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Serological investigation into the association between *Streptococcus bovis* and colonic cancer

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Abstract

***Aims*—To determine if there was an increase in antibody titre to *Streptococcus bovis* in patients with colonic cancer, and if this might be a useful marker of the presence for colonic cancer.**

***Methods*—Serum samples from 16 patients and 16 age matched controls were tested by immunoblot and enzyme linked immunosorbent assay (ELISA) against antigen preparations from two strains of *S bovis* and one strain of *Enterococcus faecalis*.**

***Results*—No distinction between cancer patients and controls could be made using immunoblots. ELISA did show an increase in antibodies to *S bovis*, but there was a greater increase in antibodies to *E faecalis*. The increase in antibody titres was greatest with antigens which had been treated with periodate, and was therefore thought not to be caused by antibody to the shared group D carbohydrate antigen.**

***Conclusion*—It may be possible to construct a test for the detection of colonic cancer based on the detection of antibody to *S bovis* or *E faecalis*, though considerable further development of this concept is required.**

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Streptococcus bovis bacteraemia (in many cases with endocarditis) is associated with colorectal tumours in 25% to 80% of cases.[1-6] *S bovis* bacteraemia and endocarditis have also been reported in association with other tumours of the gastrointestinal tract and with non-neoplastic colonic lesions, but the association with colorectal tumours is stronger, and the finding of any form of *S bovis* infection necessitates rigorous exclusion of colonic neoplasia.[1 2 4]

The mechanism underlying this association is not known, but may be due to a specific increase in the incidence of *S bovis* bacteraemia derived from the gut in patients with colonic cancer. Colonic cancer is associated with a roughly five-fold increase in the faecal carriage rate of *S bovis*,[7] which may in part explain the increased incidence of bacteraemia with this organism. In patients with coincidental cardiac valve lesions bacteraemia could lead to endocarditis, but in most patients the bacteraemia would be transient and clinically silent.

Bacteraemia with *S bovis* may therefore be more common than is realised in colorectal cancer, and if this is the case there might be an increase in antibodies to the organism. Detection of such an antibody response might be useful as a marker of colonic neoplasia.

We therefore set out to measure antibody to crude preparations of *S bovis* in serum samples from patients with colonic cancer and age-matched controls, using immunoblots and ELISA. To distinguish a specific antibody response from non-specific increase in antibody to colonic bacteria we also measured antibody to *Enterococcus faecalis* and to a cocktail of Gram negative lipopolysaccharide core antigens. *S bovis* and *E faecalis* share the group D carbohydrate antigen (which is sensitive to periodate[8]) and preparations treated with periodate were therefore used in ELISA to aid the detection of antibodies to species specific protein antigens.

Methods

For most experiments we used NCTC 8133 (*S bovis* biotype I); 10b167 (a local blood culture isolate of *S bovis* biotype II from a patient with endocarditis and colonic adenoma); and NCTC 10449 (*E faecalis*). Other strains were blood culture isolates. Identification and biotype of the *S bovis* isolates was confirmed by the Streptococcus Reference Unit, Central Public Health Laboratory, Colindale, London. Bacteria were grown overnight at 37°C in Todd Hewitt broth (THB; Oxoid) in a shaking incubator, harvested by centrifugation at 3000 × *g* for 10 minutes, washed, and resuspended in distilled water.

A positive control serum was obtained, during the course of treatment, from a patient with *S bovis* endocarditis. Serum samples from 16 patients admitted for primary surgery for colonic cancer were collected, and age matched control sera were selected from samples which had been submitted to this department to be tested for rheumatoid factor and anti-nuclear factor and found to be negative for both. All sera were heat inactivated at 56°C for 30 minutes and stored at −20°C.

