Inhibition of Hematopoietic Growth Factor–Induced Proliferation by Adenosine Diphosphate–Ribosylation Inhibitors

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The effects of adenosine diphosphate (ADP) ribosylation inhibitors on hematopoietic growth factor–induced proliferation were examined. Significant inhibition of interleukin-3 (IL-3), colony-stimulating factor 1, and lung conditioned media–induced clonal agar growth of normal murine hematopoietic cells by 10 mmol/L nicotinamide (NAM), 10 mmol/L 3-aminobenzamide (3AB), and 5 mmol/L N\textsuperscript{3}-methyl nicotinamide (1MN) was noted. Nicotinic acid, a related compound that does not inhibit ADP ribosylation, failed to inhibit the growth factor–mediated proliferation. NAM (10 mmol/L), 3AB (10 mmol/L), and 1MN (5 mmol/L) also prevented IL-3 and phorbol ester–stimulated \textsuperscript{3}H-thymidine incorporation into the IL-3–responsive FDC-P1 cell line. Exposure of FDC-P1 cells to 10 mmol/L NAM led to a significant decrease in nuclear poly-(ADP-ribose) levels. Exposure of FDC-P1 cells to 6 mmol/L 1MN did not affect evidence supporting a role for ADP ribosylation in cell differentiation but not cell proliferation.

To investigate the possible role of ADP ribosylation in the mediation of hematopoietic growth factor induced–proliferation, we studied the effects of three different ADPRT inhibitors (3-aminobenzamide [3AB], N\textsuperscript{3}-methyl nicotinamide [1MN], and nicotinamide [NAM]) on CSF-1–, IL-3–, and GM-CSA–stimulated clonal agar growth of normal murine hematopoietic cells and on IL-3–induced proliferation of FDC-P1 cells. The changes in nuclear ADP-ribose levels in FDC-P1 cells in response to IL-3 in the presence and absence of the ADPRT inhibitors and the interaction of these agents with PK-C activation in FDC-P1 cells as well as in mouse thymoma EL4 cells were examined. Our findings demonstrate that (a) active ADP ribosylation inhibitors interfere with growth factor–induced proliferation of murine hematopoietic cells and (b) the inhibition occurs at a step that follows the activation and translocation of PK-C and is more closely linked to DNA synthesis.

The purification of the murine hematopoietic growth factors interleukin-3 (IL-3), colony-stimulating factor 1 (CSF-1), and granulocyte-macrophage colony–stimulating activity (GM-CSA) has permitted the evaluation of the intracellular events that mediate the proliferative response to these agents. Studies using an IL-3–responsive cell line (FDC-P1) have suggested that activation of protein kinase C (PK-C) occurs after exposure to IL-3. The mechanism by which the interaction of IL-3 with its receptor and the subsequent activation of PK-C lead to cell proliferation is not known.

Adenosine diphosphate (ADP) ribosylation of nuclear proteins, a reaction catalyzed by ADP–ribosyl transferase (ADPRT), plays an important role in the recovery from DNA damage induced by alkylating agents. Conflicting data exist regarding the possible role of this reaction in DNA replication. Some studies suggest that ADP ribosylation stimulates DNA synthesis, and others support the opposite conclusion. Experiments using ADPRT inhibitors have shown that these agents interfere with mitogen-induced activation (DNA synthesis) of human peripheral blood lymphocytes. These findings and the lack of a significant effect of these agents on the proliferation of the human T lymphoblastoid cell lines Molt 4 and CEM have been interpreted as

