Elevated Production of Active Oxygen in Bloom’s Syndrome Cell Lines

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Abstract

Based on our previous evidence indicating that the elevated sister chromatid exchange that characterizes Bloom syndrome (BS) cells may arise in response to elevated production of active oxygen, we have quantitated the levels of active oxygen in two control, two BS and one BS revertant cell lines. Luminol-dependent chemiluminescence was used as a measure of active oxygen production following treatment of the cells with the calcium ionophore A23187 or the chemotactic tripeptide N-formylmethionylleucyl-phenylalanine. A peptide factor present in plasma was required for priming the cells to undergo the oxidative response. As determined with A23187, active oxygen production was elevated in BS cell lines by 48.6% above control. Using N-formylmethionylleucylphenylalanine, active oxygen production was found to be increased by 250–314%. Chemiluminescence was inhibited in a dose-dependent manner by diphenylene iodonium, which specifically binds to and inhibits membrane-associated NADPH oxidase activity. This compound inhibited oxygen radical production nearly 3 times more effectively in control cells than in BS cells. The capacity to produce elevated levels of oxygen radicals may contribute to the spontaneous chromosomal instability of BS cells and to the associated high incidence of neoplasia in individuals with BS.

Introduction

BS3 is a rare autosomal recessive disorder that exhibits numerous clinical features including sun sensitivity, growth retardation, and immunodeficiency leading to infections of the respiratory tract (1). The most prominent feature is a marked predisposition towards the spontaneous chromosomal instability observed in BS. It has been suggested that oxygen radicals may cause the spontaneous chromosomal damage observed in BS cells. It has been suggested that oxygen radicals may cause the spontaneous chromosomal damage observed in BS cells either through their excessive production (6) or through a deficiency in their removal (7). Indirect evidence that superoxide radicals are overproduced in BS cells has been provided previously. Thus, in all six BS cell lines studied we found superoxide dismutase activity to be elevated over that of controls (7). That finding has now been confirmed by another laboratory (8). Further, α-tocopherol, an inhibitor of lipid peroxidation, has been shown to inhibit the formation of SCEs in BS cells in a dose-dependent manner (7); finally, SCEs have been generated in control lymphoblastoid cell lines when the superoxide-generating compound, paraquat, was administered at a high concentration that induces SOD activity (7). Since SOD activity mirrors the cellular oxidative state in these cells, high dismutase activity is considered to reflect the level of oxidative stress (9). An elevated rate of formation of endogenously generated superoxide radicals acted on by an increased concentration of SOD is expected to result in high levels of hydrogen peroxide. Without a parallel induction of the catalase and peroxidase activities that would remove the hydrogen peroxide formed, considerable cellular damage is likely to occur.

Neutrophils possess a bactericidal activity attributable, in part, to their ability to generate oxygen radicals including superoxide, hydrogen peroxide, and hydroxyl radicals (10). Superoxide is produced primarily through the activation of a plasma membrane-bound NADPH oxidase system (11). The contribution that superoxide and its dismutation products make to the bactericidal capacity of competent cells is demonstrated by the susceptibility to infection of patients with chronic granulomatous disease, in whom there is present a defect in respiratory burst oxidases (12). Other cell types, including EBV-transformed B-lymphoblastoid cell lines, can also undergo an oxidative burst (13), although at a much lower rate than neutrophils. The function of the oxidative burst in these cells is not presently clear (13).

In this study we assessed the capacity of various BS cell lines to produce active oxygen following activation with either of two agonists, the calcium ionophore A23187 and the chemotactic tripeptide fMLP. We also examined the participation of NADPH oxidase in the oxidative burst occurring in these cells.

Materials and Methods

Cell Lines. The EBV-transformed B-lymphoblastoid BS cell line GM3403, control B-lymphoblastoid cell lines GM3299 and GM1953, and BS revertants GM4408 were obtained from the Human Mutant Cell Repository (Camden, NJ). The lymphoblastoid cell line HG1525 was a gift from Dr. James German (New York Blood Center, New York, NY).

Plasma Preparation. Fresh blood drawn from normal individuals prior to each assay was allowed to stand for 2 h, after which it was centrifuged for 10 min at 180 × g. The plasma layer was removed by pipetting and was again centrifuged at 400 × g for 10 min. The supernatant was once again removed, leaving behind any remaining cells pelleted to the bottom of the tube.

