Kill and cure: dietary augmentation of immune defences against colon cancer

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At its most fundamental, cancer is a genetic disease resulting from inherited or acquired mutations in tumour suppressor genes and proto-oncogenes. Environmental factors, including ingested food components, interact with genetic inheritance to determine individual cancer risk. There is growing evidence that the immune system exerts selective pressure during neoplastic development. Tumour cells that evade this immunosurveillance because they are non-antigenic or because they defend themselves successfully against immune attack have a survival advantage. Effective chemopreventative agents will include dietary components that enhance the immune system's ability to identify transformed cells and to target them for apoptosis.

Cancer: Immunosurveillance: Polysaccharides

Cancer arises through genetic alterations in stem cells that result in loss of the normal controls on proliferation, migration and apoptosis. In addition, as solid tumours become established they promote the formation of their own blood vessels to supply O_2, nutrients and growth factors, and to remove unwanted products of metabolism. An important feature of tumorigenesis is evasion of the body's systems for immunosurveillance. Each of these key processes (cell proliferation, migration, apoptosis, angiogenesis and immunosurveillance) is a rational target for dietary or pharmaceutical chemopreventative agents. Dietary components that enhance the ability of the immune system to detect and target for apoptosis cells with transformed characteristics are likely to play an important role in cancer prevention.

Epidemiology of colo-rectal cancer

Colo-rectal cancer (CRC) is the second highest cause of cancer mortality in many Western societies, accounting for 11 000 deaths annually in England and Wales, and affecting men and women equally (Department of Health, 1998). Although dominantly-inherited gene mutations are responsible for 10–15 % of CRC cases, the 15-fold range in age-standardized incidence throughout the world, together with the striking changes in incidence which accompany migration from an area of low risk to one of high risk, or vice versa, argue strongly for a major influence by environmental factors, particularly diet. This hypothesis is supported by secular changes, e.g. the fall in CRC incidence in the UK during the 1940s and 1950s attributed to the dietary changes subsequent to food rationing, and the rise in incidence in Japan since 1950 coincident with Westernization of Japanese lifestyles, including diet (Department of Health, 1998). Conventional epidemiology, however, hampered as it is by the lack of reliable data on intakes and composition of foods, is a rather blunt instrument for dissecting out which components of diet are responsible for raising or lowering CRC risk. The recent Committee on Medical Aspects of Health Policy report (Department of Health, 1998) drew the cautious conclusion that there 'is moderately consistent evidence that diets with less red and processed meat and more vegetables and fibre are associated with reduced risk of colorectal cancer'.

Colo-rectal cancer as a genetic disease

Inherited or acquired mutations in tumour suppressor genes (resulting in loss of control of cell proliferation) or in oncogenes (resulting in gain of abnormal function by otherwise normal genes) within intestinal stem cells are fundamental to the initiation and development of CRC. There is strong support for the belief that loss of function of both alleles of the tumour suppressor gene adenomatous

Abbreviations: APC, *APC*, *apc*, adenomatous polyposis coli; CRC, colo-rectal cancer.
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polyposis coli (*APC*) is the key event and that *APC* may be regarded as the gastrointestinal gatekeeper gene (Reale & Fearon, 1996). Following inactivation of *APC*, 'initiated' stem cells have a growth advantage, probably via increased intracellular concentrations of β-catenin which acts as a nuclear signal in association with the transcription factor Tcf-4 (Behrens *et al*. 1996) and, over time, acquire mutations in several other key genes including *K-RAS* and *p53*. These mutations give the tumour cells greater 'fitness' in a Darwinian sense, allowing them to ignore signals controlling growth, differentiation and migration provided by adjacent cells (Ilyas *et al*. 1999). An additional selective advantage would be failure to express tumour-specific cell-surface antigens, so making the initiated cells invisible to the immune system (see below). Mutations in one of the six genes encoding the DNA mismatch repair system are responsible for the autosomal dominant condition known as hereditary non-polyposis colon cancer which accounts for about 5 % of CRC cases (Burn *et al*. 1998). Failure to detect and repair DNA base pair mismatches, introduced by DNA polymerases during replication especially where there are nucleotide repeat sequences, increases the chance of spontaneous mutations occurring in tumour suppressor genes or proto-oncogenes. Dietary components may interact with these genetically-driven processes by altering the extent of DNA damage, augmenting or diminishing the effectiveness of DNA repair or, where repair is inappropriate, targeting of the cell for apoptosis. The recent demonstration *in vitro* that exposure to aspirin of CRC cell lines with mutations in DNA mismatch repair genes results in suppression of the mutator phenotype and selection of clones of cells with genetic stability (Rüschoff *et al*. 1998) may be a paradigm for the mechanism of action of salicylate and other dietary constituents *in vivo*.

