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Cell cycle-dependent intervention by benzamide of carcinogen-induced neoplastic transformation and *in vitro* poly(ADP-ribosyl)ation of nuclear proteins in human fibroblasts

(cell synchronization/S phase/anchorage-independent growth)

ERNEST KUN*, EVA KIRSTEN*, GEORGE E. MILO†, PONNAMMA KURIAN†, AND H. L. KUMARI†

*The Cardiovascular Research Institute and the Department of Pharmacology, University of California, San Francisco, CA 94143; and †Department of Physiological Chemistry, and Comprehensive Cancer Center, Ohio State University, Columbus, OH 43210

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ABSTRACT **Human fibroblasts were subjected to nutritionally induced G_1 block, followed by release and subsequent entry into S phase, and exposed to nontoxic concentrations of carcinogens in early S phase. Cell transformation occurred as determined by early morphologic cell alterations, anchorage-independent colony formation, cell invasiveness, and augmentation of Ab 376 human malignancy-specific cell-surface antigenic determinant. Methylazoxymethanol acetate was the most potent transforming agent at doses that were negative in toxicity tests. Benzamide (10 μM intracellular concentration), a specific inhibitor of poly(ADP-ribose) polymerase, prevented transformation in a cell cycle-specific manner, maximal prevention coinciding with early S phase, also characteristic of maximal susceptibility to transformation. Neither an interference of carcinogen deoxyguanosine nucleoside adduct formation nor a chemical reaction between benzamide and carcinogens was detected. Methylazoxymethanol acetate at transforming but nontoxic dose partially inhibited poly(ADP-ribosyl)ation to about the same extent as benzamide. However, simultaneous exposure of cells to both agents in early S phase, resulting in the prevention of transformation, augmented poly(ADP-ribosyl)ation above the controls. Enzymatic activities ran parallel with the formation of DNA-associating polymer-nonhistone protein adducts that are assumed to regulate the physiological function of chromatin at the structural level.**

A significant increase in poly(ADP-ribosyl)ation of nonhistone proteins was found in an early precancerous state of hamster liver (1) and in regenerating rat liver (2), suggesting that cellular regeneration may be a common reason for the accelerated enzymatic rates. Cellular regeneration is known to augment the number of cells in S phase and this has been correlated with the facilitation of neoplastic transformation in organs (3) and in cultured C3H/$10^1/_3$ CL8 mouse fibroblasts (4). Poly(ADP-ribosyl)ation also exhibits a cell cycle-dependent oscillation, a maximum coinciding with S phase after a release of G_1 block (5). These observations tend to suggest an as yet undefined connection between (poly ADP-ribosyl)ation, cell cycle, and neoplastic transformation.

Ontogenic development (6), the effect of developmental hormones (7–9), and differentiation (10) also coincide with changes in rates of poly(ADP-ribosyl)ation of mainly nonhistone proteins (11, 12) that are thought to regulate selective gene expression (13).

Identification of poly(ADP-ribose) as a unique nucleic acid (14, 15) that is covalently bound to presumably DNA-associated proteins may provide a molecular model of chromatin regulation. Ionic environment-dependent helical polymer chains of poly(ADP-ribose) (15) may act as crosslinking agents between regulatory proteins and, depending on their nature and localization, could alter chromatin conformations, expressed as karyological changes that accompany the cell cycle in normal and malignant cells (16, 17). The poly(ADP-ribosyl)ation-dependent variation in nucleosomal structures (18) tends to support the proposed regulatory mechanism.

We examined the possible participation of poly(ADP-ribosyl)ation of chromatin proteins in carcinogen-induced oncogenic transformation of synchronized human fibroblasts (19–21). This model was chosen because we intended to obtain information that could be relevant to human neoplasia. If poly(ADP-ribosyl)ation of certain chromatin proteins in the S phase plays a role in the regulation of carcinogen-induced oncogenic transformation, then a selective inhibitor of poly(ADP-ribose) polymerase, benzamide (22), would be expected to alter transformation. A preliminary report has appeared (23).