Chemiluminescence. Cells were grown in RPMI 1640 (GIBCO, Grand Island, NY) containing penicillin, streptomycin, and 10% fetal calf serum (HyClone, Logan, UT). Log phase cells were adjusted to a concentration of 4 × 105 cells/ml in fresh media. Aliquots (0.5 ml) of this cell suspension were placed into a cuvet, to which 50 μl of fresh centrifuged plasma and 15 μl of luminol (10 nm in 0.1 w borate buffer) were added. The cell preparation was incubated for 2 min and 1.0 to 5.0 μl of A23187 or fMLP (dissolved in dimethyl sulfoxide) was added. Incubation proceeded for the length of time required for each agonist to effect the maximal level of chemiluminescence. With the calcium ionophore A23187 (0.5–8.0 μM) maximum output was attained in 6 min, and with fMLP (2.5–600 nm) 3 min were required. The assays were conducted at 37°C with continuous stirring. For studies using the NADPH oxidase inhibitor DPI (17.5–210 nm) (obtained from Dr. O. T. G. Jones, University of Bristol, Bristol, United Kingdom), the cells were preincubated for 2 min with inhibitor prior to the addition of agonist. Apparent kinetic constants (Vₐ and Kₐ) for DPI on chemiluminescence were calculated using the non-linear least squares computer program NFTT (Island Products, Galveston, TX). Nondenatured and heat-denatured proteinase K (30 μg) (Boehringer Mannheim, Indianapolis, IN) was preincubated with plasma in the assay. SOD (10 μg) and catalase (20 and 40 μg) were also tested for inhibition of chemiluminescence both individually and in combination. Peak chemilu-
minescence was obtained by monitoring continuously with a Chrono-Log Model 800 Lumi-Aggregometer (Havertown, PA).

Results

Plasma Requirement. BS, BS revertant, or control EBV-transformed B-lymphoblastoid cell lines did not generate an oxidative burst without first being exposed to a small quantity of fresh plasma obtained from normal individuals (Table 1). This result is in contrast to the finding that under similar conditions EBV-transformed B-cells required only agonist for expression of oxidase activity (13). Lymphocytes isolated from whole blood were also incapable of undergoing an oxidative burst without addition of plasma (data not shown). The level of the oxidative burst obtained varied with the source of plasma, but the difference between the oxidative response of BS and control cells remained constant.

Characterization of the Oxidase-stimulating Plasma Component. Plasma obtained from freshly centrifuged blood demonstrated little or no capacity to stimulate NADPH oxidase activity when added to cells alone or in combination with agonist (Table 1). Allowing the blood to stand at ambient temperature for 2 h prior to centrifugation induced full oxidase activity (Table 1). Activated plasma stored in the cold for 2 days or heated at 60°C for 10 min lost its oxidase activity. Treatment of the plasma with 30 μg of proteinase K for 10 min also resulted in the loss of all oxidase activity (Table 1). These experiments indicate that the plasma component required for the activation of NADPH oxidase is a protein, which is either released from blood cells or processed in the plasma during standing at room temperature.

SOD and Catalase Inhibition of Chemiluminescence. Luminol-dependent chemiluminescence offers a highly sensitive means of detecting active oxygen, although it is not specific towards any one species of reactive oxygen. SOD (10 μg) inhibited 81% of the chemiluminescence, whereas catalase (40 μg) inhibited only 47% of the activity (Table 2). A catalase concentration (20 μg) that, by itself, was essentially inactive, complemented SOD in eliminating all chemiluminescence. These data indicate that superoxide is the major species of active oxygen generated and that hydrogen peroxide may subsequently be formed, likely through the dismutation of the superoxide radical.

Stimulation of Oxidase Activity by A23187 and fMLP. Activation of luminol-dependent chemiluminescence in response to the calcium ionophore A23187 demonstrated a 48.6% increase in oxidative activity in two separate experiments using one BS and one control B-lymphoblastoid cell line (Fig. 1, A and B). In order to compare their priming effect, plasma from two separate donors was used. Although the level of chemiluminescence attained differed slightly between the two experiments (Fig. 1, A and B), the response was consistently higher in BS cells. A sigmoidal dose-response curve is suggested for the cells stimulated with A23187.

Activation of all four B-cell lines by fMLP using plasma from the same donor consistently yielded an approximately 3-fold higher level of chemiluminescence in BS cell lines as compared to control cell lines (Fig. 1C). A repeat experiment using a different source of plasma showed a 2.5-fold increase in the maximal chemiluminescence in the BS cell line GM3403 as compared to control cell line GM3299 (Fig. 1D). The BS cell line HGI525, when compared to control cell line GM1953, also yielded an approximately 3-fold increase in active oxygen following activation by fMLP and another source of plasma (data not shown). Thus, the plasma is not responsible for the differential oxidative response displayed by BS and control cells. Activation of NADPH oxidase by fMLP generated a hyperbolic curve in contrast to A23187, which generated a sigmoidal curve. These results indicate that these two compounds activate NADPH oxidase by different mechanisms.

Oxidase Activity of a Revertant BS Cell Line. A B-lymphoblastoid cell line of BS origin, GM4408, is typical of BS cells that exhibit low SCE levels and are considered to be revertant cells. It has previously been demonstrated that this cell line also contains normal SOD (7) activity. We find (Fig. 1E) that normal levels of active oxygen are produced in this cell line following stimulation by fMLP.

Inhibition of Oxidase Activity by DPI. Evidence for participation of plasma membrane-bound NADPH oxidase in the oxidative burst that occurred after stimulation of cells was provided by the fact that the enzyme was inhibited by DPI (14). Control cells were inhibited nearly 3-fold more effectively than were BS cells, controls exhibiting an apparent $K_i$ of 40.1 nM and BS cells, 113.3 nM (Fig. 1F). This differential inhibition by DPI indicates a potentially aberrant protein present in the BS cells.

