Brief Definitive Report

LYMPHOCYTE DYSFUNCTION AFTER DNA DAMAGE BY TOXIC OXYGEN SPECIES

A Model of Immunodeficiency

By DENNIS A. CARSON, SHIRO SETO, AND D. BRUCE WASSON

From the Department of Basic and Clinical Research, Scripps Clinic and Research Foundation, La Jolla, California 92037

Immune attrition may occur in association with severe chronic infections and neoplastic diseases (1). Mechanisms of immunodeficiency in these disorders have not been elucidated precisely. During inflammatory states, activated neutrophils release toxic oxygen species into the extracellular environment (2). The oxygen products can damage the DNA of adjacent cells (3).

The accumulation of DNA strand breaks markedly stimulates the activity of the chromatin-bound enzyme poly(ADP-ribose) synthetase (4). The addition of poly(ADP-ribose) to nuclear proteins has been reported (5) to facilitate DNA ligation. However, the rapid synthesis of the ADP-ribose polymer can also deplete cellular pools of NAD^+ (6).

Recently (7), the unrestrained synthesis of poly(ADP-ribose) in lymphocytes with unrepaired DNA strand breaks has been postulated to be a possible cause of immune deficiency in children who lack adenosine deaminase. Herein, we document the central role of DNA strand breakage and the programmed synthesis of poly(ADP-ribose) in the genesis of lymphocyte dysfunction induced by exposure of normal resting human PBL to toxic oxygen species.

Materials and Methods

Lymphocyte Isolation and Culture. PBL were isolated from the heparinized peripheral blood of normal volunteers by Ficoll-Hypaque centrifugation. Cells were suspended in nicotinamide-free RPMI-1640, prepared from a Selectamine Kit (Gibco, Grand Island, NY), supplemented with 10% autologous plasma, 2 mM L-glutamine, and antibiotics (regular medium). Monocytes were depleted by adherence to plastic for 1 h at 37°C.

To assess the effects of oxidant damage, the lymphocytes were incubated at 37°C in 95% air, 5% CO_2 at a density of 1–2 × 10^6 cells/ml in nicotinamide-free RPMI-1640 containing 1% autologous plasma and 100 μM hypoxanthine. In some cases, the media were supplemented further with 3-aminobenzamide (3-ABA), nicotinamide, nicotinic acid, or other compounds, as indicated. Oxidation was initiated by the addition of xanthine oxidase to a final dilution of 100 mU/ml (Sigma Chemical Co., St. Louis, MO). 30–60 min later, the cells were washed and resuspended in regular medium at a density of 10^6 cells/ml. PHA 2.5 μg/ml (Sigma Chemical Co.), was added to some cultures, either immediately or 24 h later. After 3–5 d, replicate cultures were pulsed for 2 h with 1 μCi/ml methyl-[^{3}H]thymidine (sp act 69 Ci/mmol, ICN Radiochemicals, Irvine, CA). The incorporated radioactivity was determined by liquid scintillation counting.

This work was supported by grants GM 23200, AM 25443, AM 07144, CA 35048, and RR 00833 from the National Institutes of Health, Bethesda, MD. This is publication number 4123BCR from the Research Institute of Scripps Clinic, La Jolla, CA.

Biochemical Methods. DNA strand breaks in resting lymphocyte cultures were estimated by the fluorometric DNA unwinding assay developed by Birnboim and Jevcak (8), modified exactly as described previously (7). In this method, cell lysates are exposed to an alkali solution at pH 12.8 for 30 min to allow for DNA unwinding. Then ethidium bromide, which fluoresces more intensely when bound to double-stranded DNA, is added to each sample. The measurement of the fluorescence intensity compared to control cultures permits one to calculate the relative percentage of double-stranded DNA remaining after alkali treatment. This value correlates inversely with the number of strand breaks or alkali-sensitive sites (8). In Results, data are presented as percent double-stranded DNA remaining after exposure to alkali.

