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The permeability of normal, adenomatous, ulcerative colitic and malignant large bowel epithelial cell membranes to inulin

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Summary. We measured the permeability of normal, adenomatous, colitic and malignant large bowel epithelial cells by immersing fragments of large bowel mucosa in radiolabelled inulin and comparing autoradiograph grain density inside and outside cells after incubation. All the carcinomas studied showed extensive uptake of inulin within 5 min, while normal, adenomatous and colitic epithelial cells completely excluded inulin for 30 min. We found no difference in the proportion of epithelial cells incorporating uridine into RNA in carcinomatous and normal mucosa, and this suggests that the increased inulin permeability of carcinoma cell membranes was not due to leakage into non-viable cells. Experiments with cytochalasin B also showed that increased pinocytosis by carcinoma cells could not account for the difference. The relative impermeability of adenomatous and colitic cells suggests that increased permeability is not caused by increased proliferation. The consistent finding of increased permeability in the plasma membranes of carcinoma cells suggests that this may be more than an epiphenomenon of malignancy. It also suggests that measurement of cell permeability may have a role in distinguishing malignant from benign epithelial neoplasms.

Keywords: colon cancer, carcinoma, adenoma, ulcerative colitis, neoplasia, membrane permeability

There is evidence to suggest that membranes of carcinoma cells may be more permeable than those of their normal counterparts. Ehrlich ascites cells have been shown to release cytosolic enzymes such as lactate dehydrogenase and glycolytic enzymes during culture (Bosch 1958; Wu 1959). Bladder mucosa has been found to become more permeable as carcinoma supervenes and this appears to be due to an increase in plasma membrane permeability rather than to leakage between cells (Caruthers & Bonneville 1980).

We decided to compare the permeability of normal, adenomatous and malignant large bowel epithelial cells. We did this by immersing fragments of normal, hyperplastic and neoplastic large bowel mucosa in radiolabelled inulin, a molecule which is unable to penetrate normal plasma membranes. Diffusion into cells was measured by frozen section autoradiography.

Methods

Inulin permeability. Colorectal carcinomas were collected from the operating theatre, opened, and thoroughly washed in sterile phosphate-buffered saline (PBS). Strips of normal mucosa were taken at least 6 cm

from the edge of a carcinoma, dissected free of underlying muscularis propria, and cut into 3-mm squares. Samples of carcinoma were taken from non-ulcerated areas and similarly cut into 3-mm fragments. Adenomas and fragments of mucosa from patients with ulcerative colitis were obtained by colonoscopy. We used only a portion of the tissue: the remainder was taken for routine diagnostic histopathology. The tissue was similarly washed in PBS.

The tissue was immersed in medium 199 (Flow) containing 10% heat-inactivated fetal calf serum (Flow), 100 iu/ml of benzylpenicillin (Glaxo) and 100 μg/ml of streptomycin (Glaxo) (199 FCS) and ^{3}H-inulin (Amersham Radiochemicals), (50 μCi/ml, >500 μCi/mmol). When cytochalasin B (Sigma) was added, it was added to the ^{3}H-inulin-labelled medium at a final concentration of 20 μg/ml. The samples were incubated at 37°C for 1, 5, 10 or 30 min before they were removed (without washing) and placed on a drop of Tissue-tek on a square of copper 4 × 4 × 1 mm. The copper plate was then immersed in isopentane cooled in liquid nitrogen and stored in liquid nitrogen until sectioned.

Coverslips were prepared for autoradiography thus: Kodak AR 10 autoradiography stripping film was immersed in water in the dark, wrapped around each coverslip and allowed to dry thoroughly. For sectioning, the copper squares were mounted on the block-holder of a Reichert Frigocut cryostat with Tissue-tek and frozen sections were cut at −30°C. The frozen sections were placed on warmed (40°C) autoradiograph film-coated coverslips. Sections treated this way melt and dry very rapidly: the water is absorbed by the gel of the film as it melts and we found that little diffusion occurred during the melting process (Figs 1 and 2). The autoradiograph films were exposed for 7 days in a light-tight box before they were developed, stained and mounted on glass slides with Depex.