Cancer and the immune system

The concept of immunosurveillance

Arguments over the existence of immunological surveillance mechanisms that detect and eliminate tumour cells have raged for at least 30 years. Thomas and Burnet (Burnet, 1976) proposed that Darwinian evolution must have evoked such mechanisms for the protection of long-lived higher vertebrates against DNA damage during replication and by exogenous agents. Conversely, Möller & Möller (1975) argued that there is no immunosurveillance because there is no need (in a Darwinian sense) for it. Since the development of a fully-malignant cell is a very rare event, occurring often only after reproductive age, the counter hypothesis is that there has been no selection pressure to favour the evolution of immunosurveillance (Currie, 1980). Evidence in support of the existence of immunosurveillance includes:

the presence of lymphoid infiltrates in certain tumours which suggests that the immune system 'sees' the tumour;

spontaneous regression of tumours which may be the result of immune action;

the greater occurrence of tumours in the neonatal period and in old age when immune function is less efficient (Roitt *et al*. 1993).

In most forms of immunodeficiency studied to date, however, there is little clinical evidence of increased risk of malignancy, except for tumours of the lymphoid system that may be associated with Epstein-Barr virus infection (Roitt *et al*. 1993) and Kaposi's sarcoma in patients with acquired immune deficiency syndrome. The observation that some transplantable tumours in mice failed to grow and that these anti-neoplastic mechanisms were absent in T-cell-deficient mice provided experimental evidence that a protective immune response against tumours was possible (Janeway *et al*. 1999). There is a growing list of candidate tumour-rejection antigens which are recognized by cytotoxic T lymphocytes ($CD8^+$ cells) and which include embryonic antigens and mutated oncogene and tumour suppressor gene products (Janeway *et al*. 1999). However, there are hints that the immune system may not always be beneficial in defending the body against malignancy. Stewart *et al*. (1997) reported no effect on the incidence of gastric cancer; colon cancer relative risk was increased (but not significantly), whilst there was a highly significant ($P < 0{\cdot}001$) reduction in the relative risk for rectal cancer from a survey of 73 076 men and women who were chronically immunosuppressed after heart or renal transplantation. In contrast, a well-controlled survey of renal transplant patients in the Nordic countries reported a significant (2–5-fold) excess risk of cancers of the colon, larynx, lung and bladder, and even higher excess risks for some rarer cancers (Birkeland *et al*. 1995), which suggests that the immune system confers a considerable benefit in combating tumorigenesis.

