Mutation Research, 164 (1986) 209–215
Elsevier

MTR 08616

An in vivo assay for small intestine genotoxicity

M.T. Goldberg * and P. Chidiac

Department of Biomedical Sciences, University of Guelph, Guelph, Ont. N1G 2W1 (Canada)

(Received 28 November 1985)
(Revision received 18 February 1986)
(Accepted 25 February 1986)

Summary

The induction of nuclear aberrations (NA) (apoptotic bodies and micronuclei) in duodenal crypts in a dose-dependent manner was associated with administration of agents known to induce tumours in the small intestine. These included X-irradiation, *N*-methyl-*N*-nitrosourea (MNU), benzo[*a*]pyrene (B[*a*]P), and 1,2-dimethylhydrazine (DMH), which were found to induce NA in cells in the proliferative region of crypts 24 h after they were given to mice. Methylurea (MU) and benzo[*e*]pyrene (B[*e*]P), which are non-carcinogenic structural analogues of MNU and B[*a*]P, respectively, did not induce NA under similar conditions. Based on these results, the ability of an agent to induce NA in the small intestine appears to reflect of its oncogenic potential in that organ.

Although tumours of the gastrointestinal tract are among the leading types of fatal cancers in humans, the small intestine is one of the organs of the body least likely to develop cancer (Lowenfels, 1973). It is unclear why so few tumours are seen in the small intestine compared with the colon and stomach. Reasons generally given to explain the paucity of tumours in the small intestine include the relative lack of flora compared with the colon, and the rapid nature of small-intestinal transit and fluid nature of small bowel contents, which would tend to reduce exposure to carcinogens (Lowenfels, 1973). It is also possible that the small intestine in humans has less ability than the large intestine to activate carcinogens metabolically.

Virtually all agents used experimentally to induce tumours in the small intestine also cause tumours in other parts of the alimentary tract, such as the colon (IARC, 1979). Small intestine carcinogens include X-irradiation (IARC, 1979), 1,2-dimethylhydrazine (DMH) (Toth and Patil, 1983), *N*-methyl-*N*-nitrosourea (MNU) (IARC, 1972), and benzo[*a*]pyrene (Chu and Malmgren, 1965).

Nuclear aberrations (NA), which include apoptotic bodies and micronuclei, are caused by carcinogenic agents in a variety of tissues (Wyllie et al., 1980; Ronen and Heddle, 1984). In the colonic epithelium of the mouse, NA have been shown to be an organ-specific indicator of colon carcinogens (Goldberg et al., 1983; Wargovich et al., 1983), with all colon carcinogens tested causing a dose-related increase in NA, and all non-carcinogens and all non-colon carcinogens, with the exception of benzo[*a*]pyrene, having no observable effect on NA incidence (Wargovich et al., 1983).

This study was undertaken to find out whether such a relationship exists between the carcinogen-

* To whom correspondence should be addressed.

icity of an agent in the small intestine and its ability to induce NA in small intestinal epithelium.

Materials and method

Animals

Male mice of an outbred strain (CD1, Charles River, Canada) and an inbred strain (C57Bl/6J, Jackson Laboratories, Bar Harbor, ME) aged 6–10 weeks were kept in groups of 5 or 6 and maintained on a 12-h light/dark cycle until they were used for experiments.

Mice were allowed Purina laboratory chow and tap water ad libitum.

Preparation of chemicals

Benzo[*a*]pyrene (B[*a*]P) (Sigma Chemical Co., St. Louis, MO) was dissolved in dimethyl sulphoxide (BDH Chemical Co., Toronto, Canada). Benzo[*e*]pyrene (B[*e*]P) (Sigma Chemical Co., St. Louis, MO), *N*-methyl-*N*-nitrosourea (MNU) (Sigma Chemical Co., St. Louis, MO) and methylurea (MU) (Aldrich Chemical Co., Milwaukee, WI) were dissolved in dimethyl sulphoxide and diluted 1 : 1 with distilled water. 1,2-Dimethylhydrazine (DMH) (Sigma Chemical Co., St. Louis, MO) was dissolved in 1 mM EDTA (Fisher Scientific Co., Toronto, Canada) and the pH was adjusted to 6.1–6.9 using 1 N NaOH.

