus albus* LY3 were treated with conjugated LY3 antiserum

which had been absorbed with LY3 organisms. Examination by blue-light fluorescence showed a slight glow visible only where the cocci were closely packed. On the other hand, three successive absorptions of conjugated LY3 antiserum with a suspension of *Escherichia coli* did not decrease the fluorescence imparted to LY3 organisms appreciably. It was concluded that the fluorescence observed was due to a specific serological reaction.

Gordon *et al.* (1962) showed that conjugates specific for bacterial antigens could be obtained by Sephadex treatment of labelled γ -globulin solutions. The column separated unconjugated dye and low molecular weight fractions from labelled protein. A conjugate of whole serum (titre 1/20,000) prepared in this way stained non-specifically. Smears of *Escherichia coli* stained as brightly as the homologous antigen *Ruminococcus albus* LY3. *Staphylococcus aureus* did not fluoresce with this conjugate. The antibody titre of the protein fraction was 1/5000. Visible fluorescence in solution did not extend beyond a dilution of 1/640, suggesting that the non-specific material was present in considerably lower concentration than the specific labelled antibody. Subsequently it was shown that complete removal of unconjugated FITC by a second Sephadex treatment did not give a specific antiserum. Two successive absorptions with liver powder were required to produce a conjugate specific for *R. albus* LY3. After this treatment the agglutination titre of labelled antiserum was 1/1300 and fluorescence in the supernatant fluid of the agglutination tubes was absent above a dilution of 1/80. Table 2 records the results obtained when the twice absorbed conjugate was used to stain smears of *E. coli* and *R. albus* LY3.

Although Sephadex G-25 is not intended to fractionate proteins, three fractions

Table 2. *Staining of Escherichia coli and Ruminococcus albus* LY3 by conjugated antiserum specific for *Ruminococcus albus* LY3

Diluent	<i>E. coli</i>	<i>R. albus</i> LY3
Phosphate buffered saline (pH 7.0)	Fluorescence just visible. Very hard to distinguish rods	Bright fluorescence of cocci against a black background
Rumen liquor	No identifiable fluorescent rods	Bright fluorescence of cocci against a faintly fluorescent background

Table 3. *Staining of Escherichia coli and Ruminococcus albus* LY3 in rumen fluid by conjugated protein fractions specific for *Ruminococcus albus* LY3

Fraction*	<i>E. coli</i>	<i>R. albus</i> LY3
1	No fluorescent rods	Bright fluorescence of cocci on a faintly staining background
2	<i>E. coli</i> visible but much less fluorescent than <i>R. albus</i> LY3 cocci	Brilliant fluorescence of cocci on a black background
3	No fluorescent rods but a few fluorescent cocci which would be present in rumen fluid were observed	Bright fluorescence of cocci on a faintly staining background

* These protein fractions are described in the text.

were recognized in the protein band: *fraction 1*, a small volume having the brightest fluorescence and travelling ahead of the main band, *fraction 2*, the main protein band, and *fraction 3*, a small volume behind the main peak and seemingly more dilute. An attempt was made to determine whether these three fractions could be bulked for use as specific labelled antiserum. Each fraction was used to stain homologous and heterologous cells in rumen liquor. The results are recorded in Table 3. It was concluded that the non-specific reaction was associated with protein travelling in the middle of the band and that this could be decreased to a satisfactory degree by two absorptions with liver powder without impairing the specificity of the antiserum.

Counting ruminococci in rumen fluid

Precision of the counting method. A sheep was sampled 10 hr after being fed 800 g. lucerne chaff. Eight parallel dilutions of the sample were each smeared once and 30 fields were counted on each smear. The resulting estimates of numbers of ruminococci were: 6.76×10^6 , 4.90×10^6 , 4.17×10^6 , 5.30×10^6 , 7.8×10^6 , 7.37×10^6 , 5.34×10^6 , 4.43×10^6 , mean 5.76×10^6 /ml., standard deviation 1.38×10^6 (corrected for the small number of observations); standard error of the mean (95 % confidence interval), $\pm 1.16 \times 10^6$ /ml.

Wilson (1935) examined the errors inherent in the Breed smear technique and concluded that it was unsuitable for low count samples since an average of at least one organism per field was required for reasonable accuracy. The main source of random error in the method was considered to be the irregular distribution of the bacteria in the smear. Subsequently Hanks & James (1940) recommended the use of circular smears, and the selection of fields at regular intervals along two diameters at right angles to each other in order to minimize these errors. In the method described here an additional source of error was the masking effect of fluorescent background material. Sources of variation in the background fluorescence included: extent of sedimentation before dilutions were prepared, thickness of the smear, evenness of drying, extent of digestion within the rumen before sampling. Samples taken after extensive digestion showed more intense background fluorescence and higher counts than those taken soon after feeding. Consequently the background fluorescence could be controlled by suitable dilution of the sample without introducing excessive statistical errors. The total number of fluorescent cocci/ml. rumen content varied between 2×10^6 and 12×10^6 . A counting method with a precision of approximately $\pm 1.0 \times 10^6$ was considered adequate to resolve the differences encountered.