MATERIALS AND METHODS

Materials

NAM, 3AB, nicotinic acid (NA), 1MN, unlabeled phorbol esters, phosphatidyl serine (PS), 1,2-diolein, magnesium acetate, calcium chloride, adenosine triphosphate (ATP), ethyleneglycol tetracetic acid (EGTA), and EDTA were obtained from Sigma Chemical Co (St Louis). Methyl \textsuperscript{3}H-thymidine (\textsuperscript{3}H-TdR, 100 Ci/mL) and [20-\textsuperscript{3}H]phorbol-12,13-dibutyrate (PDB, 12.5 Ci/mmol in ethanol) were obtained from New England Nuclear (Boston). \textsuperscript{[\gamma-\textsuperscript{32}P]}ATP, 0.15 to 1.0 Ci/mmol, was obtained from the Diabetes Research Center Core Laboratory at the University of Virginia where it was synthesized by the method of Johnson and Walseth. Purified IL-3 was kindly provided to us by Dr J. Ihle, National Cancer Institute, Frederick, MD. Purified CSF-1 was obtained from Dr R. Shaddock, Pittsburgh. Lung conditioned medium (LCM) was used as a source of GM-CSA. FDC-P1 cells developed by Dexter et al \textsuperscript{9} (provided by Dr J. Ihle, Frederick, MD) were grown at 37°C in RPMI 1640 (GIBCO, Grand Island, NY) with 10% fetal calf serum (FCS) (HyClone, Logan, UT) and 25% conditioned medium from WEHI-3 cells (WEHI-CM) as a source of IL-3 under a 5% CO\textsubscript{2} atmosphere. Phorbol ester–sensitive EL4 cells \textsuperscript{11} were maintained in RPMI 1640 with 5% heat-inactivated serum as previously described. CBA-J
mice (Jackson Laboratories, Bar Harbor, ME) were used as a source of bone marrow cells.

**Methods**

**Bone marrow culture.** Eight-week-old CBA-J mice were killed and their femurs and tibias were collected. Marrow from these bones was flushed into McCoy’s medium containing 15% FCS. Cells (10^7/mL) were plated into 35-mm plates containing different stimuli (IL-3, 25 U/mL; CSF-1, 400 U/mL; LCM 10% dilution) and in the presence and absence of 10 mmol/L NAM, 10 mmol/L 3AB, 10 mmol/L NA, or 5 mmol/L 1MN. Each condition was set up in triplicate, and each experiment was done twice. Poly-(ADP-ribose)-specific nucleoside derivative 1-N’-ethenoribosadenosine was quantified by fluorescence detection as described previously.14

**Phorbol ester (PDB) binding assay in FDC-P1 cells.** FDC-P1 cells were washed three times in RPMI 1640 with 5% FCS, resuspended to a concentration of 1–2 × 10^7/mL, and in three experiments, incubated in RPMI 1640 and 10% FCS for two hours (this incubation step was omitted in two other experiments). The inhibitor 1MN at a final concentration of 5 mmol/L or vehicle was added to portions of the cells, and they were incubated at 37°C and 5% CO2 for 30 minutes. They were washed with RPMI 1640 and ruptured in 2 mL of homogenization buffer (20 mmol/L Tris, pH 7.4, 0.33 mmol/L sucrose, 2 mmol/L EDTA, 0.5 mmol/L EGTA, 10 µg/mL leupeptin, and 50 µmol/L 2-mercaptoethanol) with 20 strokes in a tight Dounce homogenizer. This preparation was centrifuged at 100,000 g for 45 minutes to yield cytosol (supernatant) and membrane (pellet) fractions. The pellet was resuspended in a volume of homogenization buffer equal to that of the cytosol and passed through a 23-gauge needle. Binding assays were done as previously described.15 Briefly, cell fractions equivalent to 1 to 3 × 10^6 cells were incubated with 40 mmol/L [20-3H]PDB plus either a 100-fold excess of unlabelled PDB or vehicle (0.02% ethanol) for two to 18 hours at 4°C in the presence of bovine serum albumin at 1.2 mg/mL, 0.5 mmol/L CaCl2, 75 mmol/L Mg acetate, and PS at 96 µg/mL. Bound PDB was separated from free by filtration through glass fiber filters, and the radioactivity remaining on the filters was determined. Specific binding represents the difference between binding in the absence and that in the presence of competitor.

**Protein kinase assay.** Calcium/phospholipid-dependent protein kinase activity in crude cytosol preparations was assayed by determining the incorporation of [γ-32P] ATP (2 to 4 × 10^6 cpm/tube) into histone H1 in the presence and absence of PS (40 µg/mL) and diolein (1.6 µg/mL). All of the incubations contained Mg acetate (5 mmol/L), CaCl2 (60 mmol/L), and ATP (0.1 mmol/L).15,16 The reaction was stopped after three minutes by spotting an aliquot of the reaction mix onto phosphocellulose chromatography paper, and the radioactivity on the filter was determined after washing three times in 50 mmol/L NaCl and once in acetone. The effect of 1MN on PK-C activity was determined by adding either the vehicle (deionized H2O) or 5 mmol/L 1MN to the cytosol preparation before the addition of the reaction mix.

**Statistical methods.** Analysis of variance with multiple comparisons (Duncan’s test) was used. Results are expressed as means ± SEM. Statistical significance was accepted for P < .05. Statistical analysis of the phorbol ester–binding assay was done by using the Wilcoxon signed rank test.