Autoradiograph grain density was measured with a Leitz photometric microscope using polarized incident light. We used incident light and inverted slides so that light travelled only through the film before returning to the photometer, thus eliminating the photometric problem of variable light absorption by the tissue section. In polarized incident light the autoradiograph grains shine white against a dark background (Fig 1). The photometer diaphragm was adjusted to measure a tissue area 5 × 5 μm. Readings were taken in pairs, the first reading over the centre of an epithelial cell, and the second over the immediately adjacent (extracellular) border showing the highest grain density. The first reading divided by the second was taken as a measure of the extent of uptake of inulin into the cell. Each point in Fig 2 represents the mean of at least 50 such individual ratios. A total of eight colorectal specimens were studied in this way.

Cell viability. This was demonstrated by ^{3}H-uridine incorporation into ribonucleic acid, and by horse-radish peroxidase exclusion. Similar fragments to those above were incubated on stainless steel grids in 199 FCS to which 10 μCi/ml of ^{3}H-uridine (Amersham Radiochemicals) had been added as previously described (Serafini *et al.* 1981). After 30 min at 37°C the fragments were washed in several changes of PBS, fixed in formalin and processed for paraffin sections. Autoradiographs were prepared as described above, with an exposure time of 4 weeks.

Horse-radish peroxidase type II (Sigma) was visualized by reaction with 3,3′-diaminobenzidine (Sigma). Horse-radish peroxidase (0.05%) was dissolved in culture medium, and incubated with fragments of normal and neoplastic large bowel mucosa for 2 h at 37°C as above. Fragments were then fixed in buffered 5% glutaraldehyde for 3 h and left overnight in 0.2 M sodium cacodylate sucrose buffer. Slices (0.5 mm thick) were cut with a razor blade and immersed in a solution of 6 mg diaminobenzidine in 20 ml Tris-saline (pH 7.6) containing 300 μl of hydrogen peroxide (BDH, 30% w/v) for 5 min. They were then washed in running tap water for 10 min, embedded in

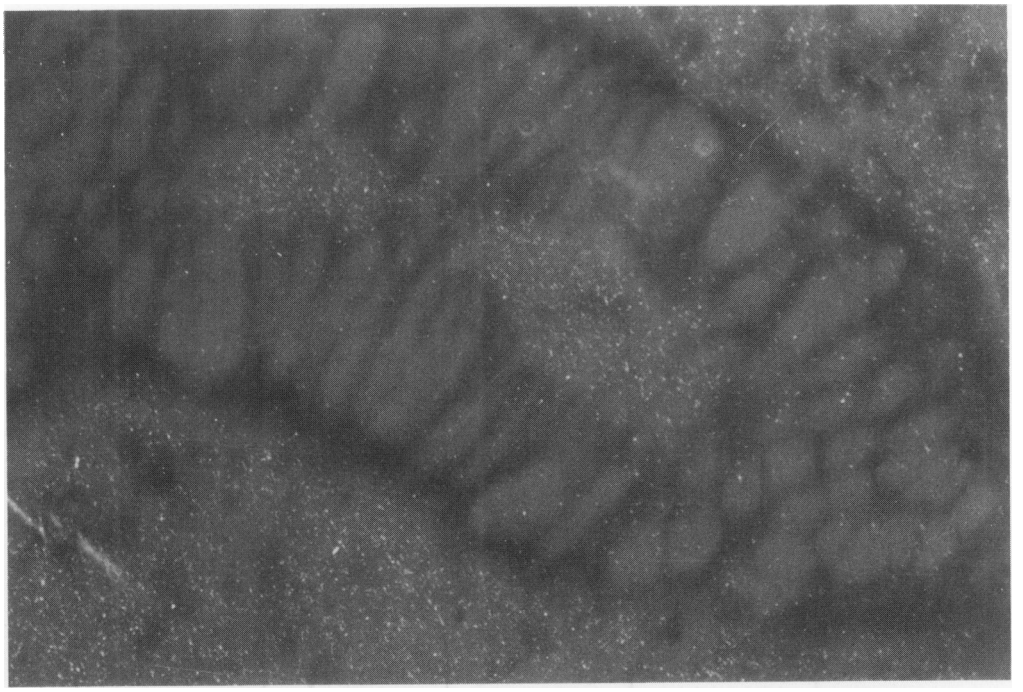

Fig. 1. Normal colonic mucosa incubated in radiolabelled inulin for 30 min. Autoradiograph grains are present on both luminal and mucosal surfaces of the crypt, but are relatively excluded from cells. Half-crossed polarizers. × 520.