Variation between sampling sites in the rumen. At intervals of several days samples were obtained from three sites in the rumen 11 hr after feeding. The regions sampled were: anterior ventral, anterior dorsal, posterior ventral. Duplicate dilutions were made from each sample and each was smeared once: 30 fields were counted on each smear. The average for each sample is recorded in Table 4. It was concluded that sometimes there were significant differences in the concentration of ruminococci at different points in the rumen. Samples intended to represent the average population should be drawn from various parts of the organ.

Effect of homogenizing rumen contents. Davies (1965) used an indirect fluorescent antibody technique to study the distribution of cellulolytic bacteria isolated from the large intestine of a horse. She concluded that 'free' and 'fixed' bacteria could be enumerated. But the present study indicated that the natural fluorescence of many

particles of ingesta was too bright to permit fluorescent bacteria on them to be distinguished. Homogenization was intended to release 'fixed' bacteria. The whole rumen content was macerated in a tissue grinder at 0° for 2 min. This caused partial destruction of food particles but viable protozoa still remained. Counts were made on homogenized whole rumen content and compared with those on untreated rumen liquor (prepared as described in Methods). Homogenized rumen content was diluted 1/10 to decrease the background fluorescence to an acceptable degree. These results are recorded in Table 5 where each figure represents the mean of duplicate dilutions smeared once. The increase in count due to homogenization is not as much as expected (Bryant, 1959; Warner, 1962*a*). This may have been due to the particular antiserum used or to the technique used, but it is interesting to note that Hungate (1947) found no increase in cellulolytic colony counts after similar treatment. Qualitative examination of rumen contents indicates that the fluorescent organisms do not occur as large colonies and are often associated with highly degraded material, consequently blending might not release many.

Table 4. *Concentration of ruminococci at selected sites in the rumen of sheep fed chaffed lucerne hay once daily and sampled 11 hr after feeding, determined by a direct fluorescent antibody technique*

Series*	Anterior ventral (10 ⁻⁶ /ml.)	Anterior dorsal (10 ⁻⁶ /ml.)	Posterior ventral (10 ⁻⁶ /ml.)
1	6.68	7.00	7.08
2	4.68	8.18	8.13
3	2.75	1.79	4.81
4	2.42	1.40	3.33
5	3.78	3.93	12.26
Average	4.06	4.46	7.10

* Successive sets of samples were collected at intervals of several days.

Table 5. *Effect of maceration on the number of fluorescent ruminococci observed by a direct fluorescent antibody technique in the rumen contents of sheep fed chaffed hay once daily*

Sheep sampled*	No. of cocci (× 10 ⁻⁶ ml.)		Ratio, homogenized: control
	Untreated Control	Homogenized	
C ₆ 12 hr after feeding	7.18	6.35	0.89
C ₁₈ 4 hr after feeding	5.43	6.78	1.25
C ₁₈ 4 hr after feeding	7.38	8.69	1.19
C ₆ 12 hr after feeding	12.05	16.37	1.36
Average	8.01	9.55	1.19

* Successive samples were collected at intervals of several days.

Diurnal variation in the concentration of fluorescent ruminococci. Results obtained with one sheep are presented here as an indication of the fluctuation in numbers to be expected (Fig. 1). Sheep C18 was fed 800 g. lucerne chaff each morning. Food was withdrawn after 2 hr. Samples of ingesta were taken before feeding and also at 3.00 p.m., 9.00 p.m. and 3.00 a.m. The samples were preserved with 1/10,000 Thiomer-sal and held at 2°. Each count represents the mean of counts made on duplicate

dilutions smeared once. Thirty fields were usually counted on each smear, but 60 or 90 fields were counted on smears from 3.00 p.m. samples because of the small number of fluorescent cocci.

The numbers of fluorescent ruminococci fluctuated in accordance with a recognizable pattern. The full extent of these fluctuations is not known, but the lowest values were always observed at 3.00 p.m. and the highest at 3.00 a.m. There was also a noticeable day-to-day variation in the concentration of cocci at a given sampling time. A decrease in total bacterial numbers after feeding has been observed by Christian & Williams (1957), Bryant & Robinson (1961) and Warner (1962*b*). The subsequent increase in

Fig. 1. Diurnal and daily changes in the concentration of fluorescent ruminococci obtained from a sheep fed chaffed lucerne hay once daily. The sheep was fed 800 g. lucerne chaff daily, between 8.00 a.m. and 12.00 noon, after it had been sampled.

numbers found here appeared to parallel the pattern of rumination (Pearce, 1965); this is in accordance with the observations of Pearce & Moir (1964) and Dehority & Johnson (1961) which indicated that trituration played an important part in the digestion of roughage.

It is probable that the use of labelled antiserum to one antigen would not reveal all the ruminococci although most strains of *Ruminococcus albus* would have been counted (Jarvis, 1967). Over a period of 39 days samples were taken on seven occasions among five sheep fed chaffed lucerne hay. A total of 14 strains were collected, only half of which were subsequently identified as *R. albus* (Jarvis & Annison, 1967). This suggests that a count restricted to *R. albus* would enumerate about half the ruminococci present.

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