Mutanolysin preparations[9] were made by mixing 100 μl bacterial suspension (in distilled water with an optical density (OD) of 10 at 660 nm) with 5 μl mutanolysin solution (Sigma, 1000 U/ml in 0·1M HEPES, pH 7·2), 5 μl sodium azide (0·4% weight/volume) and 5 μl phenylmethylsulphonyl fluoride (0·02M in ethanol). The mixture was

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incubated at 37°C for 18 hours and stored at −20°C. French press extracts were made from the growth obtained in 1 litre THB, washed, and resuspended in 30 ml phosphate buffered saline (PBS) (0·05M sodium phosphate, 0·15M sodium chloride, pH 7·4). The organisms were fragmented in an ice cooled cell at a pressure of 8000 to 9000 pounds a square inch, repeated until more than 80% breakage was achieved, as determined by phase-contrast microscopy. The supernatant fluid, after centrifugation at 10 000 × *g* for 10 minutes, was collected and the protein content measured.[10]

IMMUNOBLOTS

Antigen preparations were solubilised with an equal volume of sample buffer (4% weight/volume sodium dodecyl sulphate, 20% volume/volume glycerol, 2% volume/volume 2-mercaptoethanol, 0·002% weight/volume bromophenol blue, pH 6·8) at 100°C for 10 minutes. Proteins (50 μg protein or 20 μl mutanolysin preparation per 5 mm track) were separated on 10% polyacrylamide gels[11] and transferred to nitrocellulose membrane (0·2 μm pore size).[12] The nitrocellulose was then washed for 10 minutes in TRIS-buffered saline (TBS; 0·02M TRIS, 0·5M sodium chloride, pH 7·5), then in 3% (weight/volume) gelatin in TBS for 45 minutes, then incubated with serum diluted in 1% gelatin in TBS for 3 hours. The nitrocellulose was then rinsed in distilled water and washed twice in Tween 20 (0·025% (volume/volume) in TBS), incubated for one hour with horseradish peroxidase anti-human IgG (Sigma, diluted one in 500 in 1% gelatin in TBS), rinsed, and washed in Tween as before, and developed over 30 minutes with horseradish peroxidase colour reagent (Bio-Rad).

ELISA

NUNC polysorb flat-bottomed eight-well strips were coated with 100 μl a well of French press antigen preparation (40 μg protein/ml) in 0·05M sodium carbonate, 0·02% weight/volume sodium azide, pH 9·6), incubated at room temperature overnight. Wells were then washed four times with wash buffer (0·05% volume/volume Tween 20, 0·05% sodium azide in PBS) in a Dynatech plate washer, shaken dry, and stored at −20°C. To prepare periodate treated antigen, strips coated with French press extract were incubated with 100 μl a well of sodium periodate (0·01M in PBS) for two hours at room temperature, then washed four times.

To perform antibody assays, 100 μl of a one in 1000 dilution of serum in antibody diluent (4% weight/volume polyethylene glycol, 0·5% weight/volume bovine serum albumin in wash buffer) was added to duplicate wells and incubated for 90 minutes at 37°C. Plates were then washed four times and incubated for 90 minutes at 37°C with 100 μl a well of alkaline phosphatase conjugated anti-human IgG (ICN) or IgM (Miles-Yeda) diluted 1 in 1000 in antibody diluent. Plates were again washed, 100 μl a well of alkaline phosphatase substrate (0·1% weight/volume *p*-nitrophenyl phosphate (Sigma) in 0·05M sodium carbonate, 1 mM magnesium chloride, pH 9·8) added, incubated for 60 minutes at room temperature, and OD measured at 405 nm using an Anthos plate reader with substraction of blank readings from wells with no added serum. All assays of IgG or IgM antibody to each antigen preparation were performed as a batch.

Anti-LPS core ELISA assays were performed by Dr GR Barclay using plates coated with equal molar quantities of rough LPS from *E coli* K12, *Klebsiella pneumoniae* M10b, *Pseudomonas aeruginosa* PAC605 and *Salmonella typhimurium* 878, complexed with polymyxin.[13] Results were expressed as median units where 100 was the median value determined from assays on 1000 blood donor sera.

