Magnetic Microcapsule Exploration in the Gastrointestinal Cavity of the Origins of Colorectal Cancer-Associated DNA-Damaging Agents in the Human Diet

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Introduction

Because the human diet (1) and gastrointestinal (GI) sites (2) have the major nontobacco roles in human carcinogenesis, the general aim of our work has been the development and use of a system capable of selectively trapping otherwise inaccessible DNA-damaging agents in the human GI tract. To facilitate the short-term molecular identification of GI carcinogens and their dietary sources, our work has involved devising not only a means of monitoring the lower GI tract but also methods to independently test the influences of single components of human diets on (model) carcinogens within the GI cavity. The work reviewed here is the development of recoverable microencapsulated targets (3), their applications in the use of carcinogens and precursors, the first use of human diets altered systematically in colorectal (CR) cancer risk components (4,5), and an introduction to the first use of these microcapsules in humans, which is reported in detail elsewhere in this issue (6).

Since the inconclusive 1987 review by Bruce (7) on postulated determinants of CR cancer, a series of important findings have been made: a) ras oncogene activation and p53 inactivation were found, respectively, in half or most of CR cases (8); b) different acetylator status was found for CR cases (9); c) the principal fecal mutagen (fecapentaene) was demonstrated as a rodent carcinogen (10,11) and CR cases were found to have a 6-fold lower stool residual concentration of fecapentaene than controls (12); d) dietary iron and phytate, respectively, were shown to enhance or decrease 1,2-dimethylhydrazine (DMH)-induced tumorigenesis (13,14), and human feces were shown to contain very high levels of hydroxyl radical generators (15), all in accord with the hydroxyl radical hypothesis of Graf and Eaton (16); e) the protein kinase C activator diacylglycerol was found to be produced by intestinal microflora (17); and f) several studies in humans confirmed the CR-protective effects of bulking fiber, antioxidant vitamins, and calcium (18-20). The last four findings involving substances or sources passing through the GI cavity (together with classical studies on dietary/microfloral manipulation of experimental CR carcinogenesis) indicate that the GI cavity is the principal source of CR carcinogenic agents.

Although entrapment within the GI cavity could reveal substances of interest, the three following conundrums nevertheless had to be faced: a) If we do not know what types of substances are to be trapped, what type of trapping system should be used? b) Since many dietary interactions are already known, yet complete human diets typical of human everyday consumption have never been used in experimental animals, how can we quantify and control the probable (major?) influences on microcapsule trapping of model carcinogens by dietary risk factors already shown by epidemiological means? c) Since GI transit time is typically several days and dietary intervention studies require at least several weeks, how can short-term monitoring be used...
to approach long-term, multistage carcinogenesis? We are considering here our most extensive environmental exposure that occurs whether or not the individual has other lifestyle or workplace exposures, and for which highly developed techniques have long been used in clinical nutrition studies. The GI milieu requires only a few transit times to adapt to a chosen diet and to produce new agents of interest, and this must not be confused with the months of dietary intervention needed to observe changes in, say, cellular proliferation rates. Hence the briefness both of adaptation and of microcapsule exposure provides realistic opportunities to correlate the changes in what microcapsules trap with a series of systematic changes in diet; CR risk factors established already through long-term epidemiological studies and medium-term intervention studies can be used as benchmarks.

Basic Features for the Monitoring System

Excretible Microcapsules within the GI tract

The basic idea is to orally administer millions of small microcapsules that disperse within the GI tract and collectively provide a large surface area for trapping reactive substances that probably would not be excreted into the feces. Subsequent fecal excretion of the microcapsules with reactive agents trapped and protected within the microcapsule interior should preclude loss of reactive agents (Table 1) by mucosal absorption or microfloral metabolism.