### RESULTS

**Effect of ADPRT Inhibitors on Clonal Agar Growth of Bone Marrow Cells**

Colonies formed by hematopoietic cells in response to IL-3, LCM, and CSF-1 were significantly inhibited by 10 mmol/L NAM, 10 mmol/L 3AB, and 5 mmol/L 1MN, whereas 10 mmol/L NA did not have an inhibitory effect (Fig 1). Inhibition of CSF-1 effects was more pronounced than the effect on IL-3–induced colony formation (P < .05). Macrophage colony formation in response to IL-3 was affected to a greater extent than granulocyte-containing colonies (of eight surviving colonies in the presence of 10 mmol/L NAM, seven were G-CFU and one was GM-CFU;
Fig 1. Effect of ADPRT inhibitors on IL-3-, GM-CSA-, and CSF-1-induced clonal agar growth of hematopoietic cells. Marrow cells were grown in agar in the presence of IL-3 at 25 U/mL, CSF-1 at 400 U/mL, or 10% LCM as a source of GM-CSA. Ten millimolar NAM, 10 mmol/L 3AB, or 5 mmol/L 1MN were added to experimental plates; sterile water (diluent) was added to control plates. Results expressed as means ± SE from two experiments (n = 6 per group).

This corresponds to a 95% inhibition of monocyte-containing colonies and a 13% inhibition of G-CFUs).

Effect of ADPRT Inhibitors on Thymidine Incorporation and Proliferation of FDC-P1 Cells

To eliminate problems in interpreting responses of mixed cell populations, we examined the effects of ADPRT inhibitors on IL-3-induced thymidine incorporation by FDC-P1 cells (a bone marrow-derived, IL-3-responsive cell line). Figure 2A shows the effects of different concentrations of NAM, 1MN, and 3AB on IL-3-induced 3H-TdR incorporation. NA at 10 mmol/L had no significant effect on IL-3-induced 3H-TdR incorporation (P > .5), whereas 10 mmol/L NAM, 10 mmol/L 3AB, and 5 mmol/L 1MN had a significant inhibitory effect (P < .0001, Fig 3A). This inhibitory effect was of the same magnitude over a wide range of IL-3 concentrations, which suggested that the inhibitors do not interfere with binding of IL-3 to its receptor (Fig 3A).

Since PK-C has been implicated in IL-3 action,1 we examined the effects of the inhibitors on proliferation supported by the direct PK-C activator PMA. Inhibition of PMA-induced 3H-TdR incorporation by FDC-P1 cells at 24 hours was noted at the same concentrations tested in the experiments using IL-3 (Fig 2B). This inhibition was also noted over a wide range of PMA concentrations (P < .0001), thereby suggesting that this effect is not due to interference of these agents with the interaction between PMA and its proposed receptor, PK-C (Fig 3B). 3H-TdR incorporation in cultures containing NA and PMA was not any different from that of those containing PMA alone (P > .5).

The time course for this inhibitory effect on IL-3- and PMA-induced proliferation of FDC-P1 cells is shown in Fig 4. Ten millimolar NAM, 5 mmol/L 1MN, and 10 mmol/L 3AB inhibited the proliferation of the FDC-P1 cells in liquid cultures containing WEHI-CM as a source of IL-3. This effect became less evident with time, mostly due to a lack of cell proliferation in the control group by day 4, with all cultures reaching similar cell densities (Fig 4A). The inhibition by 1MN of PMA-induced proliferation of FDC-P1 cells was evident over a four-day period. Cell viability was the same in experimental and control cultures containing WEHI-CM during the first three days of culture, which suggested that the observed effects are not due to cell death.

Effect of ADPRT Inhibitors on Nuclear Poly-(ADP-Ribose) Levels

A significant decrease in the nuclear poly-(ADP-ribose) levels was detected within 20 minutes of exposure of FDC-P1 cells to 10 mmol/L NAM, thereby demonstrating that at the concentration tested these agents have one of their proposed biochemical effects, namely, inhibition of ADPRT (Fig 5).
Fig 3. Effects of ADPRT inhibitors on (A) IL-3- and (B) PMA-induced incorporation of FDC-P1 cells. Serial dilutions of IL-3 and PMA were made in separate cultures containing fixed concentrations of tested substances: 10 mmol/L NAM, 10 mmol/L 3AB, 5 mmol/L 1MN, and 10 mmol/L NA. Results are mean incorporation at 24 hours from three experiments for A and two experiments for B.