The approximate 95% confidence interval (CI) of median values was calculated, as described by Campbell and Gardner.[14] All p values are derived from Mann-Whitney two sample tests calculated using Epi Info (Georgia, USA; USD Incorporated).[15]

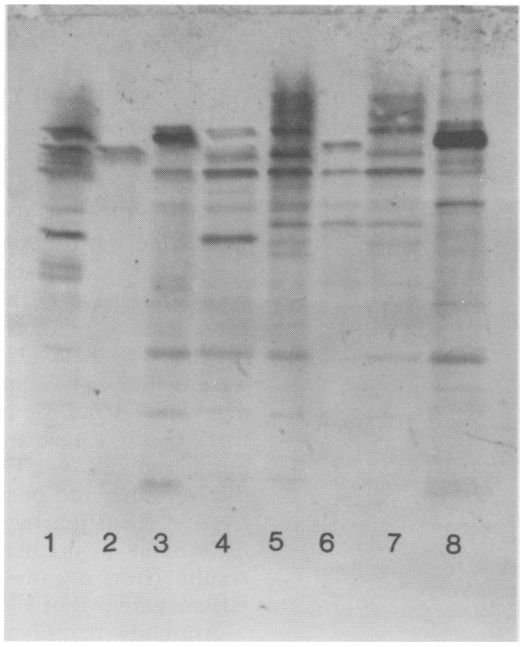

Figure 1 Immunoblot of antigen preparations from seven isolates of S bovis with serum from a patient being treated for S bovis endocarditis. All antigens are mutanolysin preparations except for lane 1, a French press preparation. Strains used are (1 and 2): NCTC 8133; (3): 11b275 (biotype 1); (4): 11b172 (biotype II); (5): 10b167 (biotype II); (6): 10b160 (biotype II); (7): 11b45 (biotype II); (8): 12b42 (biotype II, from the same patient as the serum).

Results

IMMUNOBLOTS

Serum from the patient with *S bovis* endocarditis reacted strongly in immunoblot with whole cell protein preparations from several *S bovis* isolates, although the pattern of bands observed varied considerably among isolates (fig 1). Reactivity of comparable strength was also observed with an isolate of *S mitior* (NCTC 10712) but not with individual blood culture isolates of *S morbillorum*, *S sanguis*, *S mutans*, or *E faecalis* (data not shown). For further experiments we chose to use the NCTC strains of *E faecalis* and *S bovis*, together with the local isolate of *S bovis* (10b 167) which had reacted most strongly (fig 1).

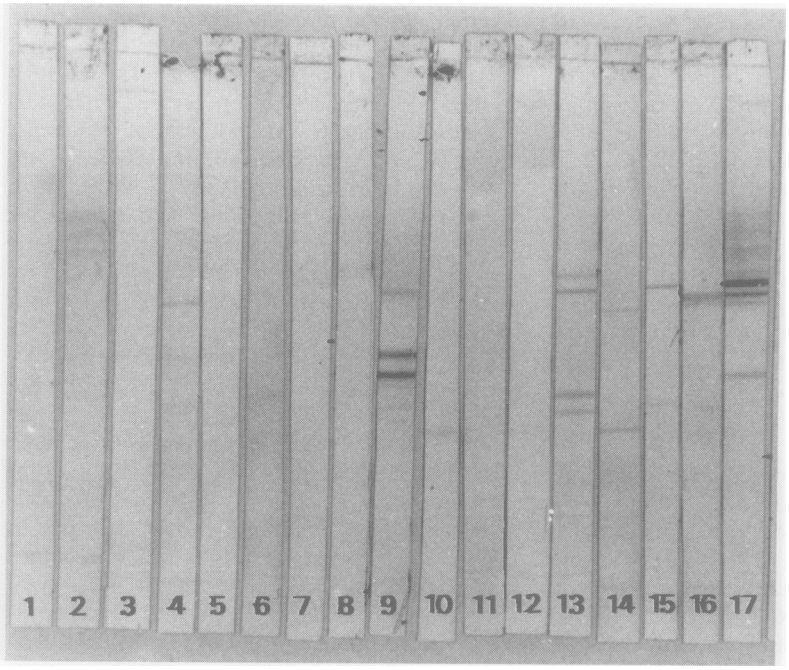

Figure 2 Immunoblot of sera from eight patients with colonic cancer (lanes 1–8), eight age matched controls (lanes 9–16), and one patient with S bovis endocarditis (lane 17), against antigens prepared by French press method from S bovis 10b167.