Treatments

All animal treatments were carried out between 10:00 a.m. and noon, after animals had been deprived of food overnight, except the animals that were irradiated, which were not starved before treatment. Unless otherwise stated. Chemicals were administered by gavage through a blunt 17- or 18-gauge stainless steel needle. DMH was injected intraperitoneally. Animals were weighed before treatment and given the appropriate dosage in 0.01 ml vehicle/g body weight. Animals were killed by cervical dislocation 24 h after treatment and dissected immediately afterwards.

Preparation of tissues

Duodenums were excised, rinsed in and flushed with Krebs (phosphate-buffered saline) solution. They were then cut lengthwise, 'Swiss-rolled' from the pyloric end, fixed, sectioned and stained for light microscopy using the DNA-specific Feulgen technique and counterstained with fast green (Goldberg et al., 1983).

Scoring of slides

Slides were coded with random numbers and scored blind to treatment. Sections were examined for aberrant nuclei (i.e., micronuclei, pyknotic nuclei, karyorrhectic bodies, vacuolated bodies). 10 crypts were scored per animal, starting as near the stomach end of the roll as possible, and moving distally. Only completely cross-sectioned crypts were scored. Initially, results were expressed on the basis of percentage of aberrant cells in a sample of 500 cells/mouse. Subsequently, it was determined that the number of cells per crypt follows a normal distribution.

Analysis of data

For each animal, the mean number of NA/crypt was calculated The results of 5 or 6 animals per experimental group were pooled. Groups were compared using one-way analysis of variance, and $p < 0.01$ was taken to be the level of statistical significance. Correlation coefficients (r values) were calculated where a dose–response effect was apparent.

Results

X-irradiation

Increases in NA in duodenal crypts were observed 24 h after whole-body X-irradiation of CD1 mice ($F = 55.12$, $df = 5, 47$, $p < 0.01$). A dose–response effect ($r = 0.89$) was apparent up to 200 rad (Fig. 1).

Benzo[a]pyrene and benzo[e]pyrene

Administration of B[*a*]P to C57Bl/6J mice was associated with a significant ($F = 16.83$, $df = 3, 15$, $p < 0.01$) dose-related increase in NA/crypt ($r = 0.87$). B[*e*]P did not induce NA (Fig. 2).

N-Methyl-N-nitrosourea and methylurea

Administration of MNU to C57Bl/6J mice led to a statistically significant increase in NA ($F = 23.48$, $df = 7, 39$, $p < 0.01$) in duodenal crypts. This effect was dose-related ($r = 0.83$ up to 10 mg/kg). MU did not induce NA (Fig. 3).

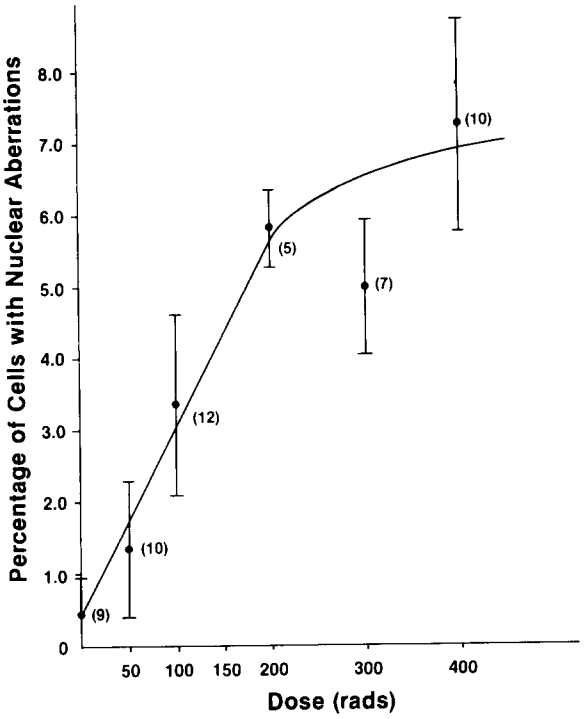

Fig. 1. Effect of X-irradiation on incidence of nuclear aberrations (±S.D.) in mouse small intestine epithelial cells. Number of observations per dose shown in parentheses.

