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MODULATION OF INFLAMMATORY ARTHRITIS BY INHIBITION OF POLY(ADP RIBOSE) POLYMERASE

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Abstract—Poly(ADPR) polymerase (PARP; EC 2.4.2.30) is a nuclear enzyme, which, when activated by oxygen- and nitrogen-radical-induced DNA strand breaks, transfers ADP ribose units to nuclear proteins and initiates apoptosis by depletion of cellular NAD and ATP pools. The present study investigates whether the oxidative stress-dependent activation of PARP plays a role in the etiopathogenesis of arthritis. The antiarthritic reactivity of the biogenic PARP inhibitor nicotinamide was tested in DBA/1 × B10A(4R) mice suffering from potassium peroxochromate-induced arthritis. Daily doses of 4 mmol/kg of NA suppressed the arthritis by 35% and inhibited the phagocytic generation of reactive oxygen species, which increases sixfold during the development of arthritis. The onset, progression, and remission of arthritis correlated positively to the phorbolester-activated respiratory burst of neutrophils and monocytes, and a dose-dependent inhibition of NADPH oxidase activity was determined with human phagocytes. Our data support the hypothesis that oxidative stress-induced alterations in cellular signal transduction pathways play a pivotal role in the development of arthritis, which can be suppressed by the simultaneous inhibition of poly(ADPR) polymerase and NADPH oxidase.

INTRODUCTION

The ubiquitous nuclear enzyme poly(ADP ribose) polymerase (PARP) catalyzes the synthesis of the nucleic acid-like homopolymer poly(ADP ribose), which consists of up to 300 repeating ADP ribosyl residues (1). The N terminus of the enzyme contains two zinc finger motifs, which assemble to nicks of DNA. The C-terminal domain binds NAD^+ and catalyzes the posttranslational modi-

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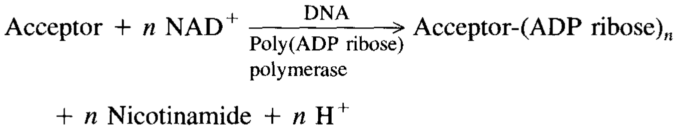
fication of nuclear proteins by covalent attachment of ADP ribosyl moieties derived from the splitting of NAD^+ to nicotinamide and ADP ribose:



$$\text{Acceptor} + n\ NAD^+ \xrightarrow[\text{Poly(ADP ribose) polymerase}]{\text{DNA}} \text{Acceptor-(ADP ribose)}_n + n\ \text{Nicotinamide} + n\ H^+$$

Preferred acceptor proteins are nuclear histones (H1, H2A, H2B, H3, H4, and H5), whose poly-ADP ribosylation induces local alterations in the architecture of chromatin domains. The induction of free DNA domains by removing histones from specific nucleosomes is a prerequisite step for all major chromatin functions, including replication, transcription, and repair of DNA. Excessive activation of PARP, due to numerous DNA strand breaks, depletes the cell of its intracellular NAD^+ pools and initiates apoptosis (2, 3). This suicide response to unrepairable intracellular stress assures that unwanted mutations do not arise.

Reactive oxygen species [ROS: superoxide ($O_2 \cdot^-$), hydroxyl radicals ($\cdot OH$), singlet oxygen ($^1\Delta g O_2$), and hydrogen peroxide (H_2O_2)] have come to the fore as powerful inducers of poly(ADP ribose) polymerase (4). Due to the partial collapse of the antioxidant system and subsequent cytokine-mediated hyperreactivity of mononuclear and polymorphonuclear leukocytes, patients suffering from inflammatory and autoimmune rheumatic diseases produce up to 30-fold increased levels of ROS and fivefold elevated levels of nitric oxides (5, 6). The subsequent induction of poly(ADP ribose) polymerase has recently been linked to synovial hypertrophy and pannus formation in rheumatic patients, and antibodies against poly(ADP ribose) and poly(ADP ribose)-anti-poly(ADP ribose) immune complexes have been detected in patients with systemic lupus erythematosus (SLE) and progressive systemic sclerosis (PSS) (7–9).

The present study investigates the antiarthritic reactivity of a biogenic PARP inhibitor in male DBA/1 × B10A(4R) mice suffering from potassium peroxochromate (K_3CrO_8)-induced arthritis (10). The in vivo decay of K_3CrO_8 to superoxide, singlet oxygen, hydroxyl radicals, hydrogen peroxide, and CrO_4^{2-} causes chronic inflammation and arthritis in mice and rats, mainly by depletion of intra- and extracellular antioxidant pools and concomitant inhibition of major antioxidases by CrO_4^{2-} (11, 12).

