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Effect of Freezing of Human Fecal Specimens upon the Isolation of Nuclear Dehydrogenating Clostridia (39998)

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There has been much interest in the possible role of intestinal bacteria in the etiology of colon cancer (1, 2). Coombs *et al.* (3) suggested that full aromatization of the bile acid nucleus would potentially yield a carcinogen metabolite based on cyclopentaphenanthrene. Goddard *et al.* (4) demonstrated the presence in the human fecal flora of nuclear dehydrogenating clostridia (NDC) which are capable of partially aromatizing a sterol nucleus.

Hill *et al.* (5) reported that a higher percentage of colon cancer patients had fecal NDC than did control patients. However, Finegold *et al.* (6) found no difference in counts of *Clostridium paraputrificum*, an organism capable of nuclear dehydrogenation, between patients with polyps and controls. Similar results were obtained by Moore and Holdeman (7) who showed that clostridial species are more numerous in the fecal floras of Africans, a low-risk population, than in the patients with polyps. Studies by Mastromarino *et al.* (8) indicate that species of *Clostridium*, although not the *C. paraputrificum* group, constituted a relatively greater percentage of fecal flora in patients with colon cancer.

The methods used by Hill *et al.* (5) for the isolation of NDC were different from those used by Mastromarino *et al.* (8), Finegold *et al.* (6), and Moore and Holdeman (7). The experimental techniques of Hill *et al.* (5) involved freezing the fecal specimens in glycerol soon after collection and performing NDC analysis at a later time, whereas methods used by Finegold *et al.* (6), Moore and Holdeman (7), and Mastromarino *et al.* (8) required fresh samples.

Freezing may be selective for NDC and thus accounts for the ability of Hill *et al.* (5) to show a correlation between colon cancer and NDC. We have, therefore, investigated the effect of freezing of fecal samples upon isolation of NDC.

Methods and Materials. Fecal samples were obtained in the New York Metropolitan area from healthy control persons and from patients with colon cancer and other colonic diseases. All of the subjects were eating a typical mixed Western diet. The feces were placed in a Gas Pack container (BBL) immediately after voiding and arrived under an anaerobic atmosphere at the laboratory for processing within 3 hr of the time that they were passed. Feces were processed under oxygen-free conditions in the laboratory. After thorough mixing of the fecal sample to obtain a homogenous sample, approximately 1.0 g wet wt of the fecal homogenate was suspended in 9.0 ml of glycerol broth (9). One milliliter of the suspension was removed, serially diluted, and plated on egg yolk lactose sugar (10). All dilutions were done under a gas mixture of 10% CO_2:5% H_2:85% N_2, and egg yolk plates were incubated under a 30% CO_2: 70% H_2 atmosphere (10). The remainder of the glycerol broth suspension was frozen and stored at $-20°$ for 2-3 weeks before processing. After thawing at room temperature, the frozen sample was processed in the same manner as the fresh samples. Several colonies, one colony of each morphological type or five colonies per plate, whichever yielded the highest number of colonies, were selected for determination of nuclear dehydrogenation ability. Colonies, after restreaking on egg yolk agar, were inoculated into Todd Hewitt broth and incubated, using the modification of Goddard *et al.* (4), with 5β-androsten-3,17-dione (Sigma) as the substrate and menadione (Sigma) as the electron acceptor. The cultures were extracted with chloroform and analyzed by thin-layer chromatography on silica gel GF plates, using solvent system A (benzene–dioxane–acetic acid 100:10:1), as described by Aries *et al.* (11). Fluorescence-

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quenching spots corresponding in location to 4-androsten-3,17-dione were eluted from the plates and the uv absorption spectrum was determined, using a Beckman DG-GT spectrophotometer. Only if the material had an absorption maximum of 240 nm, characteristic of androstene, was the organism scored as being capable of nuclear dehydrogenation.

Results. Freezing the fecal samples in the glycerol broth medium affected both the kind and number of organisms obtained from the suspension. Lecithinase activity in all the fresh samples was very pronounced on the egg yolk agar. Because of overlapping zones of lecithinase activity, lecithinase-positive and lecithinase-negative colonies could not be differentiated. After freezing, the number of lecithinase-positive organisms was greatly reduced, with most samples having no lecithinase-producing colonies present. If lecithinase-producing colonies were present, they were a minor component of only 1 to 5% of the total colonies on the plate. The number of colony-forming units was also reduced by freezing by a factor of 20- to 50-fold. In cases where there was a change in the number of different types of colonies, as based upon morphology, freezing resulted in an increase in morphological types rather than in a decrease.

There was also a greater frequency of isolation of nuclear dehydrogenating bacteria from frozen as compared to fresh stool samples, as shown in Table I. This table shows the number and percentage of persons from whom NDC have been isolated and the number of persons in whom no NDC were detected. In comparing results from fresh samples and the same samples after freezing and thawing, there is a significant increase in the number and percentage of persons from whom NDC were isolated (Table I). The mean concentration of NDC isolated from fresh feces is 2.4×10^7 bacteria/g wet wt and 1.3×10^5 bacteria/g wet wt from frozen samples.

Colonies most likely to possess nuclear dehydrogenation activity were flat irregularly round colonies the color of which closely matched that of egg yolk agar medium and a round concave colony with a bright pink color. Not all colonies matching these descriptions proved to be capable of dehydrogenating the androstan substrate used for assay purposes.

TABLE I. COMPARISON OF NUCLEAR DEHYDROGENATING CLOSTRIDIA IN FRESH AND FROZEN STOOL SAMPLES[a]

	NDC^+	NDC^-	NDC (%)
Fresh[b,*]	2	11	15
Frozen[c,*]	11	2	85

[a] NDC^+: Number of persons from whom NDC were isolated; NDC^-: number of persons from whom no NDC were isolated. NDC (%): percentage of group who harbor NDC.
[b] Sample assayed fresh upon arrival in laboratory.
[c] Sample assayed after freezing in glycerol broth.
* Significantly different by χ^2 test, $P < 0.0005$.

Discussion. Several changes occurred as a result of freezing fecal samples in glycerol broth. There was a 20- to 50-fold decrease in the number of colony-forming units, lecithinase activity was virtually eliminated, and there was an increase in the frequency of isolation of NDC from the primary egg yolk agar plates. The lecithinase-producing bacteria appear to be very sensitive to freezing. Although the NDC were not completely resistant to freezing, they were more resistant to freezing than were many of the other bacteria present in the fresh samples. Thus, freezing appears to be selective for NDC, resulting in a higher frequency of isolation of NDC after freezing.

Too little data have been collected at this time to form any conclusions about the effect of freezing upon correlation of NDC and colon cancer. We have found that freezing affects the frequency of isolation of NDC and are continuing to collect data to determine how freezing and NDC correlate with incidence of colon cancer.

Summary. Fresh and frozen human fecal specimens were used for the isolation of nuclear dehydrogenating clostridia (NDC). The total bacterial count and the number of lecithinase-positive bacteria were lower in frozen samples than in fresh samples, but the frequency of isolation of NDC from frozen specimens was higher than from fresh specimens.

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