Araldite and sectioned for light and electron microscopy.

Results

When mucosal fragments are completely immersed in inulin the epithelial cells are exposed both from the luminal aspect and from the lamina propria, by diffusion from the edges and base of the fragment. The epithelial cells are thus exposed to inulin from all sides. Despite this we found that normal epithelial cells were impermeable to inulin even after 30 min of incubation (Fig 1). The extracellular concentration of inulin varied somewhat, being higher at the periphery of the specimen than at its centre. The ratio of the counts inside a cell to the counts over the adjacent extracellular region with the greatest grain density was taken to reflect the concentration gradient across the intervening plasma membrane. For normal cells this ratio remained constant at about 10% throughout the time of the experiment (Figs 2 and 3). Because this ratio did not increase with time of incubation the intracellular count presumably reflects the degree of diffusion inherent in the soluble isotope technique itself (i.e. diffusion of isotope during the preparation of autoradiographs).

In contrast to the exclusion of inulin by normal epithelial cells, the cells of all the carcinomas tested showed uptake of inulin into their cytoplasm. Inulin uptake by carcinoma cells proceeded rapidly and by 30 min the mean grain density over carcinoma cells was approaching that seen outside.

To see if this increased inulin uptake was a reflection of increased cell proliferation, we measured the uptake of epithelial cells in the mucosa of patients with ulcerative colitis in remission, and in adenomas. [Ulcerative col-

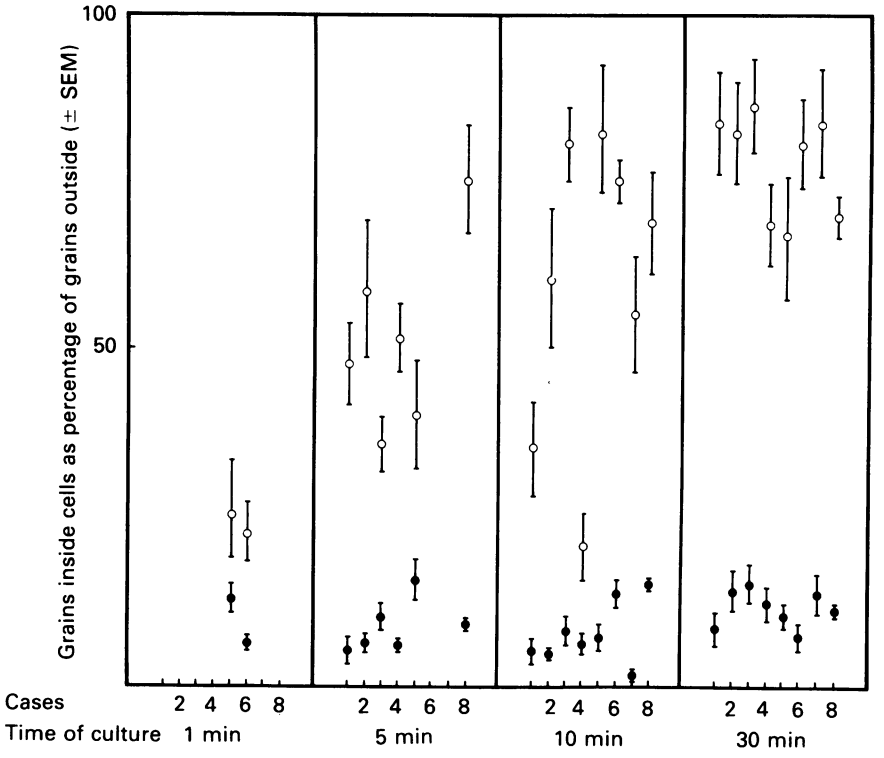

Fig. 2. Ratio of grains over cells to grains over adjacent extracellular space in normal (●) and malignant (○) mucosa incubated in radiolabelled inulin (eight patients).

itis is associated with increased cell proliferation even in remission (Serafini *et al.* 1981)]. We found no significant increase in inulin uptake in epithelial cells in mucosa from either of these sources (Fig 4).