While the onset and progression of K_3CrO_8 arthritis is effectively modulated by NADPH oxidase inhibitors and active center analogs of Cu_2Zn_2 superoxide dismutase (13, 14), the influence of PARP inhibition on the course of arthritis has not been determined so far. To investigate the antiarthritic reactivity, the biogenic vitamin B-group PARP inhibitor nicotinamide was chosen for its well-known pharmacological safety and low toxicity both in humans and experimental animals (15). Nicotinic acid, which does not inhibit poly(ADP ribose) polymerase (3) served as a control. Disease activity was quantified by whole blood

chemiluminescence and compared to overt arthritic symptoms as judged by the standard arthritis index (10, 16). Potential antioxidant effects of poly(ADP ribose) polymerase inhibitors, which may influence the ROS–PARP network by decreasing oxidative stress-dependent DNA damage, were tested ex vivo by lucigenin-amplified chemiluminescence (10). The role of imbalanced pro- and antioxidant levels and poly(ADP) ribosylation reactions in the etiopathogenesis of rheumatic diseases is discussed in light of novel therapeutic strategies targeted to the simultaneous inhibition of PARP and NADPH oxidase.

MATERIALS AND METHODS

Animals and Housing. Male DBA/1 × B10A(4R) mice, weighing 25–30 g, were kept on a standard laboratory diet ad libitum. The animals were housed in groups of 10 in wire-topped polycarbonate cages with a layer of sawdust as bedding. The cages were located in a room routinely checked for specific pathogen-free conditions. The facility had controlled lighting (light: 0700–1900 h), temperature (22°C), and relative humidity (50%).

Chemicals. Unless otherwise indicated all chemicals were purchased from Sigma-Aldrich, Deisenhofen, Germany. Potassium peroxochromate (K_3CrO_8) was synthesized from H_2O_2, CrO_3, and KOH following standard procedures, with minor modifications (17): 200 ml of a 12.5% (w/v) solution of KOH in deionized water and 25 ml of a 50% solution of CrO_3 (w/v) were mixed, cooled to −4°C, and 30 ml of H_2O_2 (30%) added dropwise under vigorous stirring, carefully avoiding the increase of reaction temperature above 0°C. One hour later, the formed crystals were filtered and washed with ice-cold ethanol (90%; v/v), air dried, and kept in an evacuated desiccator at room temperature. Yield: 50% of $[H_2O_2]$.

Induction of K_3CrO_8 Arthritis. K_3CrO_8 was dissolved in 0.01 N NaOH. At alkaline pH, the aqueous solution of K_3CrO_8 is stable for a mininum of 2 h, but decays rapidly to superoxide, hydroxyl radicals, singlet oxygen, hydrogen peroxide, and chromate(VI) at physiological pH (17). The K_3CrO_8/NaOH solution was diluted with an equal volume of phosphate-buffered saline (PBS), pH 7.4, within a syringe and 3 μmol/kg of K_3CrO_8 administered immediately, by intraplantar application into the left hind-paws of mice anesthetized with diethyl ether. Solvent-treated animals served as controls.

Determination of Arthritis Index. The physical symptoms of arthritis were judged by a standard grading system routinely used to assess arthritis (18): 0 = normal paws; 1 = erythema of toes; 2 = erythema and swelling of paws; 3 = swelling of ankles; 4 = complete swelling of the whole leg and inability to bend it. The maximum achievable score is thus 16.

Assessment of Arthritis by Whole Blood Chemiluminescence (CL). Blood was collected from the median tail artery of mice into polysytrene tubes coated with 10 mM EDTA (Greiner, Frickenhausen, Germany) from five animals per group on alternate days. Human blood from healthy volunteers was obtained by venipuncture after informed consent. The formation of superoxide was determined by chemiluminescence in a Berthold LB 953 (Wildbad, Germany) luminometer in the presence of lucigenin, within 2 h of collection, on duplicate samples. Each 1-ml sample contained 100 μl EDTA-anticoagulated blood, 100 μM lucigenin, and 100 μM diethyldithiocarbamate in RPMI 1640, pH 7.4 (Gibco, Paisley, Scotland) without phenol red. The mixture was equilibrated for 10 min at 37°C and the reaction started by the automated injection of 0.5 μM 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) in deionized water. The resulting chemiluminescence was recorded for 60 min at 37°C and integrated.