MATERIALS AND METHODS

Cells and Culture Conditions for G_1 Block/Release. Primary human fibroblasts were prepared (24) and cultured as reported (25). These cells have a finite replicative capacity of 35 ± 7 population doublings, which is 22.4 hr, plating efficiency between 95 and 100%, and relative colony (defined as 50 cells)-forming efficiency of 20–21%. For each series of experiments the average yield of cells prior to the soft-agar growth test was $3 \times 10^6 \pm 20\%$ per flask (75 cm^2 each) and for biochemical studies the number of cells was scaled up to about 20×10^6. G_1 block (26) was induced as described (19). It is critical that the number of population doublings at the initiation of G_1 block must not exceed 5 and the doubling time 23 hr; otherwise resistance to transformation by carcinogens may develop (24) and the variations in poly(ADP-ribosyl)ation will differ from results reported here. In G_1 block (19) radiolabeling fell to 0.1% of controls within 24 hr. The G_1 block was released by refeeding with the minimal essential medium, which also contained 0.5 unit of insulin per ml (see Fig. 4). After two washings with minimal essential growth medium, from which bovine serum was deleted, cultures were divided (1:2) and culturing commenced after the addition of 2× concentrated essential vitamins, 9× concentrated essential amino acids, 0.2% $NaHCO_3$, 50 μg of gentocin per ml, and 20% fetal serum until confluence was approached and serial passages were continued (1:10) in the enriched minimal essential medium (see Fig. 4). Seeding (4×10^5 cells per plate) into soft agar (19) was carried out after 16–20 population doublings.

Exposure to Carcinogens and Benzamide. Exposure to carcinogens and benzamide was done 10 hr after the release of the

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Abbreviation: RCF, relative cloning frequency.

metabolically induced G_1/S block (Fig. 4) and exposure lasted 10 hr, followed by three washings and refeeding with fresh media (25).

Cellular Toxicity. Cellular toxicity, tested at least at six concentrations of drugs, was determined by effects on cloning frequency (26). Relative cloning frequency (RCF_1) was defined as the ratio between cloning efficiency of controls and cultures exposed to drugs, and RCF_{50} was defined as the concentration of drugs that caused a 50% decrease of cloning frequency. Toxicity was the same in random and synchronized cultures. The time of exposure to drugs was 24 hr, even though in the transformation experiments (Fig. 4) this was only 10 hr, providing an extra margin of safety for the determination of nontoxic doses, which were further tested by the absence of effects on plating efficiency and doubling time.

Criteria for Phenotypic Transformation. In addition to colony counts on soft agar (19, 25), criteria for phenotypic transformation were cellular invasiveness (27), determined in six parallel tests per experiment, and the immunofluorescence test for human malignancy-specific monoclonal cell-surface antigenic determinant Ab 376 (28, 29) on cells obtained from soft agar clones (unpublished data). The tumor take in *nude* mice (19) was identical with the incidence of tumor formation by surgically obtained human fibrosarcomas, cultured parallel with transformed fibroblasts, and the low incidence (20–30%) is probably explained by genetic differences between the human cells and the host (30).

Biochemical Procedures. Biochemical procedures related to poly(ADP-ribose) were the same as published (1, 2, 6, 11, 12, 14, 15, 31–33). Intracellular benzamide (^{14}C-labeled) was determined after removal of the adsorbed drug, lysis in NCS tissue solubilizer, methanol extraction, and reversed-phase chromatography on a C_{18} (Bondpak) column with a 0–60% methanol/H_2O linear gradient. Fibroblasts were permeabilized by lysophosphatidylcholine (34), which did not interfere with enzyme assays.

