The Effect of Oxidant Stress on Human Lymphocyte Cytotoxicity

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We have studied the effect of oxidant stress on human lymphocyte cytotoxicity. Lymphocyte cultures maintained good viability after incubation with xanthine and xanthine oxidase, an enzyme system known to generate several highly reactive oxygen compounds. We demonstrated that these lymphocytes were viable after exposure to an in vitro oxidant stress but had a significant impairment in both antibody-dependent and nonantibody-mediated cytotoxicity against the CEM lymphoblast target cell. Specific scavengers of reactive oxygen species were examined for a potential protective effect on lymphocyte function. Cultures supplemented with catalase demonstrated highly significant but incomplete protection from oxidant injury. In contrast, cultures supplemented with superoxide dismutase and mannitol were not protected. The impairment of lymphocyte function that resulted from the in vitro exposure to the xanthine and xanthine oxidase was mediated primarily through the generation of hydrogen peroxide. The scavenger studies indicated that superoxide, hydroxyl radical and singlet oxygen were probably not involved in producing the oxidant injury. Incubation of lymphocytes directly with hydrogen peroxide produced a similar functional impairment. Heat inactivation or chemical inhibition of the enzyme xanthine oxidase with allopurinol prevented oxidant injury and implied that the intact enzyme was necessary to enhance radiation injury possibly secondary to their metabolic by-products. These experiments were presented in part in abstract form at the American Society of Hematology meetings New Orleans, La., December 1978.

Human granulocytes and monocytes generate several highly reactive oxygen compounds during phagocytosis. These molecules, including hydrogen peroxide (H$_2$O$_2$), superoxide (O$_2^-$), and possibly singlet oxygen (O$_2$) and hydroxyl radical (OH$^-$), may be the biochemical mediators of the tissue damage resulting from inflammation. These highly reactive oxygen species may also mediate the tissue injury that results from ionizing radiation. Consistent with this hypothesis is the fact that radiation-induced injury is enhanced by exposure to oxygen. In addition, specific chemotherapeutic agents, including daunomycin and doxorubicin, enhance radiation injury possibly secondary to their generation of reactive oxygen species.

We have utilized the enzyme system xanthine-xanthine oxidase, which is known to generate these four highly reactive oxygen compounds in vitro, to study the effect of oxidant injury on lymphocyte function and have recently reported that the enzyme system xanthine-xanthine oxidase could impair lymphocyte function. The viability of the lymphocytes exposed to oxidant injury was preserved, as demonstrated by the cells' exclusion of trypan blue dye and the maintenance of normal resting glucose metabolism. We, therefore, concluded that the generation of hydrogen peroxide in vitro by xanthine-xanthine oxidase could impair the peroxide metabolism. We now present data that demonstrate that oxidant injury impairs additional lymphocyte functions. The generation of hydrogen peroxide in the present study impaired both the antibody-dependent and the nonantibody-dependent cellular cytotoxicity of human lymphocytes.

**MATERIALS AND METHODS**

**Preparation of Lymphocytes**

Lymphocytes were obtained from defibrinated venous blood of normal healthy volunteers. This blood was incubated with iron dextran (300 mg/10 ml blood) and mixed on a rotating wheel for 40 min at 37°C, and then separated by Ficoll-Hypaque density centrifugation. The mononuclear cells were harvested and washed twice with Seligmann's balanced salt solution. They were resuspended in complete RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 0.3 mg/ml l-glutamine, penicillin 50 U/ml, and streptomycin 50 U/ml. This preparation of effector cells, which consistently demonstrated greater than 97% lymphocytes, was incubated in the appropriate test system to determine if oxidant injury could impair lymphocyte function.