Preparation and Basic Properties of Microcapsules As Traps for Alkylating Agents

Although microencapsulation has permitted the controlled release of pharmaceuticals, pesticides, and other agents through the destruction of the membrane encapsulating the active principal, we needed to produce microcapsules that are stable and selectively entrap low molecular weight substances that enter through a semipermeable membrane (3). Several major advantages are realized with a membrane that both excludes enzymes, bacteria, and macrophages, which could destroy the entrapped carcinogen, and also retains a water-soluble macromolecular target inside the microcapsules. Because most covalent-binding reactions of DNA (not necessarily those most important in causing miscoding) are on the nitrogen atoms of guanine and adenine, we used polyethyleneimine (PEI) as a first approach to a DNA surrogate. For simple use, we arranged a quick method of recovery from feces by including magnetite in the microcapsules. There is substantial art in producing microcapsules and minor changes in production conditions commonly give inexplicable and profound alterations to microcapsule batches; nevertheless, the conditions were optimized for membranes of poly (hexamethylene-terephthalalalimide) grafted onto PEI so as to enable trapping of [14C]-N-nitrosomethylurea (NMU) in vivo (21, 22).

Several key features were demonstrated during the early work: 
a) microcapsules were capable of entrapping substances up to a molecular weight of about 1000 in vitro; b) use of hemoglobin instead of PEI resulted in microcapsules that were proteolytically unstable, presumably due to hemoglobin molecules within the membrane being available to enzymic attack; c) rats showed no evidence of distress or harm from gavage treatments with the microcapsules; d) microcapsules could be recovered intact by simple magnetic means from the feces; e) the microcapsules could be broken ultrasonically after treatment in vivo with 14C-labeled NMU or methyl iodide, to show a core versus membrane distribution of PEI; f) which in turn was radically altered by changes in membrane formulation; g) microcapsules after GI transit unexpectedly became stronger (more resistant to ultrasonic rupture).

In another study (23), entrapment in vivo was shown for an IP-administered carcinogen requiring metabolic activation, dimethylhydrazine (DMH). The time course of trapping showed that microcapsules administered more than 2 hr after injection of DMH trapped relatively much less of the DMH dose presumably because they were not in position for biliary-excreted metabolites. For [14C]-NMU administered intra-cotally, only microcapsules given by gavage within the time 4 to 8 hr showed any trapping. That is, the microcapsules had to reach the rectum to be in position to intercept the electrophilic species.

| Reactant                           | In vitro | In vivo | Diet effect | Microcapsule type¹ | Assay type² | Substances               |
|-----------------------------------|----------|---------|-------------|--------------------|-------------|-------------------------|
| Direct-acting carcinogen          | x        | x       | –           | 1,2,3              | RA          | NMU, MMS, CH₃I          |
| electrophiles                     |          |         |             |                    |             |                         |
| Carcinogen with host activation   | –        | x       | x           | 1,2,3,4            | RA          | IQ, BaP, PhIP            |
| Planar structure                  | x        | x       | –           | 1,4                | RA, C       | IQ, BaP, PhIP, Glu-P-1, endogenous |
| carcinogen                        |          |         |             |                    |             |                         |
| N-nitrosating agents              | x        | x       | –           | 1,5                | TEA         | Nitrite/drinking water  |
| Cross-linking agents              | x        | x       | x           | 4,5                | M/T         | Many, endogenous        |
| Reactive oxygen species and       | x        | x       | x           | 5                  | RA          | H₂O₂, endogenous        |
| precursors                        |          |         |             |                    |             |                         |

Abbreviations: NMU, N-nitrosomethylurea; MMS, methylmethane sulfonate; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; BaP, benzo[a]pyrene; PhIP, 6-phenyl-2-amino-1-methylimidazo[4,5-f]pyridine; DMH, dimethylhydrazine; Glu-P-1, 2-amino-6-methylidipyridol[1,2-d]imidazole; endogenous, as-yet unidentified endogenous agents.