It seemed possible that the increased uptake of inulin which we observed in carcinoma cells could be due to increased pinocytosis. We therefore incubated fragments of carcinoma in radiolabelled inulin in the presence of 20 μg/ml of cytochalasin B. This concentration effectively abolishes pinocytosis (Wagner *et al.* 1971). Inulin uptake by carcinoma cells was reduced from 78 to 56% at 30 min (Fig 5). Part of the increased uptake of inulin by carcinoma cells compared to normal is thus due to increased pinocytosis by tumour cells. However, the uptake of inulin remained high even in the absence of pinocytosis, and increased pinocytosis alone could not account for this raised inulin uptake.

Carcinomas are known to contain a proportion of necrotic cells, and when cells die their plasma membranes become more permeable. We were always careful to choose areas for our counts which appeared histologically viable. The increased pinocytosis by our tumour cells is evidence for their viability. We also confirmed viability by incorporation of ^{3}H-uridine and by peroxidase impermeability. We found that at least 90% of the carcinoma cells were capable of incorporating uridine into ribonucleic acid. These sections had been processed by formalin fixation and paraffin embedding; radiolabel persisting after this treatment is thus insoluble, and therefore presumed to have been polymer-

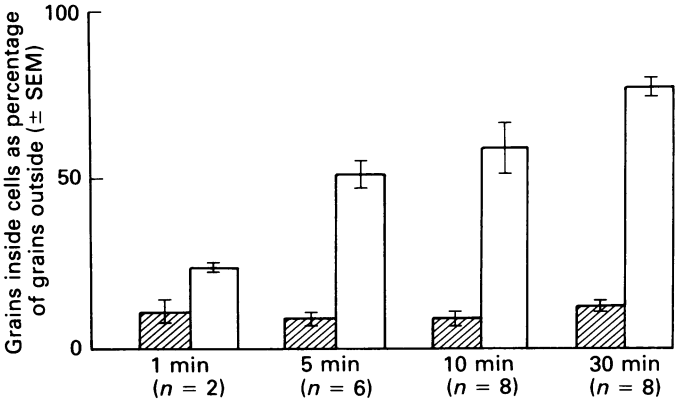

Fig. 3. Mean grain count over cells as proportion of grain count outside cells. ▨ Normal; □ carcinoma.

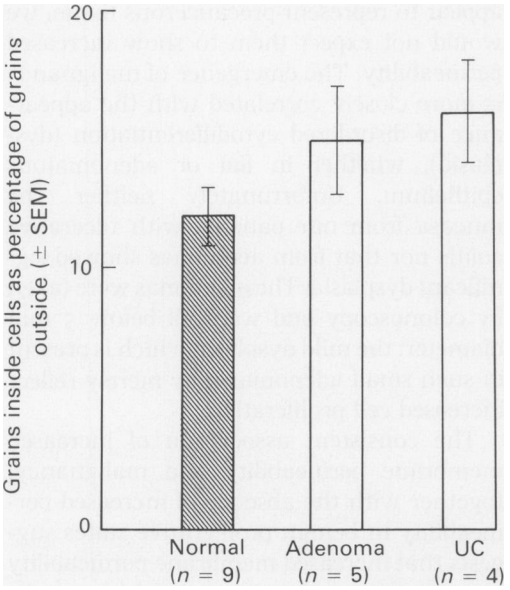

Fig. 4. A comparison of radiolabelled inulin uptake by normal, adenomatous and ulcerative colitis mucosal cells.