**Exposure to Oxidant Injury**

The first in vitro system utilized to test the effect of oxidant injury on lymphocyte function involved the incubation of these effector cells in the presence of the enzyme system xanthine-xanthine oxidase. This enzyme system is known to generate several highly reactive oxygen compounds in vitro. Lymphocytes were incubated for 4 hr at 37°C in a humidified 95% air, 5% CO$_2$ atmosphere under the following conditions: (A) in the presence of the complete RPMI, 10% fetal calf serum (FCS) medium alone, (B) in the...
presence of the complete RPMI, 10% FCS supplemented with 0.1 mM xanthine (Sigma, St. Louis, Mo.) alone, (C) in the presence of the complete RPMI, 10% FCS supplemented with 0.0075 U/ml xanthine oxidase (Sigma, grade 1, 0.75 U/mg protein) alone, (D) in the presence of both 0.1 mM xanthine and 0.0075 U/ml xanthine oxidase, and (E) in the presence of this complete enzyme system and known specific scavengers of highly reactive oxygen species. The scavengers utilized individually in the incubation mixtures included 500 U/ml catalase (Sigma, from beef liver, 36,000 U/mg protein), 10 μg/ml superoxide dismutase (Sigma, Type I, from bovine blood, activity approximately 3000 U/mg protein), and 40 mM mannitol (Merck, Sharpe, Dohme).

Lymphocytes were also incubated under the following test conditions to determine the importance of the functional integrity of the enzyme system in the production of the oxidant injury: (A) the cells were incubated with xanthine and xanthine oxidase, which had been previously autoclaved for 45 min to heat inactivate the enzyme system, and (B) in the presence of xanthine oxidase, which had been preincubated with allopurinol (Sigma) prior to supplementation with the substrate 0.1 mM xanthine. After the lymphocytes were removed from the various test incubation mixtures, they were washed twice in the complete RPMI medium with 10% FCS prior to being utilized in the cytotoxicity assay.

**Lymphocytes Exposed to Hydrogen Peroxide**

The second system utilized to test the effect of oxidant injury on lymphocyte function involved the specific incubation of these effector cells in 100 μM H₂O₂ in RPMI with 10% FCS at 37°C, and in 5% CO₂, for variable time periods as indicated in the results. After these effector cells were removed from this incubation mixture, they were washed twice in RPMI with 10% FCS prior to their use in the cytotoxicity assay.

**Preparation of the Target Cells**

The target cell utilized in the cytotoxicity assay was the CEM lymphoblast. This T-cell line was derived from a child with leukemia and lacks demonstrable Epstein-Barr virus genome. Lymphocytes Exposed to Hydrogen Peroxide

The target cell utilized in the cytotoxicity assay was the CEM lymphoblast. This T-cell line was derived from a child with leukemia and lacks demonstrable Epstein-Barr virus genome. This line was maintained in complete RPMI with 10% heat-inactivated FCS and was subcultured every 4 days. The target cells, which were utilized in the antibody-dependent cellular cytotoxicity (ADCC) assay, were coated with a specific rabbit anti-CEM antibody prior to use. The target cells used in the nonantibody cellular cytotoxicity assay (non-ADCC) were processed identically except for the incubation step with the anti-CEM antibody. In preparation for the cytotoxicity assay, the CEM lymphoblasts were washed once with Tris buffer solution, and 2 x 10⁶ cells were resuspended in a small volume (0.2 ml) of the buffer for labeling with ⁵¹Cr. This incubation with 100 μCi ⁵¹Cr proceeded for 60 min at 37°C. Both target cell populations were then washed 5 times with medium and resuspended to a final concentration of 3 x 10⁶ cell/microwell for the cytotoxicity assay. Viability of the CEM target cells was greater than 95% in each experiment as demonstrated by trypan blue dye exclusion.

**Production of a Specific Rabbit Anti-CEM Antibody**

A female New Zealand white rabbit was injected intravenously on day 1 with 10⁶ CEM lymphoblasts. One week later, this rabbit was injected subcutaneously into both flanks with an additional 10⁶ CEM lymphoblasts. Prior to this subcutaneous injection, these target cells had been emulsified with complete Freund's adjuvant. This subcutaneous injection was repeated after a 3-wk interval. After an additional 2 wk, serum was harvested from this rabbit, heat inactivated at 56°C for 30 min, and utilized as the source of anti-CEM antibody for labeling of target cells for the ADCC assay. Antibody-coated target cells were prepared by incubating 10⁶ CEM cells with 100 μl anti-CEM antibody at 37°C for 30 min.