Statistical Analysis. All data were analyzed with Mann-Whitney's nonparametric test using the Instat 2.01 statistics program (GraphPad, San Diego, California) for Apple/Macintosh and are presented as means ± standard deviations (*SD*). $P < 0.05$ was considered significant. Pearson's correlation coefficient (r) was used for the linear regression of data.

RESULTS

Groups of DBA/1xB10A(4R) hybrid male mice (which were part of a larger study on the genetics of resistance to arthritis) were intraplantarily injected with 3 μmol/kg of K_3CrO_8, and treated daily with 1, 4, or 16 mmol/kg of nicotinamide by the methods described above, and scored for visible signs of arthritis over a period of three weeks. Nicotinamide was chosen for use as an inhibitor of poly(ADP ribose) polymerase because of its low toxicity and well-defined pharmacological properties in man (15). Samples of blood were examined for their ability to generate chemiluminescence upon stimulation with TPA. In addition, the antioxidative capacity of various poly(ADP ribose) polymerase inhibitors (most of them too toxic for use in animals) was examined in unseparated human blood, and the concentration of inhibitor required to reduce the chemiluminescent phagocytic response by 50% (IC_{50}) was determined graphically.

Arthritis Index. The disease course shown in Figure 1 is representative of three other experiments, which gave similar results, with only minor variations of disease severity. Within 3 h of intraplanar application of potassium peroxochromate, 100% of the animals developed an acute inflammation, which persisted for more than three weeks. While the edema formation at the site of K_3CrO_8 injection peaked at days 2–3 and started slowly to decline, a secondary swelling developed in noninjected hind-paws, starting at day 8 and persisted for the complete period of observation. When 1–4 mmol/kg of nicotinamide were injected intraperitoneally one hour after the induction of arthritis and this treatment repeated daily, a 25–35% reduction of arthritis was observed. Higher concentrations of nicotinamide (16 mmol/kg) had no additive effect in this model. The acute inflammatory response (day 1) was immediately inhibited by nicotinamide and differed significantly from the disease controls ($P < 0.001$). Starting from the second application, the arthritis index declined readily. Nicotinic acid, which does not inhibit poly(ADP ribose) polymerase, reduced the primary inflammatory response (days 1–3), but exacerbated the secondary immunological response, which started at day 8.

Whole Blood CL. For the chemiluminescence assay, as started in Materials and Methods, duplicate samples were read within 2 h of collection, with an integration time over the oxidative burst from 0 to 60 min. Thus, 100-μl samples of whole, EDTA-stabilized murine blood, were checked for the generation of chemiluminescence upon stimulation with TPA, which initiates the

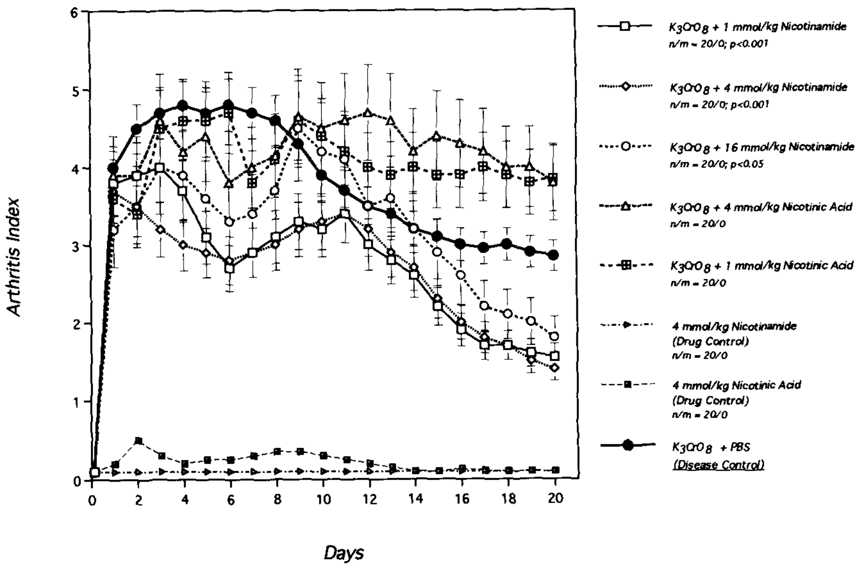


Fig. 1. Inhibition of potassium peroxchromate-induced arthritis by nicotinamide. Male DBA/1xB10A(4R) mice were intraplantarily injected with K_3CrO_8 and overt arthritic symptoms judged daily by the arthritis index. The curves were integrated and P determined with Mann-Whitney's nonparametric test. The data are presented as mean $\pm$ standard deviations. n = number of mice; m = mortality.