Two batches of sera, each consisting of eight patients with colonic cancer, eight controls, and serum from the patient with endocarditis, were blotted against whole cell protein preparations from the selected *S bovis* and *E faecalis* strains, at a dilution of 1 in 20. No consistent pattern was detected. Some sera from each group reacted with some bands from each organism, and no band could be identified with which the cancer sera regularly reacted. Figure 2 shows the results of one such inconclusive experiment. Blots were subsequently carried out with selected sera from each group at a range of higher dilutions (1 in 40, 1 in 80, and 1 in 160), but titres of reactivity could not be demonstrated by this approach (data not shown).

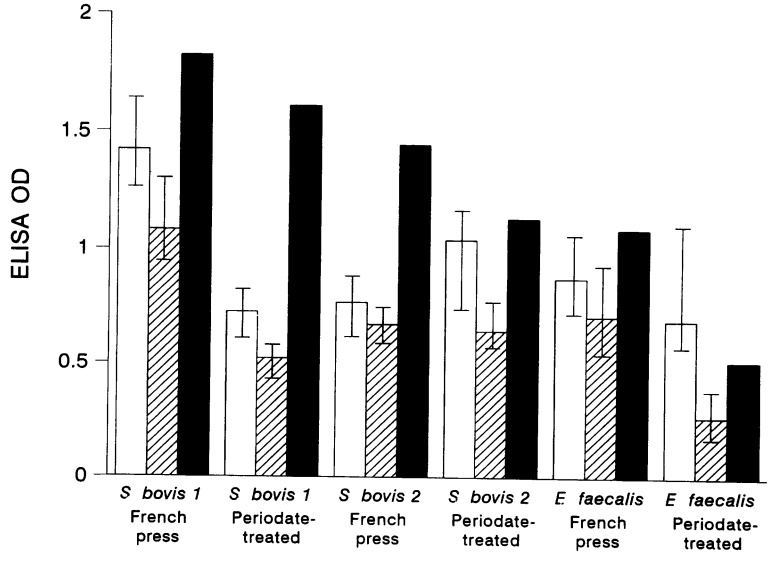

Figure 3 IgG antibody (ELISA OD) to six antigen preparations in 16 patients (open bars), 16 age matched controls (hatched bars), and one patient with S bovis endocarditis. Bars represent median values and error bars represent the 95% confidence intervals of the median.

ELISA

Preliminary ELISA experiments were carried out with plates coated with French press extracts of *S bovis* NCTC 8133 to determine suitable antigen coating concentration, serum dilution, and assay conditions to maximise the difference between the serum from the patient with *S bovis* endocarditis and one of the sera from the control group which showed no bands in the immunoblot. Using these conditions, IgG and IgM ELISAs were performed against French press and periodate treated French press antigen preparations from each of the three selected bacterial strains.

Serum from the patient with endocarditis gave the highest reading in all of the assays with *S bovis* antigens. In this serum sample the IgG antibody to periodate treated *S bovis* antigen was almost as high as the antibody to native *S bovis* antigen, suggesting that most of the antibody was directed against protein antigens (fig 3). The difference in IgG antibody to *E faecalis* periodate treated and native antigens was much greater with this serum, suggesting that much of the antibody to *E faecalis* in this serum was directed against carbohydrate antigens such as the group D antigen. Periodate treatment seemed, therefore, to have destroyed much of the common carbohydrate antigen but left the more species specific protein antigens. Periodate treatment also led to a substantial reduction in the detection of IgM antibody, which would be expected to be directed against carbohydrate antigens to a greater extent than is IgG.