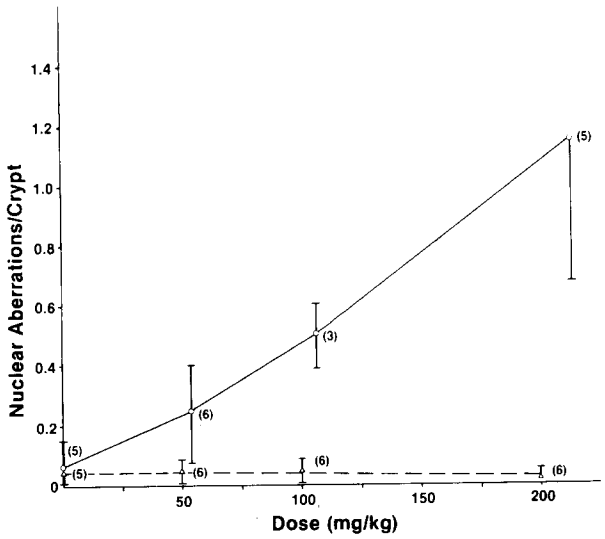

Fig. 2. Effect of benzo[*a*]pyrene (B[*a*]P) (solid line) and benzo[*e*]pyrene (B[*e*]P) (broken line) on incidence of nuclear aberrations (±S.D.) in mouse small intestine. Number of observations per dose shown in parentheses.

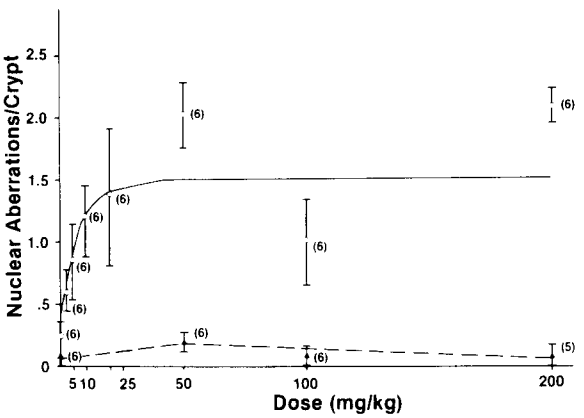

Fig. 3. Effect of *N*-nitroso-*N*-methylurea (MNU) (solid line) and methylurea (MU) (broken line) on incidence of nuclear aberrations (±S.D.) in mouse small intestine. Number of observations per dose shown in parentheses.

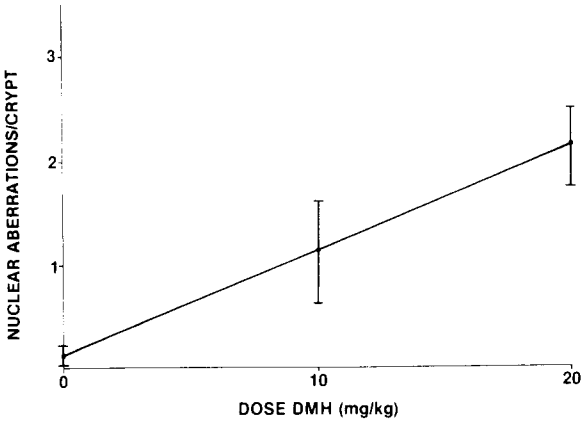

Fig. 4. Effect of 1,2-dimethylhydrazine (DMH) on incidence of nuclear aberrations (±S.D.) in mouse small intestine. $n = 6$ observations per group.

1,2-Dimethylhydrazine

Intraperitoneal injection of DMH to CD1 mice was associated with a significant increase in NA ($F = 31.46$, $df = 2, 12$, $p < 0.01$). The incidence of NA increased as the dose of DMH increased ($r = 0.92$) (Fig. 4).

Discussion

NA were induced in a dose-dependent manner in epithelial cells of the duodenum by X-irradiation, B[*a*]P, MNU and DMH, all of which are known to be carcinogenic to the small intestine

(Chu and Malmgren, 1965; IARC, 1972, 1979; Rostom et al., 1978; Toth and Patil, 1983). MU, a non-carcinogenic structural analogue of MNU, did not induce NA in duodenal epithelium. B[*e*]P, a non-carcinogenic isomer of B[*a*]P, also failed to induce NA in the present study. From these results it appears that the ability of an agent to induce NA is indicative of its carcinogenic potential in the small intestine.