**The Microcytotoxicity Assay**

The lymphocyte ADCC and non-ADCC assays were always performed in triplicate in 300-μl wells containing complete RPMI with 10% FCS. The effector to target cell ratio was maintained at 10:1 in all experiments. Thus, 3 x 10⁶ effector cells were added to the individual microwells that contained either the antibody-coated or the non-antibody-coated target cells (3 x 10⁶ cell/microwell). Microwells containing target cells alone were prepared to determine the spontaneous release of ⁵¹Cr from the individual target cell preparations. The microtest plates were then centrifuged at 50g for 5 min to initiate cell contact. The assay was then incubated at 37°C in a humidified atmosphere of 95% air, 5% CO₂ for 4 hr. After completion of incubation, the microtest plates were again centrifuged at 50 g prior to sampling 100 μl of the supernate to determine the amount of ⁵¹Cr released from the target cells. The percent cytotoxicity was then expressed as the percent ⁵¹Cr released into the supernate. Spontaneous release of ⁵¹Cr from the target wells over the 4-hr incubation in the assay was consistently 6%. The percent cytotoxicity was calculated by the following formula:

\[
\text{Percent cytotoxicity} = \frac{\text{CPM released in a test well} - \text{Spontaneous release}}{\text{CPM total added to each well} - \text{Spontaneous release}} \times 100
\]

**Statistical Analysis**

The data were analyzed using the Wilcoxon signed rank test and the Mann-Whitney U test.

**RESULTS**

The results depicted in Table 1 demonstrate that the in vitro exposure of human lymphocytes to xanthine-xanthine oxidase in 29 consecutive experiments resulted in a highly significant (p < 0.001) impairment in lymphocyte antibody-dependent cellular cytotoxicity. Although this specific function of the lymphocytes was significantly impaired, viability of these effector cells after exposure to this oxidant stress was consistently greater than 95% as determined by trypan blue dye exclusion. In 13 of those experiments, catalase (500 U/ml) was added to the incubation
medium containing the xanthine–xanthine oxidase. When catalase was present during the period of oxidant stress, it protected the lymphocytes ($p < 0.001$) from the functional impairment induced by this enzyme system. The data in Table 1 likewise demonstrate that the non-antibody-mediated cytotoxicity against CEM lymphoblasts was significantly impaired ($p < 0.001$) by exposure of the effector cells to the xanthine–xanthine oxidase system in 29 consecutive experiments. Significant protection was again afforded by including catalase in the incubation system ($p < 0.001$). Heat inactivation of the catalase afforded no protection from oxidant injury in either lymphocyte functional assay.

The protection from oxidant injury provided by catalase in both the ADCC and non-ADCC functional assays was significant ($p < 0.001$) but incomplete. Superoxide dismutase (a scavenger of O$_2^-$) and mannitol (a scavenger of OH$^-$) were used to determine if reactive oxygen species other than hydrogen peroxide contributed to the functional impairment of the lymphocytes. Lymphocytes exposed to either superoxide dismutase (10 μg/ml) or mannitol (40 mM) alone maintained greater than 95% viability and had no impairment in cytotoxicity. Addition of these scavengers to lymphocytes, incubated with xanthine–xanthine oxidase, afforded no protection from oxidant injury for these effector cells in the ADCC and non-ADCC assays (Table 2).

