

Relative carriage rates of nuclear dehydrogenating clostridia in two populations of different colorectal cancer risk

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SUMMARY Carriage of nuclear dehydrogenating clostridia has been associated with colon cancer and implicated in its aetiology. This study has compared the carriage of these organisms in a British population at high risk for the development of colon cancer with a low risk Nigerian population. Clostridia were found in all of the stools from both populations. Nuclear dehydrogenating clostridia were only found in the stools of the British subjects (32%). These results support the suggestion that the carriage rate of nuclear dehydrogenating clostridia in a population is related to the risk of colon cancer.

Cancer of the large bowel is primarily a disease of western countries¹ and has been strongly associated with diet. The epidemiology of the disease has been reviewed recently by Correa and Haenszel.² Current theories on its aetiology have suggested that it is caused by carcinogens or tumour promoters produced in the colon by bacterial metabolism^{3,4} and particular interest has centred on the possible role of bile acids as substrates.⁵ One of the possible classes of metabolites of interest is the unsaturated bile acids. A large screening study of more than 1000 strains of intestinal bacteria by Goddard *et al*⁶ showed that certain types of clostridia were the only intestinal bacteria able to dehydrogenate the steroid nucleus at the Δ^4 position to yield an unsaturated product. An association between carriage of these nuclear dehydrogenating clostridia (NDC) and large bowel cancer was demonstrated by Hill *et al*⁷ in a case control study and this has been confirmed by Borriello (unpublished observations, 1977) and Blackwood *et al*.⁸ Populations with a low incidence of large bowel cancer rarely carry the species that most commonly produce unsaturated bile acids.^{6,9} However, the strains of clostridia examined in this and a later study in Hong Kong¹⁰ had been isolated some time before the testing of their dehydrogenating activity. Thus, loss of activity or selection of inactive strains may have occurred during storage.

As part of a study of infant malnutrition in Nigeria there was an opportunity to collect and study faecal samples from infant and adult Nigerians. Large bowel cancer is rare in Nigeria^{11,12} and so the opportunity was taken to assay these samples for NDC.

Material and methods

STOOL SAMPLES

Stools were collected from 53 British adults of long-standing residence in the UK and from 17 adults from Gamzago, a hamlet in Malumfashi district, a rural savannah area 70 miles north of Zaria, Nigeria. None of the subjects included in the study had any history of previous gastrointestinal malignancy or of any gastrointestinal disease known to be associated with colorectal cancer. The stool samples from the UK subjects were either stored frozen at -20°C ,¹³ or a small amount of stool (approximately 0.5 g) was placed in a preweighed 5 ml screw-capped bottle containing 4.5 ml of glycerol transport broth (GTB). The added stool was emulsified with the aid of a Vortex mixer and then frozen and stored at -20°C .¹³ A small weighed sample of the stool specimens from Nigerian subjects was diluted tenfold in 10% GTB within 45 min of collection and frozen in liquid nitrogen stored in a portable field refrigerator. All samples were processed within 1 month of receipt. The Nigerian samples were collected during the months of April and May (dry season). Those from the United Kingdom were collected over the period January to June of the previous year.

MICROBIOLOGICAL ANALYSIS

All manipulations were performed in an anaerobic cabinet. Serial tenfold dilutions of the stool samples were made in Brain Heart Infusion broth. The prepared samples were heated to 75°C for 20 min on a dry block heater to select for clostridial spores. Aliquots (0.1 ml) of these dilutions were seeded onto an egg-yolk base

Table 1 Comparison of faecal clostridia of Nigerian and UK subjects

| Subject (No) | Mean log ₁₀ No./g (Range) Median log ₁₀ No./g | | | |
|---------------|---|-------------------|-------------------|----------------|
| | Total clostridia | Lecithinase +ve | Lecithinase -ve | NDC |
| Nigerian (17) | 6.0 (3.0-7.8) 6.2 | 5.9 (3.0-7.8) 6.2 | 1.1 (ND-5.1) 0 | ND |
| UK (53) | 5.3 (3.0-7.8) 5.3 | 3.1 (ND-7.0) 3.9 | 5.1 (3.0-7.8) 5.1 | 1.7 (ND-7.4) 0 |

NDC = Clostridia capable of dehydrogenating the steroid nucleus at the Δ^4 position.
ND = Not detected.

medium. The seeded plates were incubated in an anaerobic environment at 37°C for 3 days. All of the techniques and conditions used have been described previously.¹⁴ After incubation total counts were estimated and 10 colonies from each stool sample were transferred to Robertson's cooked meat medium (RCM) for further analysis. Colonies were chosen from plates consisting of 30 to 100 colonies. The choice of colonies was random.