The activity of poly(ADP-ribose) synthetase in lymphocyte cultures was estimated by the permeabilized cell assay of Berger et al. (4). Briefly, lymphocytes were incubated for 15 min at a density of 2×10^6 cells/ml in a permeabilization buffer containing 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 4 mM $MgCl_2$, and 30 mM 2-ME. The cells were then centrifuged to a 50 μl volume of buffer and combined with 25 μl of a reaction mixture containing 100 mM Tris-HCl, pH 7.8, 120 mM $MgCl_2$, and 1 μCi adenine-2,8-[^{3}H]NAD^+ (sp act 25 mCi/mmol; ICN). 30 min later, the cells were collected on glass fiber filters, washed with ice-cold 20% TCA and ethanol, and counted. All experiments were performed at least in duplicate.

To measure intracellular nucleotide pools, lymphocytes were washed and extracted on ice with 0.4 M perchloric acid. After neutralization and removal of precipitates, extracts were frozen at −20°C for up to 1 wk. NAD^+ levels were measured by an enzymatic cycling assay that was linear up to 150 pmols (9). ATP levels were quantitated by HPLC.

Results

DNA Strand Breaks. To test the effects of toxic oxygen products on lymphocyte function in a controlled, model system, normal resting PBL were exposed in vitro to hypoxanthine and xanthine oxidase. The oxidation of hypoxanthine and xanthine by the enzyme produces superoxide and hydrogen peroxide (10, 11), which may react further with compounds in the medium and the cell membrane to yield other toxic oxygen products, and uncharacterized clastogens capable of damaging DNA (11, 12). As shown in Fig. 1, a 30-min exposure of resting PBL to xanthine oxidase and hypoxanthine dose-dependently induced DNA strand breaks, as measured by the fluorometric alkaline unwinding assay. In control experiments, treatment of the cells with enzyme or hypoxanthine alone did not cause DNA strand breakage.

Poly(ADP-ribose) Synthesis. To estimate the activity of poly(ADP-ribose) synthetase, permeabilized cells were incubated with [^{3}H]NAD^+, and the incorporation of the radioactive ADP moiety into acid-precipitable material was measured. Table I shows that treatment of resting PBL with a combination of xanthine oxidase and hypoxanthine stimulated NAD^+ incorporation by >10-fold. 3-ABA and nicotinamide are competitive inhibitors (with respect to NAD^+) of poly(ADP-ribose) synthetase (13). Continuous supplementation of the medium with 3-ABA or nicotinamide (5 mM each) suppressed poly(ADP-ribose) formation by 99% and 92%, respectively. The inhibitory actions of both compounds were reversible. However, sufficient 3-ABA remained in the culture after a single washing step to inhibit poly(ADP-ribose) synthesis by 37%. In control experiments, none of the compounds altered the extent of DNA strand breakage after exposure of lymphocytes to xanthine oxidase and hypoxanthine. Furthermore, neither 3-ABA nor nicotinamide changed the amount of superoxide released, as estimated by cytochrome C reduction (10).

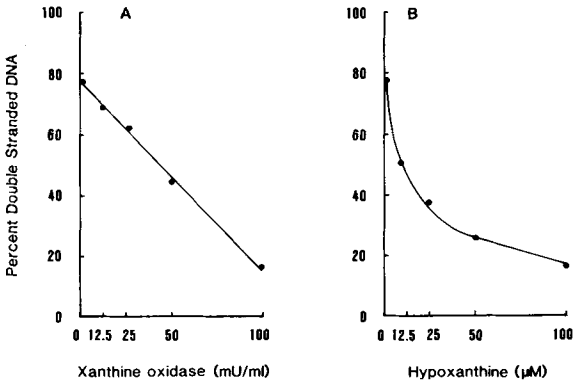

FIGURE 1. DNA damage in resting PBL exposed to toxic oxygen species. Resting lymphocytes were exposed to (*A*) xanthine oxidase (100 mU/ml) or (*B*) hypoxanthine (100 μM) for 30 min at 37°C. DNA strand breaks and alkali-labile sites were measured by the fluorometric DNA unwinding assay. DNA was treated with alkali solution for 30 min.