Since uric acid is generated by xanthine–xanthine oxidase, lymphocytes were incubated with a concentration of uric acid that could maximally be produced in our experimental xanthine–xanthine oxidase system. As seen in Table 3, incubation of the lymphocytes in 0.1 mM uric acid for 4 hr at 37°C in a humidified 95% air, 5% CO$_2$ atmosphere did not impair their function. Therefore, the production of uric acid by this system could not be implicated in the observed impairment of function. When experiments were done with xanthine oxidase, which had been either heat-inactivated or preincubated with allopurinol, addition of xanthine produced no impairment in lymphocyte cytotoxicity, indicating that active xanthine oxidase was required for impairment in lymphocyte function.

We confirmed that hydrogen peroxide in vitro was capable of impairing both lymphocyte ADCC and non-ADCC by incubating these effector cells directly with 100 μM H$_2$O$_2$. This direct incubation did not lower the viability or number of lymphocytes. Lymphocytes thus exposed to hydrogen peroxide were significantly impaired ($p < 0.002$) in both functional assays (Table 4). This impairment was maximal after exposure of the lymphocytes to this large dose of H$_2$O$_2$ for 30 min and remained for at least 48 hr after reculturing in fresh medium. Exposure of lymphocytes to the continuous production of a low concentration of hydrogen peroxide generated by the xanthine oxidase enzyme system resulted in a functional impairment that was also irreversible after 48 hr of reculturing these viable lymphocytes in fresh medium (Table 5).

**DISCUSSION**

The present study demonstrates impairment of two functions of normal human lymphocytes exposed to reactive oxygen species. The impairment in both the antibody-dependent cellular cytotoxicity and the non-antibody-mediated cytotoxicity was primarily a result of the hydrogen peroxide generated by the in vitro enzyme system as shown by the protective effect of catalase. Incubation of lymphocytes directly in hydrogen peroxide produced a similar lesion and thus supported the importance of hydrogen peroxide as a

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Sky which was also 6.5 - 1 standard deviation of 4 experiments.

<table>
<thead>
<tr>
<th>Antibody-dependent cellular cytotoxicity (ADCC)*</th>
<th>Control</th>
<th>X/XO</th>
<th>X/XO/SOD 10 μg/ml</th>
<th>X/XO/mannitol 40 mM</th>
<th>Non-antibody-dependent cytotoxicity (Non-ADCC)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>70.9 ± 6.5</td>
<td>12.1 ± 5.4</td>
<td>17.2 ± 9.9</td>
<td>7.1 ± 10.1</td>
<td>Control</td>
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<tr>
<td>X/XO</td>
<td>25.3 ± 3.5</td>
<td>3.8 ± 3.1</td>
<td>3.4 ± 3.3</td>
<td>3.2 ± 3.8</td>
<td>X/XO</td>
</tr>
<tr>
<td>X/XO/SOD 10 μg/ml</td>
<td>2.3 ± 3.5</td>
<td>3.8 ± 3.1</td>
<td>3.4 ± 3.3</td>
<td>3.2 ± 3.8</td>
<td>X/XO/mannitol 40 mM</td>
</tr>
<tr>
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<td>3.2 ± 3.8</td>
<td>X/XO/mannitol 40 mM</td>
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</tbody>
</table>

*Value represents the mean percent cytotoxicity ± 1 standard deviation of 4 experiments.

†X/XO, xanthine, 0.1 mM/xanthine oxidase 0.0075 U/ml.
‡SOD, superoxide dismutase.

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**Table 4. The Effect of Direct Exposure of Lymphocytes to Hydrogen Peroxide**

<table>
<thead>
<tr>
<th>Antibody-dependent cellular cytotoxicity (ADCC)*</th>
<th>Control</th>
<th>H$_2$O$_2$ (100 μM)</th>
<th>H$_2$O$_2$/catalase</th>
<th>Non-antibody-dependent cytotoxicity (Non-ADCC)*</th>
<th>Control</th>
<th>H$_2$O$_2$ (100 μM)</th>
<th>H$_2$O$_2$/catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>53.2 ± 12.1</td>
<td>16.0 ± 8.7</td>
<td>54.2 ± 10.7</td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H$_2$O$_2$ (100 μM)</td>
<td>10.2 ± 3.7</td>
<td>1.7 ± 0.9</td>
<td>9.2 ± 3.6</td>
<td>X/XO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H$_2$O$_2$/catalase</td>
<td>2.3 ± 3.5</td>
<td>3.8 ± 3.1</td>
<td>3.4 ± 3.3</td>
<td>X/XO/mannitol 40 mM</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Value represents the mean percent cytotoxicity ± 1 standard deviation of 6 separate experiments.
THE EFFECT OF OXIDANT INJURY ON LYMPHOCYTE FUNCTION