[^{14}C]Benzamide (1C) (4.29 μCi/mmol, 5 μCi/ml of solution; 1 Ci = 3.7×10^{10} Bq) was purchased from Pathfinder Labs, St. Louis, MO. [1,4-^{14}C]Methylazoxymethanol acetate (specific activity, 115 mCi/mmol) was kindly provided by F. Cazer (Ohio State University). The monoclonal antibody to Ab 376 was a generous gift of S. Ferrone and the secondary reagent (fluoroisothiocyanate-conjugated goat anti-mouse IgG) was purchased from Miles.

RESULTS

Prevention of Transformation. Transforming (RCF_1) and toxic (35) (RCF_{50}) doses of methylazoxymethanol acetate were 7 and 27 μM, respectively, and for benzamide the doses were 1 mM (added externally to cell cultures) and 4.75 mM, respectively. These values for the carcinogen or benzamide did not change if the two agents were combined, as under conditions that prevented transformation. Identical toxicity analyses were performed with five additional carcinogens (Table 1). Benzamide (added externally at 1 mM, corresponding to 10 μM intracellular concentration) counteracted transformation. Benzoate at 1 mM had no influence on the transformation nor did it have an effect of its own on fibroblasts (not shown).

Evidence of Cellular Transformation. The characteristic morphology of fibroblasts (Fig. 1, group 1) was markedly altered when cell cultures were exposed to RFC_1 doses of carcinogens after five cell doublings. Benzamide in cell cultures inhibited this phenotypic change (Fig. 1, group 3), whereas the cell morphology shown in Fig. 1, group 1, remained unaltered by benzamide alone. Cells shown in Fig. 1, group 3, have become resistant to transformation by reexposure to another cycle and G_1 block/release and carcinogen (in S phase) in the absence of a new dose of benzamide, and this resistance is likely to be due to a modified genetic trait because residual benzamide was not present after three or four cell doublings. Cellular invasiveness (27) of transformed colonies grown in soft agar is shown in Fig. 2, and the fluorescence-antibody binding test (29) performed with the Ab 376 monoclonal antibody (28) is shown in Fig. 3*B*.

Table 1. Inhibition by benzamide of carcinogen-induced cell transformation as determined by colony counts on soft agar

Carcinogen	Experiments, no.	Experimental conditions	Transformed cell colonies, no. per 10^5 cells
1	5	Methylazoxymethanol acetate (7.0 μM)	758 (±58)
	5	+ Benzamide	1 (±0.3)
2	2	*N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (0.7 μM)	39 (±6)
	2	+ Benzamide	0 (±0.2)
3	2	7β,8α-Dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzopyrene (0.33 μM)	26 (±2)
	2	+ Benzamide	0
4	2	β-Propiolactone (28 μM)	28 (±3)
		+ Benzamide	0
5	1	1,1-Dimethylhydrazine (167 μM)	103 (±9)
	1	+ Benzamide	1 (±0.2)
6	2	3-Hydroxy-1-propanesulfonic acid γ-sultone (122 μM)	43 (±5.5)
	2	+ Benzamide	1.0 (±0.3)
7	5	No additions	0
8	5	Benzamide	0

Both carcinogens and benzamide were present in RCF_1 concentrations.

Time Course of Exposure of Fibroblasts to RCF_1 Doses of Benzamide and Methylazoxymethanol Acetate. The time course of exposure of fibroblasts to RCF_1 doses of benzamide and methylazoxymethanol acetate as related to G_1/S block and its release is illustrated in Fig. 4. The partial antagonism by benzamide, added during G_1 block and in early S phase (lines A and B) is most probably explained by the partial retention of benzamide in cells even after several washings (see *Materials and Methods*).