¹(x) Successful demonstration, (−) not demonstrated.
²Microcapsule types were 1, PEI; 2, poly(vinyl alcohol triethylene) (PVA-TET); 3, PVA-TET acetylated; 4, PEI CPT; and 5, [14C]-labeled PEI.
³Assay types were RA, radioactive counting; C, HPLC; TEA, thermal energy analyzer; M/T, large alteration to ratio membrane/total label distribution.
from the fast-decomposing NMU. They were still able to per-
form such trapping after hours of admixture with intestinal
contents.

A more detailed investigation (24) in trapping [14C]ben-
zeo[α]pyrene (BaP) metabolites showed that a single dose of BaP
given by gavage yielded trappable metabolites mostly in the first
48 hr. The pattern of 14C activity in an extraction sequence (A,
methanol-NH4OH desorption; B, acid hydrolysis; C, solvent ex-
traction; D, inextractable) was unlike either that from simple
mixing of microcapsules with feces containing BaP metabolites
or in vitro treatment with 7,8-dihydroxy-9,10-epoxy-7,8,9,10-
tetrahydrobenzo[α]pyrene. Of the HPLC-identified metabolites,
the 1,6- and 3,6-diones were predominant, and desorbable
metabolites were mostly unconjugated.

A further study (25) with the protein pyrolysates 2-amino-
3-methylimidazo[4,5-f]quinoline (IQ) and 6-phenyl-2-amino-1-
methylimidazo[4,5-f]pyridine (PhIP) has shown both substances
to be trapped in vivo. Thus, for all alkylating agents administered
by different routes, trapping within the GI cavity was dem-
onstrated provided that the microcapsules were present at a time
desired to trap electrophilic products could be expected to be present in
the local GI segment. The products entrapped are thereby
derived from a brief exposure period, and this feature has the poten-
tial for providing a better temporal discrimination of their
sources than, say, long-term integration by adducts on blood
proteins.

Endogenous Cross-Linking and Nitrosating
Agents

Further investigation of the surprising finding that microcap-
sules became stronger in the GI tract concluded that this was
caused by cross-linking of available PEI amines, leading to both
intramembrane reactions and core-membrane cross-linking
(26). Such effects could be produced in vivo with recognized
cross-linking agents, and with fecapentaene-12 and 4-hydro-
xyonenal, which are of interest as potential endogenous car-
cinogens. This work showed that endogenous cross-linking
agents are present extensively within the rat GI tract and were
present in both gastric and large bowel sections of chow-
consuming F344 rats. These effects were readily detected by
measuring the core-to-membrane shift of 14CH3 label in PEI
microcapsules, yielding acid-resistant cross-links in the large
bowel. A recent study has shown the presence of extensive cross-
linking agents presence in humans (6), with even larger effects
obtained by 48 hr anaerobic incubation with fecal slurries. Cross-
linking agents are inherently of considerable interest, as they
would seem to be bifunctional alkylating, membrane-penetrating
agents. Many cross-linking agents are carcinogens (27,28), able
to activate fos oncogene (29), and are particularly potent mu-
tagens in Drosophila (30). Although we are not yet able to iden-
tify them (indeed there is no other detection system for cross-
linking agents), their presence deserves further investigation.

The detection of endogenous nitrosating agents through
urinary N-nitrosoprine excretion (31) initiated many studies in
this field. Because the pKₐ of the PEI homologue piperazine
renders it much more susceptible to nitrosation than the PEI
homologue of proline (32), the nitrosation of PEI microcapsules
was studied (33). The capsules were found to be nitrosated with
a pH profile similar to amides rather than amines, which was
of interest because the major nitrosatable substrates at gastric pH
are believed to be amides (34). In vivo nitrosation was dem-
onstrated (33) and preliminary results suggest that they are much
more effective in trapping nitrosating agents than proline.