Some tumours fight back

It seems probable that many tumours, particularly perhaps in their early stages, evade the immune system because they do not express cell-surface antigens that are recognized as 'non-self'. Other tumours produce cytokines e.g. transforming growth factor-β that can inhibit immune responses (Roitt *et al*. 1993; O'Connell *et al*. 1999). Activated T-cells express Fas ligand which, when it binds to its receptor Fas, induces apoptosis of the Fas-bearing cell, e.g. a tumour cell. However, some cancer cells counter-attack by expressing Fas ligand on their cell surfaces, which is an apoptotic death signal to which activated T-cells are inherently sensitive (O'Connell *et al*. 1996; Zeytun *et al*. 1997). This Fas ligand-mediated apoptosis as a means of evasion by tumour cells appears very similar to the mechanisms underlying immune privilege in tissues such as the eye, brain and testis (Griffin & Ferguson, 1997). Fas ligand-mediated apoptosis may be responsible for the depletion of anti-tumour natural killer cells in the vicinity of tumours as part of the defence against tumour-infiltrating lymphocytes (O'Connell *et al*. 1999). Another strategy, identified in a large number of lung and colon tumours, is the production of a soluble decoy receptor (DcR3) that binds to Fas ligand and inhibits Fas ligand induced apoptosis (Pitti *et al*. 1998).

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Modulations of immune responses by dietary factors

The commonest cause of immune deficiency worldwide is malnutrition (Janeway *et al*. 1999). The fact that inadequate supply of a very wide range of micronutrients, in addition to protein and lipids, compromises immune function has long been recognized (Ferguson, 1993). More recently, the concept has arisen that varying the intake of specific nutrients, e.g. fatty acids, in adequately-nourished individuals can induce major functional changes in lymphocytes (Calder, 1998) and other elements of the immune system (Grimble, 1998) which may be of clinical significance (Belch & Muir, 1998). There is some evidence from animal studies of reduced risk of CRC with diets enriched with fish oil (Reddy & Maruyama, 1986) or certain *n-3* fatty acids (Cave, 1991; Oshima *et al*. 1995) compared with the pro-inflammatory *n-6* fatty acids. This effect may be due to altered gene expression (Fernandes *et al*. 1998). Rather less is known about the effects of specific polysaccharides on immune function, although the type and amount of polysaccharide in the diet modulates intestinal cancer risk in rodent models (Young *et al*. 1996; Zoran *et al*. 1997; Williamson *et al*. 1999). Lim *et al*. (1997) found that the type of NSP included in rat diets could have marked effects on the intestinal immune system, and they reported alterations in circulating concentrations of immunoglobulins and in $CD4^+ : CD8^+$ in mesenteric lymph node lymphocytes according to the type of NSP fed.

Dietary polysaccharides and intestinal tumorigenesis in *Min* mice

Min mice have a nonsense mutation at codon 850 in the *Apc* gene which predisposes them to development of adenomas throughout the intestinal tract (Su *et al*. 1992). These mice rarely live longer than 5 months because of the secondary effects of the tumours, including severe chronic anaemia and intestinal blockage (Moser *et al*. 1990). *Min* mice have proved to be a useful model for both fundamental studies of *Apc*-driven tumorigenesis (Shoemaker *et al*. 1997) and of modulation of adenoma formation by pharmaceutical (Jacoby *et al*. 1996) and dietary agents (Wasan *et al*. 1997; Vallance *et al*. 1999).

We are engaged in a series of studies of the influence of dietary polysaccharides on intestinal tumorigenesis and on biomarkers of risk using both *Min* and *Apc*1638N mice (Williamson *et al*. 1999); in the latter, *Apc* function has been knocked out by the introduction of a chain-termination mutation at codon 1638 (Fodde *et al*. 1994). As illustrated in Table 1, tumour multiplicity is readily manipulated by alteration of the nature of the polysaccharide component of the diet fed to *Min* mice, with more than twice the number of adenomas when the indigestible polysaccharide was guar gum than when it was cellulose. Sources of α-amylase-resistant starch gave intermediate results (Table 1). It is not yet known whether these changes in tumour multiplicity are the result of alterations in immune surveillance, but preliminary studies carried out in rats with one of the resistant starch sources suggest that this process may occur. After 34 d of feeding semi-purified diets differing only in the proportions of Hylon VII and conventional maize starch, lymphocytes isolated from the spleens of animals given 300 g Hylon VII/kg diet had reduced proliferative responses to mitogenic challenge, whilst those given 100 g Hylon VII/kg diet had an enhanced response (F Armstrong and JC Mathers, unpublished results).