Discussion

Numerous DNA repair and processing defects have been proposed to explain the chromosomal instability associated with BS cells (15). Early studies reported relatively normal rates of DNA repair (16–20) in response to various types of photo- and chemically induced DNA damage. Recent studies report a defective uracil-DNA glycosylase enzyme (15, 21) and reduced DNA ligase I activity (22). However, a defect in the encoding glycosylase gene has not been reported and the DNA ligase I gene is normal and is normally transcribed (22). From the early studies emerged the proposal that an endogenous mutagenic factor produced in BS cells is the cause of the chromosomal damage (23). A small molecular weight clastogenic factor isolated from the media of BS cells or from the plasma of BS patients was reported to have caused chromosomal damage in normal cells (6). Addition of SOD reduced the level of chromosomal damage induced by the clastogenic factor (6). It was suggested thereby, that a deficiency in the removal of oxygen radicals plays a role in the spontaneous chromosomal damage observed in BS cells. However, several lines of indirect evidence have been advanced to support the hypothesis that spontaneous oxidative stress in BS cells rather than a deficiency in the removal of active oxygen is responsible for the observed chromosomal damage (7).

In the present study, luminol-dependent chemiluminescence was used as a measure of oxygen radical production following the activation of the cells with several agonists. The calcium ionophore pro-

### Table 1 Oxidase-stimulating activity of plasma in B-lymphoblastoid cells stimulated with fMLP under various incubation conditions

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<th>Treatment</th>
<th>Activity</th>
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<td>+</td>
<td>-</td>
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<td>+</td>
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<td>+</td>
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* Activity is 4.25 mV/2 × 10^5 cells.

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### Table 2 Active oxygen species released in fMLP stimulated B-lymphoblastoid cells as determined by SOD and catalase treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inhibition (%) ± SE</th>
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<tbody>
<tr>
<td>10 μg SOD*</td>
<td>81.5 ± 11.5</td>
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<tr>
<td>20 μg catalase b</td>
<td>5.5 ± 13.2</td>
</tr>
<tr>
<td>40 μg catalase</td>
<td>47.4 ± 11.5</td>
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<tr>
<td>10 μg SOD + 20 μg catalase</td>
<td>100</td>
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</tbody>
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* 3100 units/mg protein of SOD were used.

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duced a 48.6% increase in oxygen radicals and fMLP produced a 250-314% increase. In addition, the tumor promoter TPA was also shown to cause excessive production of oxygen radicals in BS cells (data not shown). In every case, BS cells produced significantly greater quantities of oxygen radicals than did control cells. It has previously been reported that activation of NADPH oxidase activity in EBV-transformed B-lymphoblastoid cell lines is not elicited by fMLP (13). The reason for the difference between our results and that of others is not clear. However, the major difference in our finding is the requirement for plasma; only when the cells are activated do they respond to a variety of agonist. Exploitation of these contrasting observations may lead to a clearer understanding of the activation process.

Participation of NADPH oxidase in the oxidative burst is strongly implicated by the dose-dependent inhibition of chemiluminescence with nanomolar concentrations of DPI. DPI has been previously shown to inhibit NADPH oxidase activity in both neutrophils (24) and in B-cell lines (25) through covalent binding to a $M$, 45,000 FAD-containing subunit of NADPH oxidase. The altered inhibition kinetics observed suggest a possible aberrant oxidase peptide. In contrast to chronic granulomatous disease, in which there exists a defect in one of several subunits of NADPH oxidase that results in reduced activity (12), we observe an increased activity in BS cells.

The suggested production of oxygen radicals at the plasma membrane is consistent with the hypothesis that SCE formation may be mediated by lipid peroxidation. This hypothesis is supported by the observation that SCE formation is inhibited by the addition of $\alpha$-tocopherol, a potent inhibitor of lipid peroxidation. We have preliminary data indicating that phospholipase A$_2$ activity is elevated in two BS cell lines and that this activity is decreased to levels present in control cell lines by treatment with $10^{-4}$ M $\alpha$-tocopherol. Phospholipase A$_2$ cleaves fatty acids including arachidonic acid from the C-2 position of phospholipids. Arachidonic acid is a well-known activator of NADPH oxidase (13, 26). Furthermore, several protease inhibitors have been...
shown to decrease SCE formation in BS cells by approximately 50% (27). Protease/protease inhibitor complexes are known to regulate NADPH oxidase-dependent superoxide production in neutrophils (28). However, the mechanism for altered regulation of NADPH oxidase activity in BS cells remains to be established.

In conclusion, the data presented in this paper represent the first direct demonstration of an increase in oxygen radical production in lymphoblastoid cell lines from Bloom’s syndrome. This increase in oxygen radical production supports the long debated notion that an endogenous mutagenic process takes place and contributes to the observed chromosomal damage that is characteristic of Bloom’s syndrome cells rather than a defect in DNA repair processing.

Acknowledgments

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References

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