TABLE I

Poly(ADP-ribose) Synthesis in Resting Lymphocytes After Exosure to Toxic Oxygen Products

Addition of compounds			NAD^+ incorporation (pmols per 106 cells)	Percent inhibition
Incubation step	Permeabilization step	Poly(ADP-ribosyl)ation step		
—	—	—	40.2	—
XOD, Hyp	—	—	623.1	0
XOD, Hyp, 3-ABA	—	—	389.7	37
XOD, Hyp, 3-ABA	3-ABA	—	78.2	87
XOD, Hyp, 3-ABA	3-ABA	3-ABA	3.6	99
XOD, Hyp, NAM	—	—	568.8	9
XOD, Hyp, NAM	NAM	—	316.6	49
XOD, Hyp, NAM	NAM	NAM	48.1	92
XOD, Hyp, NIC	NIC	NIC	582.4	7

Lymphocytes were treated with 100 mU/ml xanthine oxidase (XOD) and 100 mM hypoxanthine (Hyp) for 30 min at 37°C (incubation step). After washing, the cells were resuspended in hypotonic solution for 15 min (permeabilization step). Subsequently, incorporation of [^{3}H]NAD^+ into acid-insoluble material was measured over a 30-min interval [poly(ADP-ribosyl)ation step]. 3-ABA, nicotinamide (NAM), or nicotinic acid (NIC), each at a concentration of 5 mM, was included in the medium during the indicated periods.

NAD^+ and ATP Levels. The excessive use of NAD^+ for poly(ADP-ribose) synthesis can deplete intracellular NAD^+ pools (6, 7). Within 15 min after exposure of the resting lymphocytes to xanthine oxidase and hypoxanthine, NAD^+ pools fell by 65% (Fig. 2*a*). Over the next 2 h, NAD^+ levels gradually returned toward normal. Intracellular ATP pools also dropped abruptly after exposure of lymphocytes to the enzymatic oxidizing system (Fig. 2*b*). However, ATP levels remained low, despite the partial restoration of NAD^+ pools.

Supplementation of the medium with 3-ABA or nicotinamide enabled the lymphocytes to preserve NAD^+ and ATP pools for at least 2 h (Fig. 2). The combination of 3-ABA and nicotinamide was no better than either alone. Nicotinic acid had no effect. Presumably, the major action of the nicotinamide was to inhibit NAD^+ consumption, rather than to promote NAD^+ biosynthesis.

Mitogen Responsiveness. A 60-min exposure of PBL to the enzymatic oxidizing system completely abolished their ability to respond to PHA (Fig. 3, *top*).

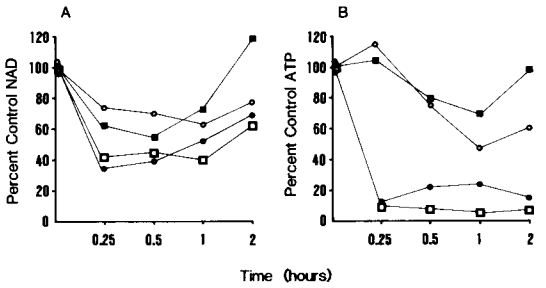

FIGURE 2. NAD^+ and ATP pools in resting lymphocytes after oxidant exposure. Resting lymphocytes were incubated in medium containing 100 mU/ml xanthine oxidase, 100 μM hypoxanthine, supplemented with (●) no additions; (○) 5 mM 3-ABA; (■) 5 mM nicotinamide; (□) 5 mM nicotinic acid. At the indicated times, NAD^+ levels (*a*) and ATP pools (*b*) were measured. Percent control = 100 × (NAD^+ or ATP in xanthine oxidase supplemented culture)/(NAD^+ or ATP in culture without xanthine oxidase). In control cultures, lymphocyte NAD^+ pools averaged 50–60 pmols per 10^6 cells, and ATP pools averaged 700 pmols per 10^6 cells. The results represent the means of three experiments.