Table 1. Tumour multiplicity (mean no. of tumours per animal) in *Min* mice given semi-purified diets containing 100 g test polysaccharide/kg for 3 months from weaning (CA Higgins, J Coaker, F Armstrong, L Karsai, M Bennett and JC Mathers, unpublished results)
(Mean values for twenty animals)

Polysaccharide …	Cellulose	Guar gum	Raw potato starch	Hylon VII	Pooled SEM
Small intestine	11·3	24·0*	14·6	17·6	3·40
Colon	0·26	0·70	0·33	0·24	0·14
Total	11·6	24·7*	14·9	17·8	3·64

Mean values are significantly different from those for the cellulose-containing diet: * $P<0·05$.

The extent of immune involvement in *Apc*-driven tumorigenesis is poorly understood. Dudley *et al*. (1996) crossed *Min* mice with others carrying the severe combined immunodeficiency (*scid*) mutation (which causes defective double-strand DNA repair and severe immunodeficiency) and examined intestinal tumour multiplicity. The doubly-mutant *Min/scid* animals had lower circulating concentrations of total immunoglobulin compared with *scid* animals and appeared less healthy, as judged by body mass at 3–4 months of age, but there was no detectable effect of the immunodeficiency on small-bowel polyp number (Dudley *et al*. 1996). The possibility of a novel role for the tumour suppressor gene *Apc* in modulating gastrointestinal immunity has been proposed by Fox *et al*. (1997), who observed decreased immune (serum immunoglobulin G), inflammatory and gastric hyperplastic responses to *Helicobacter* infection in *Apc*1638 mice compared with wild-type controls. This immune involvement for *Apc* may arise through its competitive binding to β-catenin (as an essential element of the wnt/Wingless signalling cascade; Wielenga *et al*. 1999) and to E-cadherin (Fox *et al*. 1997). Mice engineered to express a mutant form of cadherin lacking an extracellular domain essential for cell–cell connections had increased bacterial infiltration in their intestinal villi but a blunted mucosal immune response (Hermiston & Gordon, 1995).

Interactions with intestinal gut bacteria

The intestinal commensal flora may play an important role in influencing CRC risk through:

(1) production of carcinogens or other agents which damage colonocytes (Kleibeuker *et al*. 1996);
(2) production of short-chain fatty acids, including butyric acid, which are thought to be anti-carcinogenic (Mclntyre *et al*. 1993);
(3) modulating the immune response.

Dove *et al*. (1997) found that *Min* mice reared in a germ-free environment had 2-fold fewer ($P < 0·003$) adenomas in the medial section of the small bowel than conventional

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controls, but tumorigenesis was unaltered in the large bowel where bacterial densities are greatest in conventional mice. The authors offered no convincing explanation for this effect of the murine autochthonous flora, but observed that it was unlikely to be due to alteration of natural killer cell activity. *Min* mice were crossed with those carrying mutations at the *beige* (*bg*) locus and back-crossed with other bg^{J}/bg^{J} mice to produce an N2 population which were heterozygous for *Min* and homozygous for *bg*. Despite the deficiency in natural killer cell activity due to the homozygous *bg* mutation (Lane & Murphy, 1972), there was no significant effect on tumour multiplicity at any intestinal site, and Dove *et al*. (1997) concluded that the effects 'of diet and microbial status on the *Min* phenotype deserves further controlled study'.