DNA Adduct Formation. DNA adduct formation (36–38) was studied with two labeled carcinogens (carcinogens 1 and 3 in Table 1) under identical conditions required for transformation. The quantities of adducts in experiment 1 were 90.6 and 86.0 pmol/mg of DNA (22×10^3 and 21×10^3 cpm of ^{14}C) and in experiment 2 were 45 and 41 pmol of methylazoxymethanol acetate per mg of DNA (11.5×10^3 and 11×10^3 cpm). Rates of transformation were similar in both cases. In each experiment the second value was obtained in the presence of benzamide. One of the labeled products of methylazoxymethanol acetate was [^{14}C]methyl-O^6-guanosine, 5–9 adducts per 10^6 bases (cf. ref. 39). Methylation of DNA with [*methyl*-^{14}C]methionine as a methyl donor was not influenced by benzamide (results not shown). Adduct formation of DNA with [$^3H(U)$]7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzopyrene (carcinogen 3 in Table 1) yielded mainly 7β-benzopyrene diol epoxide I-deoxyguanosine (4–8 adducts per 10^6 bases) identified by chromatography (33). The quantities of these major and also of

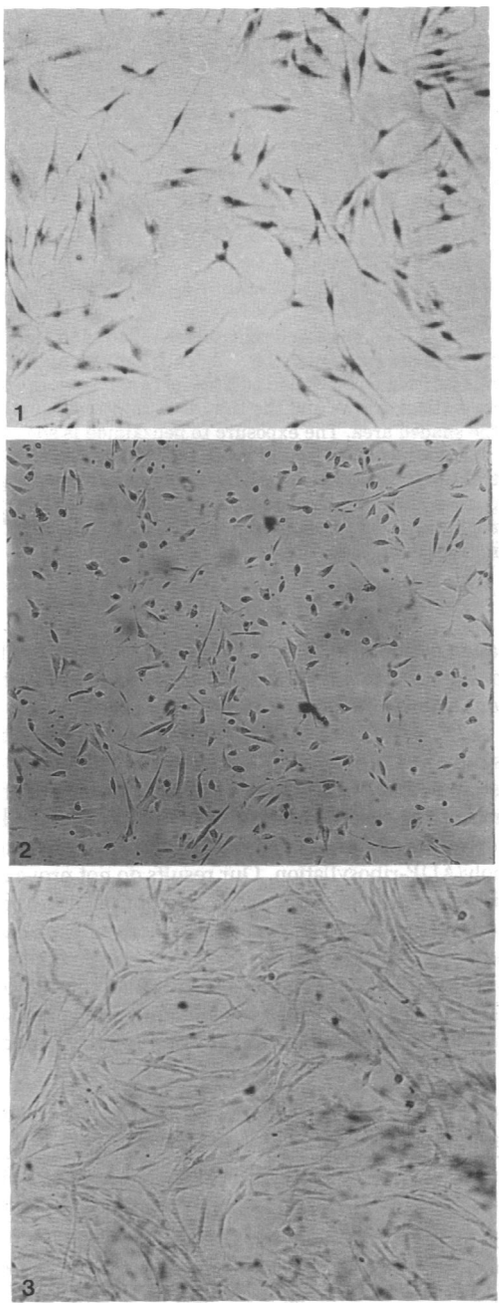

FIG. 1. Cellular morphology of normal (group 1), methylazoxymethanol acetate-treated (group 2), and benzamide- and methylazoxymethanol acetate-treated cells (group 3) as seen under phase optics. (×175.) Treatment with benzamide alone resulted in cells that were indistinguishable from group 1; therefore, they are not shown. The same morphologic effects were seen between 5 and 30 population doublings.

minor products were uninfluenced by benzamide, supporting previous results (33). Thus, the same quantities and types of carcinogen adducts occur in resistant and transformable cell cultures. Benzamide and carcinogens and their degradation products were reisolated by high-performance liquid chromatography (33), excluding chemical artefacts. Autoradiography indicated a tight association of benzamide with nuclear membrane structures.