Pilot studies indicated that 24 h was a suitable time to allow NA to appear: With MNU, NA incidence was found to reach a maximum between 20 and 30 h after treatment, while with B[*a*]P, peaks to NA incidence were observed at 24 and 36 h. This is in agreement with previous studies of the time course of appearance of NA in the colonic epithelium (Maskens, 1979; Goldberg et al., 1983).

The relationship between the oncogenicity of an agent in a given organ and its ability to induce NA or micronuclei at the same site has already been established in other organs, including the colon (Wargovich et al., 1983), the liver (Tates et al., 1980), the oral mucosa (Stich et al., 1982), the bladder (Nolte and Goldberg, 1984), the hair follicle (Goldberg and Goldberg, 1983) and the breast epithelium (Sharkey and Bruce, 1985).

The results of this study are in agreement with a number of previous studies. The induction of NA (or apoptotic bodies) in the epithelium of the small intestine after X-irradiation or other types of ionizing radiation has been reported by a number of investigators (Potten, 1977, 1983; Hendry and Potten, 1982; Hendry et al., 1982; Duncan et al., 1983; Ijiri and Potten, 1983; Ronen et al., 1983; Ronen and Heddle, 1984). DMH and MNU have also been shown previously to induce NA in the small intestine (Ronen et al., 1983; Ronen and Heddle, 1984).

Wargovich et al. (1983) evaluated 19 agents in the colon of the mouse for ability to induce NA. All 5 colon carcinogens tested (including DMH and MNU) led to a dose-related increase in NA in proliferating crypts cell populations. All 6 non-carcinogens tested (including B[*e*]P and MU) were negative in the colonic NA assay (Wargovich et al., 1983). Benzo[*a*]pyrene, which is not known to induce tumours in the colon, was positive in the colonic NA assay (Wargovich et al., 1983). The authors suggested that the colonic NA assay could be more sensitive to the genotoxic effects of B[*a*]P than the cancer bioassays (Wargovich et al., 1983). B[*a*]P, which induced NA in the small intestine in the present study, is not considered a small intestine carcinogen in mice (IARC, 1973), but it has been reported to induce carcinomas in this tissue in hamsters (Chu and Malmgren, 1965).

Whereas micronuclei are known to arise from chromosomal fragments or chromosomes that are not included in the daughter nuclei at cell division (Heddle et al., 1984), the sequence of cellular events in the process of apoptosis is not fully understood (Wyllie, 1981). Apoptotic bodies made up the majority of NA observed in this study. This is in agreement with previous results (Ronen and Heddle, 1984).

Sister-chromatid exchange in the small intestinal epithelium has also been developed as an assay of genotoxicity (Neal and Probst, 1984; Blakey, 1985). However, the nuclear aberration assay has two advantages over SCE analysis; the speed with results can be obtained and the inclusion of apoptotic endpoints. Apoptosis is indicative of cytotoxicity and for this reason is an important aspect of the nuclear aberration assay. Cell death in rapidly dividing intestinal cell populations induces a subsequent burst of hyperplasia among surviving cells (Maskens, 1979) and this may be necessary to induce permanent alterations in the DNA sequence. There are many reports in the literature that suggest cell proliferation is a prerequisite for initiation of carcinogenesis (Pound, 1968; Warwick, 1971; Scribner and Susa, 1978; Columbano et al., 1981; Farber, 1982). Thus, apoptosis in the nuclear aberration assay serves as an indicator of the extent of cytotoxicity and, by implication, the ability of the agent tested to induce post-apoptotic regeneration.