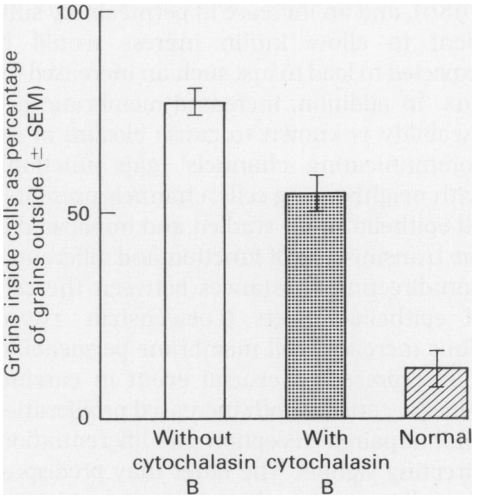

Fig. 5. The effect of cytochalasin B on grain density over carcinoma cells.

ized into ribonucleic acid. This was confirmed by the localization of the label predominantly over the nucleus.

Discussion

Inulin is a carbohydrate polymer with a molecular weight of 5200, corresponding to 32 hexose molecules. Non-toxic and physiologically inert, it is unable to cross normal cell membranes and is often used as a marker for the extracellular space. We have found an increased uptake of inulin by carcinoma cells. This increased uptake could not be accounted for by pinocytosis and therefore represents increased diffusion of inulin across plasma membrane. The carcinoma cells appeared fully viable as measured by morphology and the incorporation of uridine into ribonucleic acid. The increased permeability could not be explained by access of inulin to a greater proportion of the surface

of carcinoma cells—normal cells excluded inulin even when surrounded by it. We thus conclude that carcinoma cells show increased plasma membrane permeability to inulin.

We found that epithelial cells in adenomas and in the mucosa of patients with ulcerative colitis were impermeable to inulin. Inulin permeability is thus not merely the result of increased proliferation. There is evidence, however, that proliferating cells have a reduced transmembrane potential, due to increased permeability to monovalent ions (Cone 1980; Rozengurt & Mendoza 1980). Indeed, cells can be induced to divide by a sustained increase in ion permeability (Cone 1980), and an increase in permeability sufficient to allow inulin ingress would be expected to lead to just such an increased ion flux. In addition, increased membrane permeability is known to cause closure of the communicating channels (gap junctions) with neighbouring cells, channels present in all epithelia so far studied and implicated in the transmission of function and differentiation-directing substances between the cells of epithelial sheets (Loewenstein 1979). Thus increased cell membrane permeability may represent a crucial event in carcinogenesis, causing both increased proliferation and impaired reception of differentiation-directing signals. The latter may predispose the cell to develop phenotypic diversification which is a feature of carcinoma cells, such as inappropriate patterns of mucin or hormone production, invasiveness and metastasis: potential attributes present in the genome but suppressed in normal epithelial cells.

Because the majority of carcinomas of the colorectum develop in pre-existing adenomas the view has arisen that adenomas represent a step in the sequence between normality and malignancy. There is strong epidemiological and clinical evidence, however, that adenomas and carcinomas are caused by different agents (Hill *et al.* 1978): one agent which causes increased proliferation (adenomas) and another which is carcinogenic. Furthermore, in ulcerative colitis, a disease in which there is a continuous high rate of epithelial cell proliferation (Serafini *et al.* 1981), carcinomas do not develop in adenomas but in flat mucosa (Morson & Pang 1967). This shows that adenomas are not a stage through which cells must pass before becoming malignant, and suggests that it is an increased proliferation rate, caused either by adenomas or ulcerative colitis, which predisposes to malignancy in the presence of a carcinogen. There is evidence for a similar relationship between papillomas and carcinogenesis in the upper alimentary tract of cattle (Jarrett 1981) and in the human cervix uteri (Zur Hausen 1982). Since adenomas do not appear to represent precancerous tissue, we would not expect them to show increased permeability. The emergence of malignancy is more closely correlated with the appearance of disordered cytodifferentiation (dysplasia), whether in flat or adenomatous epithelium. Unfortunately neither the mucosa from our patients with ulcerative colitis nor that from adenomas showed significant dysplasia. The adenomas were taken by colonoscopy and were all below 5 mm diameter: the mild dysplasia which is present in such small adenomas may merely reflect increased cell proliferation.

The consistent association of increased membrane permeability and malignancy, together with the absence of increased permeability in benign proliferative states suggests that increased membrane permeability may represent a characteristic which may be of clinical use in distinguishing malignant from benign lesions.

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