assembly and activation of the ROS-producing membranous enzyme NADPH oxidase and activates the chromatin-bound poly(ADP ribose) polymerase (1, 13). When compared to healthy mice, sixfold elevated levels of phagocytic superoxide production were detected in mice suffering from K_3CrO_3-induced arthritis. The CL measurements of the disease group differed significantly from healthy animals ($P < 0.001$). The CL curves showed biphasic kinetics. At day 3, the acute inflammatory response peaked with an average of 1.4×10^6 counts per hour (cph). The acute CL response then declined progressively until day 6. A secondary response followed, which peaked on day 12. The CL curve then began to wane slowly. The secondary CL response correlated to the development of arthritis in noninjected paws. The CL curves of healthy animals stayed at low levels of about 0.2×10^6 cph and did not vary significantly over the observed period. A 25–40% reduction of CL was monitored in both NA- and NAA-treated animals (Figure 2).

Correlating CL and Arthritis Index. The arthritis index and whole blood CL of K_3CrO_8-treated mice correlated positively with an overall correlation coefficient of $r = 0.863$. The arthritis index and Cl correlated best to the primary acute inflammatory phase (days 1–3) and to the onset of the secondary immu-

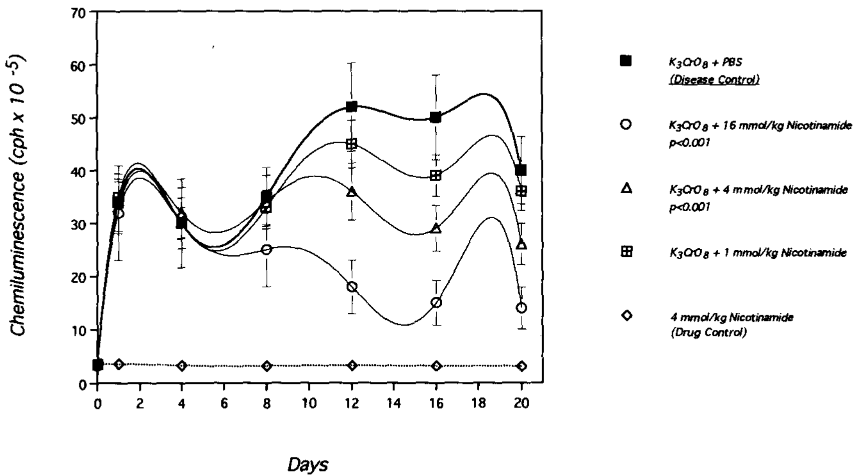


Fig. 2. Inhibition of whole blood chemiluminescence by nicotinamide in mice suffering from peroxochromate-induced arthritis. The chemiluminescence scale indicates counts per hour over the 60-min integration period. The data are presented as mean $\pm$ standard deviations.

nological response (days 7–12). In the presence of nicotinamide, the correlation coefficients declined linearly with increasing concentrations of NA, suggesting the dependence of arthritis progression on the activity of poly(ADP) ribose polymerase.

Nicotinamide-Dependent Inhibition of TPA-Stimulated ROS Production of Phagocytes in Whole Human Blood. When TPA-activated human blood was incubated with increasing concentrations of nicotinamide, the NADPH oxidase-dependent formation of superoxide was inhibited in a dose-dependent manner (Figure 3). The titrations were repeated twice and the IC_{50} determined graphically as 7.8 mM for nicotinamide. A 25% reduction of CL was obtained with 3.5 mM of nicotinamide, resembling the dose required to obtain a 25% inhibition of arthritis (Figure 1). The alternative inhibitors of poly(ADP ribose) polymerase, 3-aminobenzamide and benzamide, needed 3.8 and 95 mM, respectively, for the 50% inhibition of the oxidative burst. For nicotinic acid 5 mM were needed, and for N-methyl-nicotinamide (the primary intracellular biotransformation product of nicotinamide), 70 mM.

