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Growth Kinetics and Chemoprevention of Aberrant Crypts in the Rat Colon

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Abstract Single and multiple colonic crypts exhibiting dysplasia that are detectable *in situ* by staining of rat colon with methylene blue are called aberrant crypts (AC) and may serve as an intermediate marker for colon cancer. In a characterization study, we have established the kinetics of AC growth and development over a period of 20 d following injection of rats with the carcinogen azoxymethane (AOM). AC are not present at 5 d post-injection, but are a constant feature at 10 d and thereafter. Multiple AC, presumably clonal, begin to evolve at 10 d and are consistent by 20 d, forming incipient microadenomata. We have examined 20 candidate chemopreventive agents for inhibition of AC. All agents were given in AIN-76 diet, at two dose levels, with injections of AOM. AC were measured after 5 weeks of growth. Among the most active AC-inhibiting agents were BHA, DFMO, quercetin, diallyl sulfide, 18ß-glycyrrhetinic acid, and ascorbyl palmitate. In a post-initiation study, the differentiating agent sodium butyrate was ineffective, but piroxicam was highly effective in modulating AC growth. Further, piroxicam inhibited AC development at all stages of growth from single to polycryptal clusters of AC. The AC assay shows marked sensitivity and specificity for screening agents for chemoprevention of colon cancer.

Key words: aberrant crypts, azoxymethane, chemoprevention, colorectal cancer, intermediate biomarker, NSAID, piroxicam

Intermediate biomarkers of colon cancer risk are now a commonalty in colon cancer prevention trials. For the most part, the development of these markers, now in clinical use, began with experimental studies in rodents. In the colon the first generation of intermediate biomarkers have focussed on aspects of cellular proliferation. Markers that effectively highlight the S phase of the cell cycle or the actively cycling population have been used to assess cancer risk and efficacy of agents that prevent cancer. With use of these markers limitations have become clear and proliferation markers have been criticized as lacking specificity for the cancer process. Recently an assay describing single colonic crypts evidencing hallmarks of dysplasia has been reported and these lesions have been termed "aberrant crypts". This article will illustrate the use of the aberrant crypt bioassay in the rat colon. The assay is a very effective method for screening the activity of putative chemopreventive agents.

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ABERRANT COLONIC CRYPTS

Preneoplastic lesions have long been noted in experimentally induced colon carcinogenesis [1,2]. In one of the first reports of the detection of aberrant crypts in carcinogen-induced colon mucosa Bird [3] described crypts of "increased size, thicker epithelial lining, and increased pericryptal zones" in freshly stained rat colon with the vital dye

methylene blue. Aberrant crypts were found to be induced very specifically by carcinogens that predominantly produce colonic tumors, thus validating them as precursors for colon cancer. Dietary factors such as pyrrolyzed protein and carbohydrates have been shown to induce aberrant crypts [4]. Aberrant crypt formation and development is only now being studied in detail. However, limited studies in humans have suggested that aberrant crypts are putative precursor lesions from which adenomas and carcinomas in the colon will develop [5,6]. Aberrant crypts exhibit many of the hallmarks of dysplasia: 1) they are basophilic upon staining, 2) they are enlarged and dilated compared to normal colonic crypts, 3) nuclei are enlarged and non-polar in orientation, 4) they exhibit increased and abnormally located mitotic activity. Ultimately careful cytokinetic, molecular, and biochemical studies will characterize the biology of aberrant crypts and define their role in the etiology of colon carcinogenesis.

ABERRANT CRYPT FOCI: A BIOASSAY FOR CHEMOPREVENTION EFFICACY

Together with the Chemoprevention Branch of the National Cancer Institute we have been engaged in the determination of chemopreventive efficacy of a number of naturally occurring phytochemicals, vitamins, minerals, anti-inflammatory drugs, antihelminthic drugs, and differentiation agents in the aberrant crypt bioassay. These agents have been selected through a comprehensive literature review where some cancer preventive effects were known, or where the candidate agent may have shown promise when examined in a number of in vitro pre-screening assays. Typical in vitro assays have included inhibition of carcinogen-induced cell transformation of epithelial cells, anchorage independent growth of mammalian cells in soft agar, and calcium intolerance assays [7-9]. In some cases initial toxicological evaluation data may be known allowing some approximation of in vivo tolerance to

TABLE 1. Compounds Tested for Inhibition of Aberrant Crypt Foci

Anti-inflammatories/Analgesics	β-glucuronidase inhbitor	Others
piroxicam	potassium glucarate	silymarin
sulfasalazine	calcium glucarate	arginine
ibuprofen	β-sitosterol	purpurin
ketoprofen		d-mannitol
indomethacin		cromolyn Na
Anti-helminthics	**Phenolic Antioxidants**	**Vitamins**
levamisole	ellagic acid	ascorbyl palmitate
oltipraz	rutin	follic acid
	propyl gallate	vitamin D_3
Organosulfur Compounds	BHA	
diallyl sulfide	curcumin	**Epicatechins**
sodium thiosulfate	quercetin	± catechin
mesna	nordihydroguaiaretic acid	
Minerals	**Indoles/Isothio-Compounds**	**Differentiation agents**
sodium selenite	benzyl isothiocyanate	dihydroepiandrosterone
sodium molybdate	indole-3-carbinol	sodium butyrate
	phenylethylisothiocyanate	18 β-glycyrrhetinic acid
		fluocinolone acetonide
		inositol hexaphosphate