Fig 4. Time course of the inhibitory effect of ADPRT inhibitors on (A) IL-3- and (B) PMA-induced proliferation of FDC-P1 cells. Results are expressed as numbers of viable cells per milliliter at each time point as determined by trypan blue dye exclusion and cell counts using a hemacytometer. Results are means ± SE from three separate experiments in A or duplicate experiments in B, [1MN] = 5 mM in B.
Effect of ADPRT Inhibitors on Subcellular Location and Activity of PK-C

Since the 3H-TdR incorporation experiments suggested that these ADPRT inhibitors specifically affect the PK-C pathway in FDC-P1 cells, we next attempted to determine the step in this pathway that is inhibited by these agents. Exposure of FDC-P1 cells to 1MN did not lead to changes in the cytosol/membrane PDB binding ratio, thereby suggesting that these agents do not interfere with PK-C/phospholipid interaction (Fig 6). Five millimolar 1MN did not alter the phorbol ester (PDB)-induced changes in the cytosolic/membrane PDB binding ratio (two separate experiments, data not shown). Nor did cytosols recovered from FDC-P1 cells that had been exposed to 1MN at 5 mmol/L for 30 minutes differ from control cytosols in their total kinase or calcium/phospholipid-dependent kinase activity (PK-C) as determined by in vitro phosphorylation of histone H1 (Table 1). The presence of 5 mmol/L 1MN in the reaction mix did not affect the calcium/PS-dependent kinase activity present in crude cytosolic preparations of FDC-P1 or EL4 cells (Table 1).

Effect of 1MN on EL4 Cell Proliferation

The evidence presented thus far suggests that these agents inhibit the proliferation of FDC-P1 cells by blocking the PK-C pathway at a step that follows activation of PK-C. To determine whether this effect was specific for the PK-C pathway or for cell proliferation, we investigated the effects of 1MN on PMA-induced effects on EL4 cells. Proliferation of these mouse thymoma cells is inhibited by PMA; this effect is also accompanied by increased IL-2 production. We found that 1.25 mmol/L 1MN had no effect on thymidine incorporation of exponentially growing EL4 cells (P > .05). In the presence of PMA, 1.25 mmol/L 1MN markedly enhanced the inhibitory effect of PMA on 3H-TdR incorporation (Fig 7). Five millimolar 1MN inhibited 3H-TdR incorporation by EL4 cells to a lesser degree than in FDC-P1 cells.

DISCUSSION

The results from the bone marrow experiments reported herein demonstrate that ADP ribosylation inhibitors signifi-
significantly interfere with IL-3-, CSF-1-, and GM-CSA-induced clonal agar proliferation of murine hematopoietic cells. Francis et al investigated the effects of three different ADPRT inhibitors (5-methylnicotinamide, 3AB, and 3-methoxybenzamide) on clonal agar growth of human marrow cells in the presence of a leukocyte feeder layer as a stimulus and found that these agents preferentially inhibited the growth of macrophage-granulocyte and pure macrophage clones over that of granulocyte colonies. On the basis of these results the authors suggested that ADP ribosylation is involved in macrophage differentiation. Dexter et al investigated the effects of two different inhibitors of ADP ribosylation (benzylaminododecylguanine hydrochloride and p-methoxybenzylaminodecamethylene guanidine sulphate) on murine hematopoiesis by using long-term marrow cultures. They found that these agents decreased the number of WEHI-CM-responsive colony-forming cells to a lesser extent than their effect on the number of CSF-1-responsive cells. We found a similar hierarchy of inhibition with the ADPRT inhibitors we studied.

The inhibition by ADPRT inhibitors of IL-3- and PMA-induced proliferation of FDC-P1 cells suggests that these agents interfere with PK-C activation or with an intracellular event that occurs as a consequence of PK-C activation. The lack of an effect on the subcellular location or catalytic activity of PK-C suggests that the inhibition is at a step that follows the activation of PK-C.