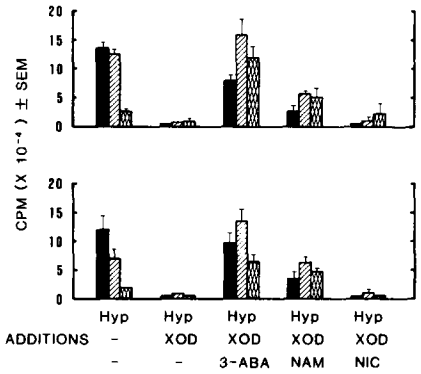

FIGURE 3. Response of oxidant-treated lymphocytes to PHA. Resting PBL were exposed to 100 mU/ml xanthine oxidase (*XOD*) and 100 μM hypoxanthine (*Hyp*) for 60 min in the presence of the indicated compounds, and then were washed and resuspended in regular medium. PHA (2.5 μg/ml) was added immediately (*top*) or 24 h after resuspension in fresh medium to allow for metabolic recovery (*bottom*). DNA synthesis was estimated by [^{3}H]-thymidine uptake at 3 d (solid bars), 4 d (hatched bars), or 5 d (crosshatched bars) after the addition of mitogen. The mean ± SD of three replicate experiments is shown. *3-ABA*, 5 mM 3-ABA; *NAM*, 5 mM nicotinamide; *NIC*, 5 mM nicotinic acid.

Depressed mitogen responsiveness was not restored by suspension of cells for 24 h in fresh medium (Fig. 3, *bottom*). However, lymphocytes responded to PHA normally if medium was supplemented with 3-ABA during exposure of cells to the oxidant stress. Nicotinamide exerted a similar protective effect, but was less potent.

Discussion

The exposure of resting human PBL to toxic oxygen species, generated by the combination of xanthine oxidase and hypoxanthine, caused massive DNA strand breakage. The DNA damage triggered a series of biochemical events that included the consumption of NAD^+ for the synthesis of poly(ADP-ribose), and an associated fall in NAD^+ and ATP pools. These events prevented the lymphocytes from synthesizing DNA after stimulation with PHA, and led to the eventual

death of the cells. However, if poly(ADP-ribose) synthesis was retarded with 3-ABA or nicotinamide, the lymphocytes responded vigorously to PHA.

Poly(ADP-ribose) formation resumed, at a slower rate, after removal of 3-ABA from the oxidant-treated lymphocyte cultures. Nonetheless, the mitogenic responsiveness of the cells was preserved for at least 24 h. These results suggest that lymphocyte inactivation required a massive and/or sustained increase in poly(ADP-ribose) synthesis. It is also possible that 3-ABA and nicotinamide exerted additional metabolic effects that protected the lymphocytes from oxidant toxicity. However, neither compound altered superoxide production nor reduced the quantity of DNA strand breaks in the xanthine oxidase and hypoxanthine–supplemented lymphocyte cultures.

Lymphocyte NAD^+ and ATP pools fell rapidly after DNA damage provoked by the cell-free oxidizing system. Using NAD^+ for poly(ADP-ribose) synthesis releases free nicotinamide. Resting PBL can convert nicotinamide to NAD^+, with the associated consumption of ATP (14). In the lymphocyte cultures treated with oxidants, NAD^+ pools eventually rose to ~60% of baseline levels. However, ATP pools remained very low. The continued use of ATP during NAD^+ resynthesis may partially explain this phenomenon. Additionally, the poly(ADP-ribosyl)ation of nuclear proteins may have impaired ATP-generating capacity in unknown ways.

In most cell types, poly(ADP-ribose) synthesis is thought to play a positive role in the DNA excision-repair process, especially at the DNA ligation step (5). However, massive poly(ADP-ribose) formation may also cause the destruction of cells with extensively damaged DNA (6, 7, 15).

One can hypothesize how such a suicide mechanism might serve a physiologic function in vivo. At sites of inflammation, phagocytes release diverse oxidant species. Activated phagocytes can induce sister chromatid exchanges, mutations, and DNA strand breaks in cultured mammalian cells (3, 11, 12). In preliminary experiments, we have noted that exposure of resting lymphocytes to activated autologous neutrophils caused massive DNA strand breakage. Perhaps some resting lymphocytes at inflammatory sites are destroyed via a programmed suicide mechanism that is dependent upon poly(ADP-ribose) synthesis.

To varying degrees, adenosine deaminase deficiency, certain DNA repair disorders (such as ataxia telangectasia and Bloom's syndrome), and severe chronic inflammatory diseases can all lead to the attrition of the immune system. The overproduction of poly(ADP-ribose), triggered by DNA strand breaks, could represent the common thread that links these apparently unrelated hereditary and acquired disorders associated with immunodeficiency.