the proposed agent. A list of agents under study are shown in Table 1. Several compounds undergoing examination in the aberrant crypt assay have proven chemopreventive activity as shown by efficacy in animal tumorigenesis assays. The most notable of these is the synthetic food additive butylated hydroxyanisole (BHA) and difluoromethylornithine (DFMO), the ornithine decarboxylase inhibitor [10,11]. As will be seen, these compounds are also effective inhibitors of aberrant crypt formation. The protocol we have employed involves the use of the F344 rat. Young rats are introduced to an AIN-76A semi-synthetic diet containing the test inhibitor at 40% or 80% of the maximum tolerated dose. These diets are fed for one week prior to receiving two injections of AOM one week apart. At the end of 5 weeks of experimentation the animals are sacrificed and the colons are assayed for frequency of aberrant crypts by staining them with methylene blue. In general, we find a frequency of 80-140 aberrant foci when two weekly injections of azoxymethane at a dose of 15 mg/kg are given and representative crypts are isolated so that dysplasia can be confirmed in them. Typical results for BHA and DFMO in aberrant crypt bioassay are shown in Figure 1. To date, we have screened over 29 natural and synthetic compounds for their chemopreventive efficacy in the assay. As a class the most effective compounds are the non-steroidal anti-inflammatory agents which include ibuprofen, ketoprofen, piroxicam, and indomethacin. Some of these agents have already proven effective in longer term experiments [12,13].

KINETICS OF ABERRANT CRYPT GROWTH

Recently we have performed several studies of the nature of aberrant crypt growth. Foci of aberrant crypts appear in the rat colon within five days of treatment with two injections of a colon carcinogen. After eight weeks of growth hundreds of foci are present in the colon and multiple aggregates of dysplastic crypts are noted. We have used this observation to test a new variation of the standard protocol for chemoprevention efficacy testing. We have begun to examine the post-initiation effects of differentiation agents in the colon. The assay endpoints, however, in addition to a measure of total aberrant crypt incidence, also contain an index of inhibition of aberrant crypt clonal expansion. In other experiments (to be reported elsewhere) we have determined that piroxicam is a highly effective compound with pronounced effects on aberrant crypt development several weeks after these lesions have become established. Surprisingly, we have also found that sodium butyrate, tested in this protocol, is *not* an effective suppressor of aberrant crypt

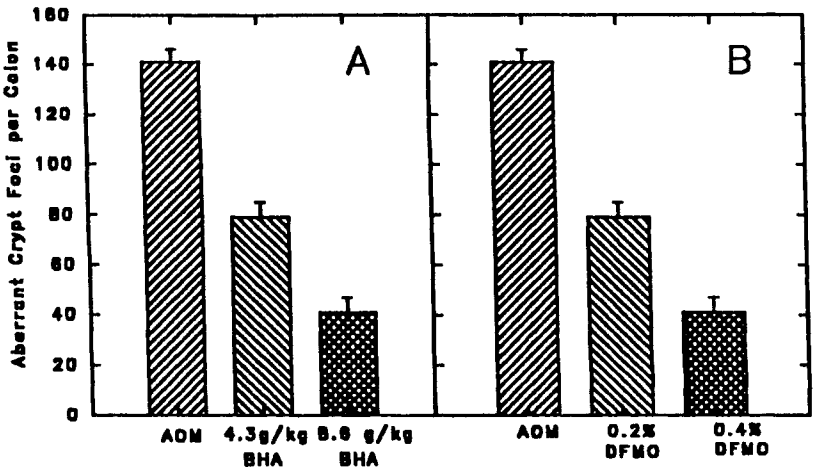

Fig. I. Inhibition of azoxymethane-induced aberrant crypt formation in the F344 rat colon by dietary BHA (A) or DFMO (B). Compounds were fed g/kg diet for (A) and as a percentage of diet for (B).

development and previous studies of the effects of butyrate in vivo have been also been equivocal in this regard.

SUMMARY AND CONCLUSIONS

As implied earlier aberrant crypts have appeal as an intermediate marker for colon cancer for two reasons. One, they harbor elements satisifying the curiosity of those who study the causes of colon cancer in that elements in diet appear to affect growth and expansion of these lesions and agents thought to be cancer preventive inhibit their development. Second, the hallmarks of neoplastic progression, as evidenced by dysplastic changes, satisify the pathologist seeking early expression of cancer in precursor lesions for colon cancer with recognizable elements retained in the tumors that will become clinical entities. The recent finding that aberrant crypts are detectable in the human colon from subjects at increased risk for colon cancer make the study of these interesting lesions all the more promising. With isolation of aberrant crypts it may be possible to discover the molecular signals underlying the dysregulation of epithelial cell transformation in the colon. Attenutation of these signals may ultimately lead to avenues of prevention and treatment for this virulent neoplastic disease.

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