Alkaline Sedimentation Profile of DNA. At 1.97 μM methylazoxymethanol acetate (colony formation in soft agar, 50–70 colonies per 10^5 cells) the alkaline sedimentation profile of DNA was the same in controls and in methylazoxymethanol acetate-

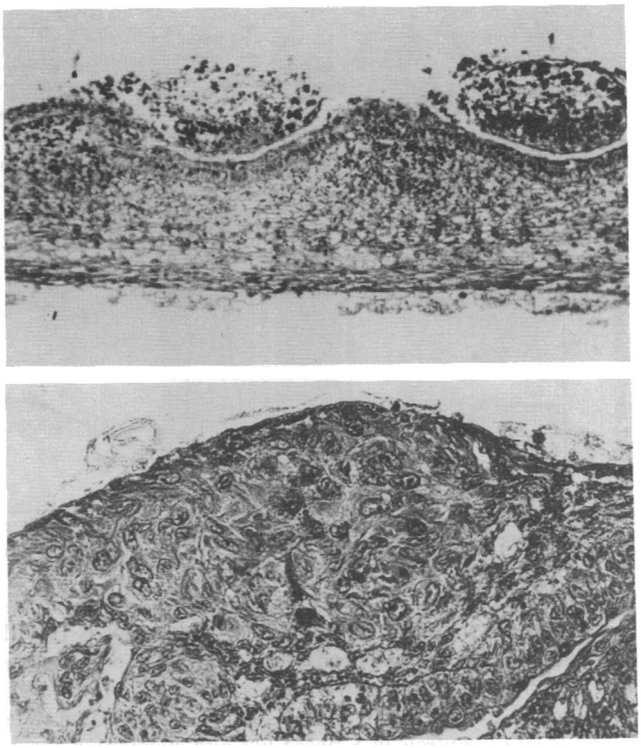

FIG. 2. Tissue invasiveness (cf. ref. 27) of methylazoxymethanol acetate-transformed fibroblasts, grown in soft agar for 20 days. (*Upper*) Normal fibroblasts placed on chicken embryo skin for 72 hr. (*Lower*) Invasive behavior after the same length of exposure to transformed cells.

or benzamide-treated cells when drugs were added separately or in combination (5.26×10^8 daltons). At RCF_1 (7 μM) methylazoxymethanol acetate induced an alkali instability in DNA size, as evident from the appearance of a second DNA molecular species of 1.52×10^8 daltons. However, prevention of transformation by benzamide did not coincide with reassociation to the larger size DNA; thus, no connection between apparent fragmentation and reassociation could be ascertained that correlated with transformation and its prevention.

Variations of Poly(ADP-ribose) Polymerase Activity and Determination of Products. After 10 hr of exposure to 1 mM benzamide in cell cultures, inhibition of enzymatic activity was similar to that observed in permeabilized cells at 10 μM benzamide. As shown in Fig. 5 methylazoxymethanol acetate at 7

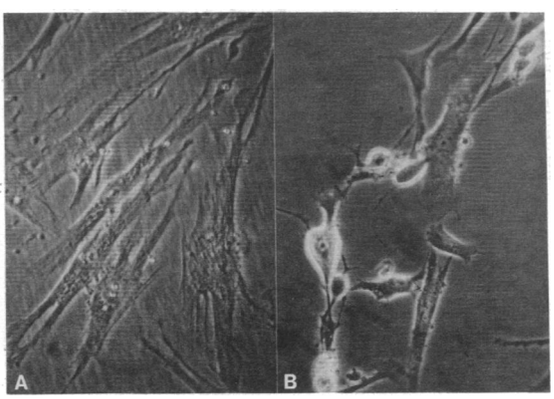

FIG. 3. Detection of common antigenic determinant of human malignancies in transformed human fibroblasts by fluorescence microscopy. (*A*) Normal; (*B*) transformed (see legend to Fig. 2). Photographs were taken with a Zeiss fluorescence microscope (excitation = 485 nm, read at 520–560 nm). (×88.)