Mutagens and Carcinogens with Planar Molecular
Structures

In examining the trapping of [14C]BaP in vivo, it was found
(24) that about 25% of total radioactivity could be desorbed with
the methanol-NH4OH mixture used for extracting adsorbed
mutagens/carcinogens with a planar molecular structure from
blue cotton (35). Because blue cotton cannot be readily ingested,
we covalently modified PEI microcapsules with the copper
phthalocyanine tetrasulfonic acid moiety (CPTS) used in blue
cotton. The resulting blue-green microcapsules have shown an
enhanced propensity to reversibly absorb both BaP (36) and the
protein pyrolysates IQ, PhIP, and 2-amino-6-methylpyridol-
[1,2-ε:3,2-ε]imidazole (Glu-P-1) (25). These were shown to
have a lower net trapping of BaP and its metabolites in vitro and
in vivo, but a higher proportion of the total could be desorbed.
These results are of interest because several large groups of car-
cinogens, including some causing intestinal tumors, have planar
structures.

Effects of Ingestion of Microcapsules in Rodents

Several studies were undertaken to demonstrate lack of harm;
should be noted that the human GI tract is exposed to huge
numbers of particles of the size range of our microcapsules both
from dust on vegetables and following the mucociliary
clearance from the lungs of inhaled particles. Following five doses of
microcapsules, F344 rats showed the same mortality pattern dur-
ing 120 weeks as historical controls (4). No translocation out of
the GI tract and a 98.7% recovery of radioactivity were found for
rodents treated with radiolabeled microcapsules (37). Chronic
dosing caused no retention in GI structures as seen when examin-
ed microscopically.

Presenting DNA-Type Targets to Identify and Discriminate Important DNA-
Damaging Agents and Their Sources

In our approach to the long-standing general problem of how
to trap and determine the structure of as-yet unidentified DNA-
damaging agents (38), we considered the following three limita-
tions: a) the only highly sensitive chemical assay procedures that
can provide structural information is mass spectrometry; b)
numerous biological/biochemical assay systems can indicate that
DNA damage has taken place, but cannot specify what caused
the damage; c) it can be expected that a large proportion of DNA-
damaging agents give no tumorigenic or mutational outcome, so
that some discrimination is needed. Therefore, as our eventual
purpose is to identify dietary risk factors that must be avoided,
the choice of both target and assay techniques for substances trapp-
ed by microcapsules should provide both biological relevance
and best chemical specificity for attribution to individual
sources. Thus, two types of trapping agent or target are needed;
one of low molecular weight that can enable identification for at
least several types of adducting substance by MS and/or 32P-
Table 2. Effects of independently altered human dietary macrocomponents of fat, beef protein, and fiber nonstarch polysaccharide on microcapsule trapping, colonic nuclear aberrations, and BaP 1,6-dione trapping arising from treatment with BaP.

| Fat, beef protein, and fiber intake | C57/B6 mice |  | F344 rats |
|------------------------------------|-------------|---|---------|
|                                    | Total BaP binding by microcapsules | Colonic nuclear aberrations, % | Total BaP binding by microcapsules | 1,6-Dione trapped in microcapsules |
| LF/LB/LNSP                         | 60          | 3.60 ± 0.25 | 0.93 ± 0.41 | 3.9 ± 2.2 |
| HF/LB/LNSP                         | 65          | 1.30 ± 0.35 | ND          | ND       |
| LF/HB/LNSP                         | 86          | 2.33 ± 0.20 | 1.04 ± 0.10 | 13.3 ± 8.8 |
| LF/LB/HNSP                         | 56          | 1.87 ± 0.31 | 0.59 ± 0.11 | 3.6 ± 2.4  |
| LF/HB/HNSP                         | ND          | ND          | 0.97 ± 0.02 | 17.2 ± 1.6 |
| Control                            | ND          | 0.45 ± 0.09f | 0.24 ± 0.01g | 2.3 ± 1.8f |

Abbreviations: BaP, benzo[a]pyrene; H, high; L, low; F, fat; B, beef protein; NSP, nonstarch polysaccharide; ND, not determined.

*Rodents treated per os with [14C]BaP.

*Expressed as pmole BaP per million microcapsules. C57/B6 mice data from pooled samples (six per group).

*Colonial nuclear aberration included apoptotic cells and micronuclei.