A variety of chemicals that are not considered small intestine carcinogens induce apoptosis in crypt cells. Agents which are carcinogenic to organs other than the small intestine, including cyclophosphamide, nitrogen mustard, actinomycin D, mitomycin C, and hydroxyurea (IARC, 1974; Weisburger, 1977; Walker and Anver, 1983; Manoharan, 1984), have been reported to induce apoptosis in small intestinal crypt cells (Philips and Sternberg, 1975; Searle et al., 1975; Ijiri and

Potten, 1983). Thus, these agents might be construed as false positives in the NA assay for small intestine carcinogens. Several agents which are genotoxic but not carcinogenic, including adriamycin (IARC, 1976), 5-fluorouracil (IARC, 1981), and isopropyl methanesulphonate (Seiler, 1977; Snyder and Regano, 1982) have also been reported to induce apoptosis in the small intestine (Ijiri and Potten, 1983). Cytosine arabinoside, vincristine, colchicine and cycloheximide induce apoptosis in the small intestine (Dinsdale, 1975; Searle et al., 1975; Ijiri and Potten, 1983), although these agents are not considered oncogenic or genotoxic (Weisburger, 1977; Duncan et al., 1985). Cytosine arabinoside is a specific inhibitor of DNA synthesis and repair (Filatov and Noskin, 1983), while vincristine and colchicine, both mitotic spindle poisons, and cycloheximide, an inhibitor of protein synthesis, interfere with the process of mitosis (Dinsdale, 1975; Sentein, 1981; Hart and Hartley-Asp, 1983). Cytosine arabinoside does not induce NA in the colon (Wargovich et al., 1983). Apoptosis is induced in crypts of the small intestine by small intestine carcinogens, as well as by some chemicals which are not known to induce tumours there. All of these agents, however, may be capable of disrupting the genome of a cell, either directly or indirectly. Thus, it is possible that apoptosis is caused by a change in the normal genetic makeup of a cell.

It has been proposed that the small intestine has an inherent ability to resist the formation and spread of neoplasia (Lowenfels, 1973). This may be partly attributable to rapid rate of cell turn-over in the small intestine (LeBlond and Messier, 1958), making it capable of efficiently deleting cells that have undergone genetic alterations which otherwise might eventually lead to tumour formation.

Previous studies have shown that a positive correlation exists in several mammalian organs between the tumour-inducing and NA-inducing abilities of a given agent (Goldberg and Goldberg, 1983; Wargovich et al., 1983; Nolte and Goldberg, 1984; Sharkey and Bruce, 1985). In the present study, small intestine carcinogens led to dose-related increases in NA in crypt cells of the small intestine, while non-carcinogenic structural analogues of intestinal carcinogens were not associated with any change in NA incidence. Thus, the NA assay as applied to the small intestine should be of use in detecting small intestine carcinogens. A review of the literature indicates, however, that a substantial number of false positive results might be expected, since the assay is sensitive not only to carcinogens, but to many agents which disrupt the integrity of the genome.

Acknowledgements

The authors wish to thank Rhonda Schop and Robert Braham for technical assistance.

This work was supported by grants from the Ontario Ministry of the Environment's Pesticides Advisory Committee and from the National Cancer Institute of Canada.

References

Blakey, D.H. (1985) Sister chromatid exchange analysis in the colonic and small intestinal epithelium of the mouse, Cancer Lett., 25, 299–305.

Chu, E.W., and R.A. Malmgren (1965) An inhibitory effect of vitamin A on the induction of tumors of the forestomach and cervix in the Syrian hamster by carcinogenic polycyclic hydrocarbons, Cancer Res., 25, 884–895.

Columbano, A., S. Rajalakshmi and D.S.R. Sarma (1981) Requirement of cell proliferation of liver carcinogenesis as assayed by three different procedures, Cancer Res., 41, 2079–2083.

Dinsdale, D. (1975) Colchicine-induced lesions in the rat duodenum, Pathol. Eur., 10, 95–104.

Duncan, A.M.V., A. Ronen and D.H. Blakey (1983) Diurnal variation in the response of gamma-ray-induced apoptosis in the mouse intestinal epithelium, Cancer Lett., 21, 163–166.

Duncan, A.M.V., J.A. Heddle and D.H. Blakey (1985) Mechanism of induction of nuclear anomalies by gamma-radiation in the colonic epithelium of the mouse, Cancer Res., 45, 250–252.

Farber, E. (1982) Chemical carcinogenesis-A biological perspective, Am. J. Pathol., 106, 270–296.

Filatov, M.V., and L.A. Noskin (1983) Sensitization of human cells by inhibitors of DNA synthesis following the action of DNA-damaging agents, Mutation Res., 110, 393–399.