The patients with colonic cancer had higher median IgG antibody titres to *S bovis* and *E faecalis* preparations than did the control patients (fig 3). This difference reached significance for both the *S bovis* NCTC 8133 preparations ($p = 0{\cdot}001$) and for the periodate treated preparations of *S bovis* 10b167 ($p = 0{\cdot}0007$) and *E faecalis* ($p < 0{\cdot}0001$). The finding of increased IgG antibody to the periodate treated antigen from both species implies that the difference is not due to cross-reactive antibody to group D carbohydrate. IgM antibody to the French press *S bovis* 2 antigen was higher in the cancer group (median 0·87, 95% CI 0·81 to 0·90) than in the controls (median 0·78, 95% CI 0·76 to 0·83; $p = 0{\cdot}006$). Significant differences among IgM antibody were not observed in any of the other assays.

Despite the differences among the groups there was a considerable overlap, and it would not have been possible to use any of these tests to predict usefully the presence of cancer. The best predictive values obtained were with the periodate treated *E faecalis* antigen where 15 of the patients with cancer and three controls gave an OD above an arbitrary cutoff value of 0·4. This translates into a predictive value for positive results (true positives ÷ (true positives + false positives)) of 83%, and a predictive value for negative results (true negatives ÷ (true negatives + false negatives)) of 93%.

Antibody to polymyxin-LPS-core cocktail

did not differ significantly between the cancer patients and controls. The IgG antibody titres was higher in the cancer patients (median 298, 95% CI 166 to 459) than in the controls (median 220, 95% CI 128 to 408). The IgM antibody titres was also a little higher in the cancer patients (median 131, 95% CI 67 to 180) than the controls (median 125, 95% CI 101 to 202).

Discussion

Previous studies of antibody response to *S bovis* and other streptococci and enterococci have found that antibody is detectable in endocarditis but not in either clinically insignificant bacteraemias[16 17] or colonic cancer.[3] These studies used immunoblotting, immunofluorescence, and crossed immunoelectrophoresis. We have also found that immunoblotting with whole cell proteins from *S bovis* or *E faecalis* was unable to distinguish between cancer patients and controls. All of these studies were limited by the use of relatively insensitive and non-quantitative methods.

In the more sensitive and quantitative ELISA we have shown an increase in IgG antibody to *S bovis* in patients with colonic cancer, and also found an increase in IgG antibody titre to *E faecalis*. The increase in IgG to *E faecalis* did not seem to be due to antibody to shared carbohydrate antigens, and may reflect increased antibody to species specific protein antigens. If IgG antibody titres reflect the incidence of bacteraemia then this implies that both *S bovis* and *E faecalis* bacteraemias are more common in patients with colonic cancer. Cases of *E faecalis* endocarditis have been associated with colonic cancer, but the association is much less strong than with *S bovis*.[18] This may be because *E faecalis* endocarditis often results from a primary focus of infection at other sites, such as infection of the urinary tract, or perhaps because *S bovis* bacteraemia is more likely to go on to cause endocarditis. It should be noted, however, that the antigen preparations used were crude and may have contained strain specific antigens or unrecognised common antigens which would confuse the interpretation of results. These questions might therefore be resolved by the use of pure antigens of known distribution among the relevant organisms.

The lack of any consistent difference in IgM antibody suggests that the increased immune stimulation in these patients has occurred over a long period of time, which is perhaps not surprising given the slow development of colonic cancer and the finding that *S bovis* infection may be associated with the preclinical stages.[1 4 18] The lack of difference in antibody to LPS-core suggests that the increase in antibody titre to *S bovis* and *E faecalis* in patients with colonic cancer patients is not simply due to a non-specific increase in antibody to gut bacteria.

There is a need for a good screening test for colonic cancer, particularly a test which could detect early lesions. Detection of faecal occult blood is neither sensitive nor specific, while carcinoembryonic antigen is regularly detectable only in advanced disease.[19] The results presented here suggest that it may be possible to develop a test to screen patients for the presence of colonic cancer by measuring IgG antibody titre to *S bovis* or *E faecalis*. Further investigation is required to identify antigens which would permit improved discrimination between the groups, and to determine whether antibody is also raised in other colonic diseases or in liver disease.