The generation of \( \cdot \text{O}_2 \) and hydroxyl radical by the xanthine oxidase enzyme system appears to require the interaction of \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \). Therefore, the fact that superoxide dismutase did not protect the lymphocytes against the effect of the xanthine oxidase enzyme system while catalase did implies that the \( \text{O}_2 \) and any \( \cdot \text{O}_2 \) or hydroxyl radicals derived from it were probably not involved in the injury caused by this system. Mannitol in concentrations as high as 40 \( \text{mM} \) likewise afforded no protection for the lymphocytes. Thus, the scavenger agents utilized in these experiments make it unlikely that superoxide, hydroxyl radical, or singlet oxygen were involved in producing the injury.

We previously have reported that a similar oxidant injury in vitro impairs the blast transformation of human lymphocytes. In that report, we showed that there was no alteration in the glucose metabolism of the unstimulated lymphocyte cultures as a result of exposure to the xanthine–xanthine oxidase system. In addition to the exclusion of trypan blue dye, that data provided further evidence that the lymphocytes were not killed as a result of the exposure to an oxidant stress. The mitogen-stimulated lymphocyte cultures, however, did not show the same increment in glucose metabolism that is characteristically seen with normal cultures. Thus, the impairment in the glucose metabolism of the mitogen-stimulated cultures was either directly related to the impairment in lymphoblastic transformation or simply reflected an additional lesion induced by the oxidant injury.

We have utilized the xanthine–xanthine oxidase in vitro system to simulate the biochemical events that might occur in vitro during an inflammatory response. Baehner et al. used a similar system to demonstrate that granulocytes not only produce highly reactive oxygen species but are also susceptible to autooxidation by hydrogen peroxide. They also demonstrated that the in vivo administration of vitamin E to human volunteers could attenuate the autooxidative injury to the granulocytes. Alteration in lymphocyte function in vivo may also occur as a result of the hydrogen peroxide generated by the phagocytic cells in inflamed tissue.

Both oxygen and specific chemotherapeutic agents are known to enhance the tissue injury induced by radiation. The tissue injury resulting from ionizing radiation is mediated through the generation of superoxide (\( \cdot \text{O}_2^- \)) and hydroxyl radical (\( \cdot \text{OH}^- \)). An existing enzymatic defense mechanism, superoxide dismutase, converts superoxide to hydrogen peroxide by the reaction: \( \cdot \text{O}_2^- + \cdot \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \). The hydrogen peroxide that is produced will likewise result in tissue injury unless it is eliminated by additional intracellular defense mechanisms.

Doxorubicin and daunomycin have recently been demonstrated to generate hydrogen peroxide in human erythrocytes in vitro. Pretreatment with the antioxidant tocopherol, before the administration of doxorubicin to mice bearing ascites tumors, markedly reduces lipid peroxidation in the heart and ameliorates cardiotoxicity without apparently impairing tumor response. The data, therefore, suggest that the enhancement of tissue injury resulting from certain combinations of chemotherapy and radiation therapy may be mediated through the production of reactive oxygen compounds. Patients with lymphoma, who have received combined modality therapy, including radiation and chemotherapy, have an impairment in their immune responsiveness. The possibility that this in vivo impairment in immune responsiveness is a result, in part, of oxidant injury to the immune effector cell is currently under investigation.

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REFERENCES

The effect of oxidant stress on human lymphocyte cytotoxicity

MR Grever, VN Thompson, SP Balcerzak and AL Jr Sagone