Goldberg, M.T., and E.A. Goldberg (1983) A novel in vivo assay for detecting topically applied genotoxins, in: Symposium on Toxic Substances in the Environment II, Kingston, Ont. (abstr.).

Goldberg, M.T., D.H. Blakey and W.R. Bruce (1983) Comparison of the effects of 1,2-dimethylhydrazine and cyclophosphamide on micronucleus incidence in bone marrow and colon, Mutation Res., 109, 91–98.

Hart, J.W., and B. Hartley-Asp (1983) Induction of micronuclei in the mouse, Revised timing of the final stage of erythropoiesis, Mutation Res., 120, 127–132.

Heddle, J.A., E. Stuart and M.F. Salamone (1984) The bone marrow micronucleus test, in: B.J. Kilbey, M. Legator, W. Nichols and C. Ramel (Eds.), Handbook of Mutagenicity Testing Procedures, 2nd Edn., Elsevier, Amsterdam, pp. 441–457.

Hendry, J.H., and C.S. Potten (1982) Intestinal cell radiosensitivity: A comparison for cell death assayed by apoptosis or by a loss of clonogenicity, Int. J. Radiat. Biol., 42(6), 621–628.

Hendry, J.H., C.S. Potten, C. Chadwick and M. Bianchi (1982) Cell death (apoptosis) in the mouse small intestine after low doses: Effects of dose-rate, 14.7 MeV neutrons and 600 MeV (maximum energy) neutrons, Int. J. Radiat. Biol., 42(6), 611–620.

IARC (1972) IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man, Vol. 1, Some Inorganic Substances, Chlorinated Hydrocarbons, Aromatic Amines, *N*-Nitroso Compounds, and Natural Products, IARC, Lyon, pp. 125–134.

IARC (1973) IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man, Vol. 3, Certain Polycyclic Aromatic Hydrocarbons and Heterocyclic Compounds, IARC, Lyon, pp. 91–158.

IARC (1974) IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man, Vol. 4, Some Aromatic Amines, Hydrazine and Related Substances, *N*-Nitroso Compounds and Miscellaneous Alkylating Agents, IARC, Lyon, pp. 145–152.

IARC (1976) IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man, Vol. 10, Some Naturally Occurring Substances, IARC, Lyon, pp. 29–49, 171–179.

IARC (1979) IARC Sci. Publ. No. 23, Pathology of Tumours in Laboratory Animals, Vol. II, Tumours of the Mouse, IARC, Lyon, pp. 174–176.

IARC (1981) IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol. 26, Some Antineoplastic and Immunosuppressive Agents, IARC, Lyon, pp. 165–202, 217–235, 365–384.

Ijiri, K., and C.S. Potten (1983) Response of intestinal cells of differing hierarchical status to ten cytotoxic drugs and five sources of radiation, Br. J. Cancer, 47, 175–185.

LeBlond, C.P., and B. Messier (1958) Renewal of chief cells and goblet cells in the small intestine as shown by radio-autography after injection of thymidine-H^3 into mice, Anat. Rec., 132, 247–259.

Lowenfels, A.B. (1973) Why are small bowel tumours so rare? Lancet, 1, 24–26.

Manoharan, A. (1984) Carcinoma of the urinary bladder in patients receiving cyclophosphamide, Aust. N.Z. J. Med., 14, 507.

Maskens, A.P. (1979) Significance of the karyorrhectic index in 1,2-dimethylhydrazine carcinogenesis, Cancer Lett., 8, 77–86.

Neal, S.B., and G.S. Probst (1984) Assessment of sister chromatid exchange in bone marrow and intestinal epithelium in Chinese hamster, Environ. Mutagen., 6, 407 (abstr.).

Nolte, A.M., and M.T. Goldberg (1984) The mutagenicity of cyclophosphamide in the urinary bladder using an in vivo assay based on nuclear aberrations, Environ. Mutagen., 6, 465 (abstr.).

Phillips, F.S., and S.S. Sternberg (1975) The lethal actions of antitumor agents in proliferating cell systems in vivo, Am. J. Pathol., 81, 205–218.

Potten, C.S. (1977) Extreme sensitivity of small intestinal crypt cells to X- and gamma-irradiation, Nature (London), 269(5628), 518–521.