*Expressed as pmole BaP 1,6-dione per million microcapsules.

*Control mice consumed LF/LB/LNSP diet but were not treated with BaP.

*Expressed as pmole BaP 1,6-dione per million microcapsules.

postlabeling, etc., and a second consisting of DNA sequences, e.g., ras and p53 oncogene sections (8) for which damage is known to be critical in human GI carcinogenesis.

Both types of target would be damaged during the vigorous acylation reactions used in forming the microcapsule membrane. An approach adopted for the first target type (low molecular weight) is to insert it into the microcapsules after membrane formation. Preliminary work with guanosine-3'phosphate coupled by carbodiimide into PEI microcapsules (Ciroussel et al., unpublished data) showed, however, that such target-attached microcapsules yielded virtually no N7-methylguanine after treatment with [14CH3]labeled NMU or methylmethane sulfonate (MMS). It appeared that the PEI microcapsule nucleophilicity, while so effective in trapping carcinogen electrophiles, was too effective in shielding this target. Consequently, we lowered the residual nucleophilicity of the microcapsule structure (to ~ 1% of PEI structures) by using a poly(vinyl alcohol)-triethylenetetramine structure and then blocking most of the amino functions (39).

A better target substance (I) has been synthesized (38). While representing the most reactive DNA site dG deoxyribose is replaced by a selectively cleavable diol linker moiety to avoid extensive depurination and/or phosphate hydrolysis with consequent loss of adducted guanine. This has been shown to react with NMU at a rate and giving O7/N7 product distribution similar to dG (McGinnis et al., unpublished data). Sodium periodate cleavage of the target linker, followed by NaBH4 reduction to give 9-hydroxyethylguanine has been shown and mass spectrometric assay of this cleaved target and several adducts demonstrated (Farmer et al. unpublished data).

With the components of the first target system in place, another task is to incorporate DNA or polynucleotides into microcapsules to provide the second target type. Encouraging progress is being made on this task. The problem of intragastric hydrolytic damage can be avoided by using enteric-coated capsules to contain the microcapsules. Such procedures may provide specific duodenal release or colonic release (40), and thereby some discrimination of the GI segment in which microcapsule trapping has occurred.

Microcapsule Trapping and Dietary Influences within the Intestinal Milieu

Although many studies have examined substances of potential carcinogenic significance in the GI cavity and the complex interactions therein, there is still the problem that altering one component of diet or microflora usually has complex and mostly unknowable consequences to which an observed effect of tumorigenesis or fecal excretion may not be directly related. A second problem is that experimental work within GI milieu derived from commercial rodent diets produces results totally unrelated to human consumption and which could be related neither to the established dietary etiology of human CR carcinogenesis nor later to human studies. Hence, diets of human foods were designed to provide all eight permutations (4; Cummings et al., submitted) of independent, 3-fold alterations in daily intakes of the three established CR risk-altering macrocomponents, namely, fat (42), beef protein (42), and dietary fiber nonstarch polysaccharide (NSP; 43).

The set of four low-fat human diets (for which available calorific intake was based on UK human food tables) were found to be well accepted and to give the same bodyweight increases in F344 rats, irrespective of fiber or beef protein changes (Cummins et al., submitted). Almost every biochemical parameter measured having relevance to carcinogenesis was greatly different from those in a chow control group (5). [14C]BaP was given by gavage, and a set of systematic alterations by fiber and beef protein was found (Table 2) for a) average trapping in microcapsules (three doses given in 48 hr), b) the disposition of 14C between cavity contents and microcapsules, and c) the pattern of microcapsule-trapped metabolites as assayed by HPLC (4, 5). A second study with the corresponding high-fat set (Cummins et al., submitted) in F344 rats showed some differences that may be attributable to a fat-fiber interaction. Overall, 3-fold alterations of intake within the normal human range caused 2- to 3-fold alterations in several parameters potentially related to the effective exposure within the GI tract. The specific trapping on microcapsules of total BaP metabolites (nmole per million) was shown to be inversely related to the 3-day fecal weight, w
(R=0.81), as one might expect from dilution of intestinal contents (25; Cummings et al., submitted). In turn, w was principally dominated by fiber intake (in accord with one hypothesis on fiber action) with w(g) = 0.37 + 0.81 (NSP) + 0.27 (beef protein). These studies of the rodent intestinal milieu have yielded semi-quantitative effects on microcapsule trapping by altering epidemiologically established dietary factors within the normal human intake range. These results indicate that such dietary factors must be controlled in seeking sources of as-yet unidentified, endogenous DNA-damaging agents.