Summary

The metabolic causes for immune impairment in patients with severe chronic inflammatory diseases have not been clearly defined. Recently, the overproduction of poly(ADP-ribose) in resting lymphocytes with unrepaired DNA strand breaks has been suggested to contribute to immune dysfunction in adenosine deaminase–deficient patients. Our experiments have determined to what extent DNA damage and poly(ADP-ribose) synthesis might also explain the impaired mitogen responsiveness of PBL exposed to toxic oxygen species. Treatment of normal resting human lymphocytes with xanthine oxidase and hypoxanthine

dose-dependently induced DNA strand breaks and triggered the rapid synthesis of poly(ADP-ribose). Subsequently, NAD^+ and ATP pools decreased precipitously. Lymphocytes exposed previously to the enzymatic oxidizing system did not synthesize DNA after stimulation with PHA. However, if the medium was supplemented with 3-aminobenzamide or nicotinamide, two compounds that inhibit poly(ADP-ribose) formation, cellular NAD^+ and ATP pools were preserved, and the lymphocytes responded vigorously to a mitogenic challenge. Excessive poly(ADP-ribose) synthesis, provoked by DNA strand breakage, may represent a common pathway that connects the immunodeficiency syndromes associated with (*a*) exposure of lymphocytes to toxic oxygen species during chronic inflammatory states, (*b*) adenosine deaminase deficiency, and (*c*) certain DNA repair disorders.

Received for publication 16 October 1985 and in revised form 16 December 1985.

References

1. Kantor, F. S. Infection, anergy and cell mediated immunity. 1975. *N. Engl. J. Med.* 292:629.
2. Klebanoff, S. J. Oxygen metabolites and the toxic properties of phagocytes. 1980. *Ann. Intern. Med.* 93:480.
3. Weitzman, S. A., and T. P. Stossel. 1981. Mutation caused by human phagocytes. *Science (Wash. DC).* 212:546.
4. Berger, N. A., G. W. Sikorski, S. J. Petzold, and K. K. Kurohara. 1979. Association of poly(adenosine diphosphoribose) synthesis with DNA damage and repair in normal human lymphocytes. *J. Clin. Invest.* 63:1164.
5. Creissen, D., and S. Shall. 1982. Regulation of DNA ligase activity by poly(ADP-ribose). *Nature (Lond.).* 296:271.
6. Skidmore, C. J., M. I. Davies, P. M. Goodwin, H. Halldorsson, P. Lewis, S. Shall, and A. A. Ziaee. 1979. The involvement of poly(ADP-ribose) polymerase in the degradation of NAD^+ caused by gamma irradiation and *N*-methyl-*N*-nitrosourea. *Eur. J. Biochem.* 101:135.
7. Seto, S., C. J. Carrera, M. Kubota, D. B. Wasson, and D. A. Carson. 1985. Mechanism of deoxyadenosine and 2-chlorodeoxyadenosine toxicity to nondividing human lymphocytes. *J. Clin. Invest.* 75:377.
8. Birnboim, H. C., and J. J. Jevcak. 1981. Fluorometric method for rapid detection of DNA strand breaks in human white blood cells produced by low doses of irradiation. *Cancer Res.* 41:1889.
9. Bernofsky, C., and M. Swan. 1973. An improved cycling assay for nicotinamide adenine dinucleotide. *Anal. Biochem.* 53:452.
10. McCord, J. M., and I. Fridovich. 1968. The reduction of cytochrome C by milk xanthine oxidase. *J. Biol. Chem.* 243:5753.
11. Fridovich, I. 1983. Superoxide radical. An endogenous toxicant. *Annu. Rev. Pharmacol.* 23:239.
12. Cerutti, P. 1985. Pro-oxidant states and tumor promotion. *Science (Wash. DC).* 227:375.
13. Purnell, M. R., and W. J. D. Whish. 1980. Novel inhibitors of poly(ADP-ribose) synthetase. *Biochem. J.* 185:775.
14. Berger, N. A., S. J. Berger, G. W. Sikorski, and D. M. Catino. 1982. Amplification of pyridine nucleotide pools in mitogen stimulated human lymphocytes. *Exp. Cell. Res.* 137:79.
15. Berger, N. A. 1985. Poly(ADP-ribose) and the cellular response to DNA damage. *Radiat. Res.* 101:4.