Potten, C.S. (1983) Stem cells in G.I. mucosa, in: E.M. Scolnick and A.J. Levine (Eds.), Tumor Viruses and Differentiation, Liss, New York, pp. 381–398.

Pound, A.W. (1968) Carcinogenesis and cell proliferation, N.Z. Med. J., 67, 88–89.

Ronen, A., and J.A. Heddle (1984) Site-specific induction of nuclear anomalies (apoptotic bodies and micronuclei) by carcinogens in mice, Cancer Res., 44, 1536–1540.

Ronen, A., J.A. Heddle and A.M.V. Duncan (1983) Site specificity in the induction of nuclear anomalies by carcinogens, in: G.M. Williams, V.C. Dunkel and V.A. Ray (Eds.), Cellular Systems for Toxicity Testing, N.Y. Acad. Sci., New York, pp. 479–482.

Rostom, A.Y., S.L. Kauffman and G.G. Steel (1978) Influence of misonidazole on the incidence of radiation-induced intestinal tumours in mice, Br. J. Cancer, 38, 530–536.

Scribner, J.D., and R. Susa (1978) Tumor initiation and promotion, Int. Rev. Exp. Pathol., 18, 137–198.

Searle, J., T.A. Lawson, P.J. Abbott, B. Harmon and J.F.R. Kerr (1975) An electron microscope study of the mode of cell death induced by cancer-chemotherapeutic agents in populations of proliferating normal and neoplastic cells, J. Pathol., 116(3), 129–138.

Seiler, J.P. (1977) Inhibition of testicular DNA synthesis by chemical mutagens and carcinogens, Preliminary results in the validation of a novel short term test, Mutation Res., 46, 305–310.

Sentein, P. (1981) Nuclear and mitotic abnormalities produced by cycloheximide in the newt egg during cleavage and their relationship to the cell cycle, Exp. Cell Biol., 49, 98–117.

Sharkey, S.M., and W.R. Bruce (1985) Breast Carcinogens Cause Apoptosis in Epithelial Cells of Mouse Mammary Gland, Canadian Federation of Biological Societies Proceedings, Toronto, Vol. 28, P. 216 (abstr.).

Snyder, R.D., and J.D. Regano (1982) DNA repair in normal human and xeroderma pigmentosum group A fibroblasts following treatment with various methanesulfonates and the demonstration of a long-patch (u.v.-like) repair component, Carcinogenesis, 3, 7–14.

Stich, H.F., J.R. Curtis and B.B. Parida (1982) Application of the micronucleus test to exfoliated cells of high cancer risk groups: Tobacco chewers, Int. J. Cancer, 30, 553–559.

Tates, A.D., I. Neuteboom, M. Hofker and L. den Engelse (1980) A micronucleus technique for detecting clastogenic effects of mutagens/carcinogens (DEN/DMN) in hepatocytes of rat liver in vivo, Mutation Res., 74, 11–20.

Toth, B., and K. Patil (1983) Enhancing effect of vitamin E on murine intestinal tumorogenesis by 1,2-dimethylhydrazine dichloride, J. Natl. Cancer Inst., 70, 1107–1111.

Walker, S.E., and M.R. Anver (1983) High incidence of neoplasms in female NZB/NZW mice treated with pulse doses of cyclophosphamide, Vet. Immunol. Immunopathol., 5, 97–104.

Wargovich, M.J., M.T. Goldberg, H.L. Newmark and W.R. Bruce (1983) Nuclear aberrations as a short-term test for genotoxicity to the colon: Evaluation of nineteen agents in mice, J. Natl. Cancer Inst., 71, 133–137.

Warwick, G.P. (1971) Effect of the cell cycle on carcinogenesis, Fed. Proc., 30, 1760–1765.

Weisburger, E.K. (1977) Bioassay program for carcinogenic hazards of cancer chemotherapeutic agents, Cancer, 40, 1935–1949.

Wyllie, A.H. (1981) Cell death: a new classification separating apoptosis from necrosis, in: I.D. Bowen and R.A. Lockshin (Eds.), Cell Death in Biology and Pathology, Chapman and Hall, New York, pp. 9–34.

Wyllie, A.H., J.F.R. Kerr and A.R. Currie (1980) Cell death: The significance of apoptosis, Int. Rev. Cytol., 68 (2), 251–305.