3-OXO-5 β -STEROID Δ^4 DEHYDROGENASE ACTIVITY
The clostridia isolated from faeces were tested for their ability to dehydrogenate the steroid nucleus at the Δ^4 position. Three drops of Todd-Hewitt broth (Oxoid) containing 0.005% wt/vol of 5 β -androstan-3-17-dione were added to each well of a microtitre tray (Sterilin Ltd). A Pasteur pipette was used to deliver two drops of a three-day culture in RCM to each well. Each well was inoculated with a different isolate. An uninoculated well served as a negative control and a nuclear dehydrogenating strain of *Clostridium paraputrificum* as a positive control. The inoculated trays were incubated anaerobically. At 48 h one drop of 5 β -androstan-3-17-dione/menadione stock solution was added (5 β -androstan-3-17-dione, 0.5 g, menadione, 0.35 g, ethyl alcohol 100 ml; filter sterilise). The trays were reincubated anaerobically for a maximum of 18 h and a minimum of 12 h. After incubation 10 μ l of the contents of each of 10 wells were simultaneously spotted onto a TLC plate (Polygram Silica Gel/UV 254 thin layer chromatography plates of 0.25 mm thickness Camlab-Cambridge) by use of a set of 10 fixed phage loops which have been previously described.¹⁵ The plates were run in benzene-dioxan-water 100:10:1 (vol/vol). All plates included a 4-androsten-3-17-dione standard. The plates were air dried and observed under ultraviolet light ($\lambda=254$ nm) for the production of 4-androsten-3-17-dione. The plates could then be sprayed with anisaldehyde reagent and the colour allowed to develop in a hot oven at 80°C.

Results

Both populations harboured clostridia in all of the stool samples analysed. The Nigerian samples yielded slightly higher counts of total clostridia with a mean of 10^{6.0}/g stool compared to 10^{5.3}/g stool for UK residents (Table 1). The Nigerian clostridial flora was composed predominantly of lecithinase positive clostridia (all of which were *C. perfringens*), whereas the UK clostridial flora was

Table 2 Carriage of clostridia capable of dehydrogenating the steroid nucleus (NDH clostridia)

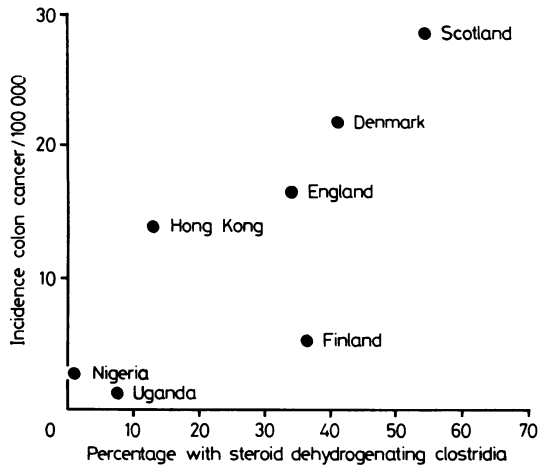
| | British adults | Nigerian adults |
|-------------------------|----------------|-----------------|
| No. of subjects | 53 | 17 |
| % carriage of NDC | 32 | 0 |
| Large bowel cancer risk | High | Low |
| Colon | 24.7* | 0.4* |
| Rectum | 15.5* | 0.4* |

*Average annual incidence per 100 000 for all age groups.¹⁷

predominantly lecithinase-negative in nature (Table 1). Clostridia with the ability to dehydrogenate the steroid nucleus at the Δ^4 position (NDC) were not detected in the stools of Nigerians but they were recovered from 32% of the stools of UK residents at a mean concentration of 10^{1.7}/g stool (Table 2). The mean number of NDC in the subjects that carried these organisms was 10^{5.1}/g stool.

Discussion

Data on the carriage of NDC by various populations are sparse. The study of Drasar *et al*⁹ concentrated upon the identity of the clostridia isolated from the faeces of populations living in England, Wales, Scotland, USA, Japan, Hong Kong, and Uganda. The most striking finding was the low carriage rate of *C. paraputrificum* in areas of low cancer incidence and the comparatively high rate in areas of high incidence; however the clostridia examined had been in culture for between five and eight years and there are no data on changes in dehydrogenating activity during such prolonged storage. Goddard *et al*⁶ in a parallel study examined the faecal clostridia isolated from these subjects, showing that *C. paraputrificum* was active in the dehydrogenation of steroids but that this metabolic activity was not restricted to a particular species, *C. indolis* and other species also being active. However, fewer active strains of all groups were represented among those strains isolated from low-incidence areas. Consideration of these studies underline the importance of examination of the metabolic activities of bacteria in epidemiological studies. These data on carriage rate of clostridia with the potential to dehydrogenate the steroid nucleus have now been corrected to give the carriage rate of clostridia that actually possessed this capability *in vitro* (Figure). Crowther *et al*¹⁰ did not show a relation between NDC carriage and large bowel cancer incidence in three socioeconomic groups in Hong Kong, all three groups having low carriage rates. A recent study found carriage rates of NDC of 40% in Danes, who



Relation between carriage of nuclear dehydrogenating clostridia and colon cancer incidence. Data of Goddard *et al.*,⁶ Crowther *et al.*¹⁰ and IARC.¹⁶

have a high incidence of colorectal cancer, and 38% in Finns, who have a low incidence of the disease¹⁶ (Figure). The carriage rate of NDC and the incidence of large bowel cancer in the Danes were similar to those of the English population studied both in this study and the case control study by Hill *et al.*⁷ The carriage rate in Finns is clearly anomalous in this respect and indicates that the relation between carriage of NDC and colorectal carcinogenesis is not a simple one (Figure).

The data reported here show that Nigerians, who have a low incidence of large bowel cancer^{12,12,17} and of potentially precancerous lesions¹⁰ also have a very low carriage rate of NDC; these results are in striking agreement with the evidence of low NDC carriage for Uganda and Hong Kong.^{6,9}

The results of this study add support to the suggestion that the carriage rate of NDC in a population is related to the risk of colorectal carcinogenesis in that population. It is hoped that prospective studies currently in progress will help clarify this possible aetiological relation.

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