DISCUSSION

The present study reports the antiarthritic reactivity of the poly(ADP ribose) polymerase inhibitor nicotinamide in DBA/1xB10A(4R) mice suffering from potassium peroxochromate-induced arthritis. Daily doses of 4 mmol/kg of NA

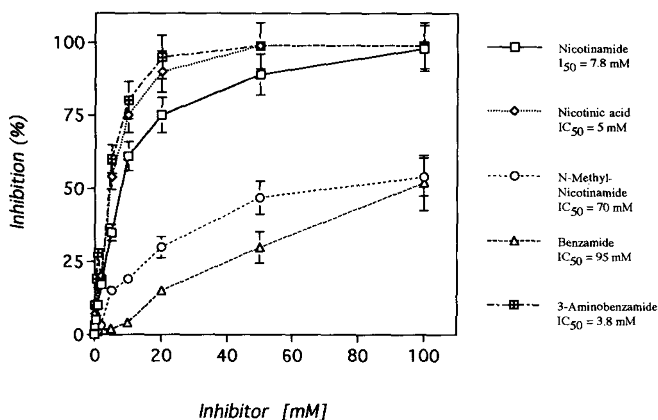


Fig. 3. Dose-dependent inhibition of phorbol-ester-stimulated whole human blood chemiluminescence. EDTA-stabilized human blood was titrated with increasing concentrations of nicotinamide, nicotinic acid, *N*-methyl-nicotinamide, benzamide, and 3-aminobenzamide, and the phorbol-ester-activated, lucigen-amplified chemiluminescence recorded for 60 min. The concentrations of inhibitor required for the 50% reduced chemiluminescence (IC_{50}) was evaluated graphically from the dose-response curves. The data are presented as mean ± standard deviations of triplicate titrations.

suppressed the arthritis by 35% and inhibited the phagocytic generation of reactive oxygen species. Nicotinic acid, which does not inhibit PARP, displayed no antiarthritic reactivity. The onset, progression, and remission of K_3CrO_8 arthritis correlated positively to TPA-activated phagocytic responses in whole blood. Poly(ADPR) polymerase-dependent adenoribosylation reactions have come to the fore to play a crucial role in cytokine-mediated signal transduction pathways. Thus, the PARP inhibitors nicotinamide and 3-aminobenzamide have recently been shown to abrogate TNF-α-mediated cytotoxicity by ADP ribosylation of a 90-kDA protein (19). TNF-α primes phagocytic NADPH oxidases to the enhanced production of oxygen radicals [13]. The prevention of neutrophil and monocyte priming by nicotinamide may partially explain the inhibition of whole blood chemiluminescence observed in our study.

TNF-α also plays a decisive role during the development of arthritis in mice (20). Transgenic mice, overexpressing this cytokine, develop arthritis at about 4 weeks of age, which can be completely suppressed by anti-TNF-α antibodies (21). Inhibition of arthritis can also be achieved with pentoxifylline, thalidomide, or $CuPu(Py)_2$, an active-center analog of superoxide dismutase (14).

In addition, ADP ribosylation is induced by nitric oxide, a potent mediator involved in the regulation of inflammation and nonspecific immunity (2). Elevated levels of nitric oxide, which are primarily produced by activated neutrophils and macrophages, have recently been demonstrated in serum of patients

with inflammatory or autoimmune arthritis (5, 6). Nicotinamide inhibits the induction of mRNA for NO synthase (22), suppresses the expression of the Ia antigen of the major histocompatibility complex in macrophages (22), and plays a role in antibody class switching of B cells (23). In addition, autoantibody formation in procainamide- or hydralazine-induced lupus was inhibited by PARP inhibitors and the histamine-induced endothelial generation of inositol phosphates repressed, thereby blocking the release of arachidonic acid and the production of prostacyclin PGI_2 (24, 25). These profound antiinflammatory effects of nicotinamide add to our findings of the antiarthritic reactivity of nicotinamide. The concomitant inhibition of poly(ADP ribose) polymerase and reduction of ROS formation by NADPH oxidase may act synergistically to diminish phagocytic hyperreactivity during arthritis and reduce excessive NAD consumption during apoptotic poly(ADP ribose) polymerase-dependent processes.

Further work is ongoing in our lab to evaluate antiarthritic mechanisms of nicotinamide in an autoimmune model of arthritis and to investigate synergistic effects of PARP inhibitors with low-molecular-weight antioxidases and TNF blockers.

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