A third study with [14C]BaP in C57/B6 mice (44) did not show such striking differences in microcapsule trapping at 24 hr after gavage, the time chosen for sacrifice in order to measure colonic nuclear alterations (Table 2). These showed large diet-dependent differences, as did the pattern of BaP metabolites extracted from the microcapsules (44), but the small fraction of BaP dose present in feces (24–39%) gives rise to caution about relative effects of the first-pass BaP metabolites versus those transiting later after duodenal absorption and enterohepatic circulation and metabolism. Dietary influences on the pharmacodynamics of BaP or other carcinogens have influenced the diet dependence of both the effective mucosal exposure and this snapshot of microcapsule trapping. Further work (Inaugurant et al., unpublished data) was undertaken to check the dependence on time, GI location, and diet of microcapsule and mucosal DNA adduct formation by [14C]BaP in F344 rats. As the microcapsules are moved by peristalsis through successive GI locations, and appear in feces, HPLC assay of extracts from microcapsules (Fig. 1) showed that BaP products within the microcapsules are progressively altered. The proportion of desorbable products decreased from 81 to 26% on passing from stomach to feces. Adduct formation within the large bowel was measured at 24 hr after gavage and the mucosal DNA adducts and those formed within microcapsules recovered from the contents of the cavity showed good correlation (r = 0.86, p < 0.005).

Diet dependence of microcapsule trapping of both total and individual metabolites has also been shown for [14C]IQ given per os (25; Turesky, unpublished data). Thus, for an ingested carcinogen, there seems little doubt that carcinogen trapping by microcapsules is strongly influenced by the close proximity to biological events and dietary influences that determine eventual DNA damage in the CR mucosa.
Studies in Humans

With total control of all the dietary consumptions and excretions of volunteers resident in a clinical nutrition suite, our first objective has been the study of the systematic alteration in levels of a sequence of dietary components before proceeding to the comparison of high versus low risk populations. The first study (6) showed lack of ill effects from microcapsules and extensive effects as apparently arising from endogenous radical oxidant precursors and cross-linking agents. Our second study is examining the effects of fiber NSP and grilled beef on several microcapsule features discussed earlier. In view of high risk for neoplasms there, we aim also to study the upper GI tract through which microcapsules move rapidly, and the required technical developments are being considered. Separation of effects from microcapsules from exposures in the upper and lower GI tract is currently being studied. A further requirement for microcapsule validation that seems attainable is the use of ethically acceptable indicators of short-term mucosal changes. Currently we can use several microcapsule end points, and we have an exceptional degree of long-term dietary control, without which human studies may be useless. We should be able to extrapolate effects of consumption of the same diets between humans and rodents in which model carcinogens can be used.

Conclusions

For a number of reasons, biomonitoring the GI tract should have importance for the etiology of GI and perhaps other cancers. After overcoming most of the technical problems with microcapsules, we have shown that microcapsules provide trapping of covalently bound and/or desorbable metabolites for all model carcinogens used. Several different end points were found. In conjunction with magnetically maneuverable substrates that permit easy recovery, clean-up, and total distinction from any endogenous substrate in the GI tract, these microcapsules provide a number of advantages. Manipulation of established dietary risk factors produces substantial alterations in trapping, as might be expected from the as-yet-unknown assumption that cavity levels of carcinogen electrophiles will directly alter GI cancer risk.

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