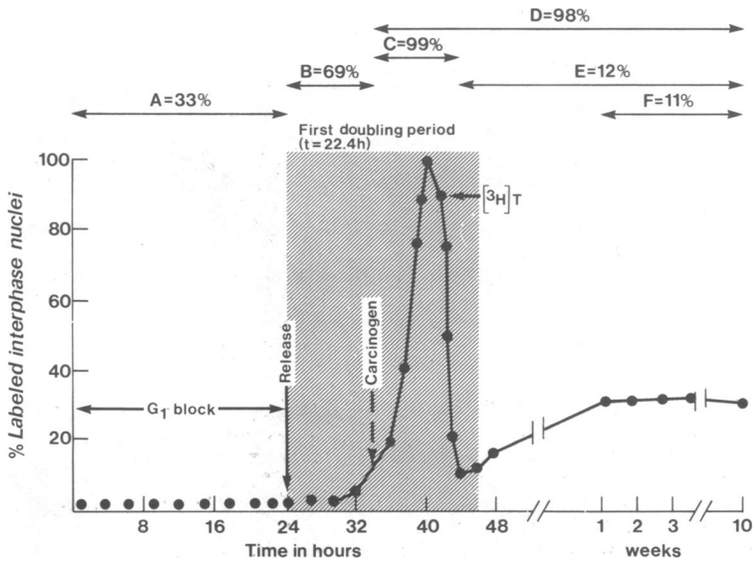

FIG. 4. G_1 block and release was followed in eight parallel cultures per experiment (4×10^6 cells per group). The times of release from G_1 block and addition of carcinogens are indicated by vertical arrows and the first doubling time is indicated by the shaded area. The exposure to benzamide is shown by horizontal arrows (top) together with the % protective effect against transformation, determined by colony counts in soft agar. Zero percent was defined when the carcinogen (methylazoxymethanol acetate) was present only. ●, [^{3}H]Thymidine ([^{3}H]T) labeling; abscissa, time (first in hours, then in weeks).

μM (Table 1, carcinogen 1) inhibited poly(ADP-ribose) polymerase activity in S phase nearly to the same extent as 10 μM benzamide, but combined exposure of cells in the S phase (see Fig. 4) to both the carcinogen and benzamide—resulting in the inhibition of transformation (Table 1)—not only restored rates to the level of controls in S phase but also augmented them. Biochemical studies (Table 2 and Fig. 5) were confined to these experimental conditions. As shown in Table 2, after incubation of permeabilized cells for 20 or 40 min, protein adducts of short chains (phenol soluble) and long chains (H_2O soluble) were isolated (40), indicating that prolonged reaction resulted in the synthesis of predominantly long chain polymer-protein adducts. Our results indicate that in S phase and after exposure to methylazoxymethanol acetate and benzamide both initiation and elongation rates increased simultaneously, consistent with an augmentation of the polymerase.

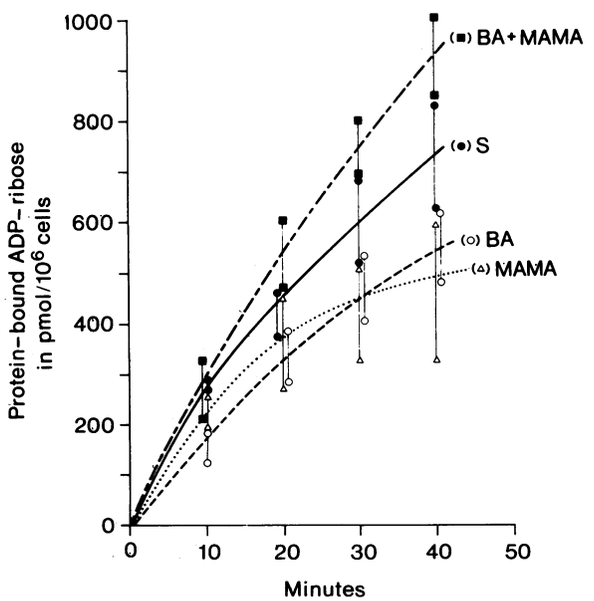

FIG. 5. Rates of poly(ADP-ribose) polymerase in synchronized human fibroblasts, assayed in the S phase. ●, S phase controls (S); ○, cells treated with benzamide (BA) (Table 2 and Fig. 4); △, 7.0 μM methylazoxymethanol acetate (MAMA); ■, simultaneous treatment with benzamide and the carcinogen (BA + MAMA). Assay conditions were the same as given for Table 2.