Previous studies by Singh et al using mouse embryo fibroblasts (C3H10T1/2) and human monocytes provide further evidence for an interaction between PMA-induced effects and ADP ribosylation. These studies suggested a role for ADP ribosylation of nuclear proteins in the modulation of gene expression induced by PMA and suggested that an oxidative burst is an intermediate step in this effect. An increase in poly-(ADP-ribose) levels and DNA breaks occurred in human monocytes within the first hour of exposure to PMA; these effects were blocked by antioxidants. It has been proposed that stimulation of phospholipase A2 by PMA, as a consequence of PK-C activation, leads to the stimulation of arachidonic acid metabolism and increased formation of hydroperoxyarachidonic acid intermediates. These unstable products decay spontaneously to

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**Fig. 7.** Effects of 1MN on 3H-TdR incorporation into EL4 cells. Results are expressed as means ± SE from three separate experiments (n = 28 per group). 1MN (millimolar) and PMA (nanomolar) were added at the same time, and 3H-TdR incorporation was determined 24 hours later. The control group consisted of EL4 cells exposed to RPMI 1640 plus 5% FCS.
the corresponding hydroxyl derivatives releasing active oxygen, the latter being highly efficient in inducing DNA breaks. Studies using P388D1 murine macrophages have shown that inhibition of DNA synthesis and activation of ADPRT occurs during the first hour of exposure to 40 \( \mu \text{mol/L H}_2\text{O}_2 \), probably as a consequence of oxidant-induced single-strand DNA breaks.

It is possible that PMA induces a similar oxidative burst in EL4 cells. Copper complexes with superoxide dismutase mimetic activity can inhibit phorbol ester–induced interleukin-2 production; however, it has not been demonstrated that the inhibitory activity of these compounds is due to their dismutase mimetic activity. Studies using human peripheral blood lymphocytes suggest that rejoining of DNA breaks is required for proceeding with mitogen-stimulated DNA synthesis and that ADPRT plays a significant role in this DNA repair. Therefore, it is possible that the 1MN-induced enhancement of the inhibitory effects of PMA on DNA synthesis in EL4 cells is due to the role of ADPRT in repairing oxidant-induced DNA breaks and the requirement for this DNA repair before resuming DNA synthesis.

One cannot conclude that the observed effects of the ADPRT inhibitors reported herein are entirely or primarily due to their effect on nuclear ADP ribosylation. These agents inhibit other nonnuclear ADP ribosylation reactions, among which there are reactions that have been associated with the adenylate cyclase system and mitochondrial calcium efflux. NAM at the concentration studied (10 mmol/L) had been reported to lower the intracellular ATP levels. 3AB also inhibits de novo synthesis of DNA precursors in lymphoblast cells, although this effect does not seem to be responsible for its cell replication effects as suggested by similar sensitivity to this agent of lymphoblast cells proficient or deficient in this pathway. The most potent inhibitor of hematopoietic proliferation was 1MN, which is the least potent inhibitor of ADPRT activity in vitro among those agents studied. 3AB at 2.5 mmol/L had minimal, if any, inhibitory effects on IL-3–induced \(^3\text{H}\)-TdR incorporation of FDC-P1 cells. This concentration of 3AB has previously been shown to inhibit ADPRT activity in permeabilized cells. Still, due to differences in intracellular metabolism between these drugs, one cannot equate in vitro potency with their effects on intact cells. The decrease in nuclear ADP-ribose concentrations after treatment of FDC-P1 cells with NAM is consistent with inhibition of nuclear ADPRT.

The lack of a demonstrable quantitative increase in nuclear poly-(ADP-ribose) levels in FDC-P1 cells after exposure to IL-3 does not necessarily imply that ADP ribosylation is not involved in the mediation of IL-3–induced proliferation. It is possible that qualitative rather than quantitative changes in nuclear ADP ribosylation occur in response to IL-3. Nuclear ADP ribosylation may be involved in cell proliferation in one of several possible ways. Histones are substrates for this reaction, and in vitro studies have shown that inhibition of DNA ligase activity by histones is abolished by ADP ribosylation. As mentioned earlier, rejoining of DNA strand breaks in resting lymphocytes has been shown to be an early event in their proliferation in response to mitogens, and ADPRT has been shown to participate in this process. In vitro studies using isolated rat pancreatic polynucleosomes have shown a direct correlation between polynucleosome relaxation, histone H1 ADP ribosylation, and activation of DNA polymerase activity. Finally, DNA topoisomerase I, an enzyme that has been implicated in the regulation of DNA replication, is ADP ribosylated in vitro by ADPRT. In vitro phosphorylation of topoisomerase II by PK-C has been reported, as has phosphorylation of topoisomerase I by other kinases. It is possible that the interaction between the phorbol ester–induced effects and the ADPRT inhibitors’ effects occur at the level of DNA topoisomerase activity.

REFERENCES

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