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1 Klein RS, Catalano MT, Edberg SC, Casey JI, Steigbigel NH. Streptococcus bovis septicaemia and carcinoma of the colon. *Ann Intern Med* 1979;**91**:560–2.
2 Friedrich IA, Wormser GP, Gottfried EB. The association of recent Streptococcus bovis bacteraemia with colonic neoplasia. *Mil Med* 1982;**147**:584–5.
3 Kaplan MH, Chmel H, Stephens A, *et al.* Humoral reactions in human endocarditis due to Streptococcus bovis: evidence for a common S. bovis antigen. *J Infect Dis* 1983;**148**:266–74.
4 Beeching NJ, Christmas TI, Ellis-Pegler RB, Nicholson GI. Streptococcus bovis bacteraemia requires rigorous exclusion of colonic neoplasia and endocarditis. *Q J Med* 1985;**56**:439–50.
5 Ruoff KL, Miller SI, Garner CV, Ferraro MJ, Calderwood SB. Bacteraemia with Streptococcus bovis and Streptococcus salivarius: clinical correlates of more accurate identification of isolates. *J Clin Microbiol* 1989;**27**:305–8.
6 Zarkin BA, Lillemoe KD, Cameron JL, Effron PN, Magnuson TH, Pitt HA. The triad of Streptococcus bovis bacteraemia, colonic pathology, and liver disease. *Ann Surg* 1990;**211**:786–92.
7 Klein RS, Recco RA, Catalano MT, Edberg SC, Casey JI, Steigbigel NH. Association of Streptococcus bovis with carcinoma of the colon. *N Engl J Med* 1977;**297**:800–2.
8 Wicken AJ, Baddiley J. Structure of intracellular teichoic acids from group D streptococci. *Biochem J* 1963;**87**: 54–62.
9 Morris EJ, Ganeshkumar N, McBride C. Cell surface components of Streptococcus sanguis: relationship to aggregation, adherence, and hydrophobicity. *J Bacteriol* 1985;**164**:255–62.
10 Lowry OM, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin-phenol reagent. *J Biol Chem* 1951;**193**:265-75.
11 Laemmli UK. Cleavage of structural proteins during assembly of the head protein of bacteriophage T4. *Nature* 1970;**277**:680–5.
12 Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979;**76**:4350–4.
13 Scott BB, Barclay GR, Smith DGE, McLoughlin F, Poxton IR. IgG antibodies to Gram-negative endotoxin in human sera. I. Lipopolysaccharide (LPS) cross-reactivity due to antibodies to LPS core. *Serodiagnost Immunother Infect Dis* 1990;**4**:25–38.
14 Campbell MJ, Gardner MJ. Calculating confidence intervals for some non-parametric analyses. In: Gardner MJ, Altman DG, eds. *Statistics with confidence.* London: BMJ, 1989:71–9.
15 Dean AG, Dean JG, Burton AH, Dicker RC. *Epi Info; Version 5.* Stone Mountain, Georgia: USD Incorporated, 1990.
16 Burnie JP, Holland M, Matthews RC, Lees W. Role of immunoblotting in the diagnosis of culture negative and enterococcal endocarditis. *J Clin Pathol* 1987;**40**: 1149–58.
17 Shanson DC, Kirk N, Humphrey R. Clinical evaluation of a fluorescent antibody test for the serological diagnosis of streptococcal endocarditis. *J Clin Pathol* 1985;**38**: 92–8.
18 Panwalker AP. Unusual infections associated with colonic cancer. *Rev Infect Dis* 1988;**10**:347–64.
19 Clark ML, Price AB, Williams CB. Tumours of the gastrointestinal tract. In: Weatherall DJ, Ledingham JGG, Warrell DA, eds. *Oxford textbook of medicine.* 2nd edn. Oxford: Oxford University Press, 1987:146–58.