Anti-neoplastic effects of butyrate

The apparent protection against CRC afforded by diets rich in 'dietary fibre' (Department of Health, 1998) may be due to the increased production of short-chain fatty acids, and particularly butyrate, with increased flow of fermentable carbohydrate to the large bowel. It is now well established that feeding wheat bran produces substantial increases in large-bowel butyrate concentration in rodent models (Walter *et al*. 1986; Cheng *et al*. 1987; McIntyre *et al*. 1993; Key & Mathers, 1993; Mathers & FotsoTagny, 1994), which may be responsible for the reduction in tumorigenesis in carcinogen-treated rats (McIntyre *et al*. 1993), although this theory is disputed in other studies (Zoran *et al*. 1997). Direct evidence for the anti-neoplastic effect of butyrate comes from a study in which butyrate was given twice daily by rectal enema to carcinogen-treated rats, resulting in substantial reductions in colonic tumour number and size (D'Argenio *et al*. 1996).

Exposure of CRC cell lines to physiological concentrations (1–5 mM) of butyrate results in suppression of proliferation, increased differentiation and increased cell death by apoptosis (Hague *et al*. 1996). When cultured in a medium containing 4 mM-butyrate, both Jurkat lymphoid and LIM 1215 colo-rectal cell lines underwent enhanced apoptosis (after a lag period of 14 h) which was strictly dependent on new protein synthesis within 10 h (Medina *et al*. 1997). This process led to conversion of the proenzyme form of caspase-3 to the catalytically-active effector protease and apoptotic death (Medina *et al*. 1997). This apoptotic response is associated with the appearance of a specific stable 90 kDa fragment of the APC protein (Browne *et al*. 1994). Recent studies by Webb *et al*. (1999) have shown that the 90 kDa fragment of APC produced by butyrate-induced apoptosis consists of an amino terminal sequence containing an intact armadillo repeat domain that is highly conserved across species from *Drosophila* spp. to human subjects and identical to that produced by the action of recombinant caspase-3. This apoptosis also results in cleavage of β-catenin, and may be responsible for the dismantling of the cytoskeletal networks which occurs in cells dying by apoptosis (Webb *et al*. 1999).

The increased differentiation and growth suppression of colon cancer cells produced by butyrate treatment appears to be mediated by histone hyperacetylation caused by inhibition of histone deacetylases (Kruh *et al*. 1994) via a protein phosphatase of unknown substrate (Cuisset *et al*. 1998). Treatment of the LS174T colon cancer cell line with 2 mM-butyrate induced dephosphorylation of the nuclear phosphoprotein retinoblastoma protein, transcription of the cyclin D-dependent kinase inhibitor p16, and cell cycle arrest at G_0/G_1 (Schwartz *et al*. 1998). Unphosphorylated retinoblastoma protein binds a number of transcription factors required for S phase progression, and phosphorylation of retinoblastoma protein in late G_0/G_1 releases a block which allows cells to progress through the cell cycle (Schwartz *et al*. 1998).

There may be important synergy between butyrate supply from the colon and the immune system which enhances the effectiveness of immunosurveillance. Concurrent treatment with both butyrate and recombinant interleukin 2 suppressed the growth of PROb colon cancer cells injected intraperitoneally into rats (Perrin *et al*. 1994). PROb cells are only weakly immunogenic, and Perrin *et al*. (1994) reported that administration of butyrate appeared to increase the immunogenicity of the cancer cells, making them more susceptible to recombinant interleukin 2-activated natural killer cells. There is recent evidence that *in vitro* several human and rat colon cancer cell lines are sensitized to Fas ligand-mediated apoptosis in the absence of a change in Fas receptor expression on the surface of the target cell (Bonnotte *et al*. 1998).

Conclusions

There is growing evidence that the immune system may exert selective pressure during neoplastic development. Transformed cells which evade immunosurveillance, for example because they do not express 'non-self' antigens on their surfaces or because they have effective defence mechanisms against immune attack, will be 'fitter' in a Darwinian sense and have a survival advantage. The mechanism of action of tamoxifen, which is the most clinically-effective chemopreventative agent to date (Fisher *et al*. 1998), may include increased immunosurveillance through increased natural killer cell densities (Berry *et al*. 1987). Dietary components that enhance the immune system's ability to identify and target for apoptosis transformed cells will have potential as effective chemopreventative agents.

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