DISCUSSION

Application of nontoxic yet biologically effective concentrations of both carcinogens and benzamide tends to insure that results represent cellular biological mechanisms. The S-phase dependence of the effectivity of transforming agents and their antagonism by benzamide and the variation of poly(ADP-ribose) polymerase activity with the cell cycle (5, 39) suggest the participation of the macromolecular metabolism (i.e., induction, turnover) of the polymerase enzyme protein as a regulatory factor in poly(ADP-ribosyl)ation. Our results do not prove enzyme induction because only kinetics and products have been determined and more specific assays for the enzyme protein (e.g., by immunological methods) are required to settle this question. However, we have repeatedly found that an inhibitor of poly(ADP-ribose) polymerase, nicotinamide, at pharmacological doses sufficient to inhibit the enzyme *in vitro* can induce a

Table 2. Distribution and quantities of total, phenol-soluble, and H_2O-soluble protein-poly(ADP-ribose) adducts

Experimental conditions	Time of reaction, min	Protein-bound ADP-ribose, pmol per 10^6 cells		
		Total	Phenol soluble	H_2O soluble
G_1 block	20	203	45	157
	40	324	35	289
S phase	20	413	126	287
	40	868	13	854
+ Benzamide	20	207	67	140
	40	294	29	265
+ Methylazoxymethanol acetate	20	279	63	214
	40	348	19	329
+ Benzamide + methylazoxymethanol acetate	20	564	245	318
	40	905	15	888

Permeabilized cells (7.5×10^6; equivalent to ≈2 mg of protein) were incubated in a total volume of 500 μl containing 100 mM Tris chloride (pH 8.0), 10 mM EDTA, 20 mM $CaCl_2$, 0.5 mM NAD [^{14}C-labeled in the adenine moiety (26,000 dpm/nmol)], and 0.1 mM phenylmethanesulfonyl fluoride at 25°C. After 20 or 40 min the reaction was terminated by addition of 0.5 ml of 20% HClO at 0°C, and poly(ADP-ribosyl)ated proteins were separated (40). Each value is the mean of three analyses with a SD of ±20%.

variety of enzymes *in vivo* (41, 42); thus, the hypothetical mechanism proposed for benzamide is not without precedent. Based on the unique structural features of poly(ADP-ribose) (14, 15), it may be assumed that a structural regulation of physiological chromatin function in S phase has been reestablished by the augmentation of certain DNA-associated poly(ADP-ribose)-nonhistone-protein adducts and this process may be causally related to the prevention of transformation. A chromatin structure-dependent control of gene regulation has been proposed earlier (43), although molecular mechanisms involved were not identified. Currently held mechanisms of neoplastic transformation by oncogene expression (44–46), gene translocations, and amplifications (47, 48) may be extended by the poly(ADP-ribosyl)ation-dependent supramolecular control of availability of DNA domains that could involve critical enhancing regions.

It was shown that 3-aminobenzamide at high doses (300–600 mg/kg) if administered to rats, 4 hr after *in vivo* pretreatment with a hepatocarcinogen, appears to augment the development of premalignant liver foci, characterized by increased γ-glutamyl transpeptidase (49). An overwhelming dose of an inhibitor of poly(ADP-ribose) polymerase when administered *in vivo* is likely to serve primarily as an enzyme inhibitor of the polymerase and not as an inducer and therefore may reinforce carcinogenicity, especially if the carcinogen has been given prior to the inhibitor. It follows that specific pharmacokinetic and cellular kinetic conditions have to be observed to reproduce the antitransforming effect of nontoxic doses of inhibitors of poly(ADP-ribose) polymerase in intact animals. A variety of inhibitors of poly(ADP-ribose) polymerase have been found to serve as antitransforming agents in fibroblast cultures if applied under conditions described here (unpublished data); therefore, the observed effect is not confined to benzamide.

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