Unna Granulocytes and monocytes generate several highly reactive oxygen compounds during phagocytosis. These molecules, including hydrogen peroxide (H₂O₂), superoxide (O₂⁻), and possibly singlet oxygen (¹O₂) 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. We have recently reported that the generation of reactive oxygen species in vitro by the xanthine–xanthine oxidase system is a prerequisite for a variety of normal lymphocyte functions. We now present data that demonstrate that oxidant injury impairs two 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% CO₂ atmosphere under the following conditions: (A) in the presence of complete RPMI, 10% fetal calf serum (FCS) medium alone, (B) in the presence of complete RPMI, 10% fetal calf serum (FCS) medium supplemented with known scavengers of the other reactive oxygen species.
Preparation of the Target Cells

This subcutaneous injection was repeated after a 3-wk interval. On day 1 with 10^8 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 10^7 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 10% FCS. The effector to target cell ratio was maintained at 10:1 in all experiments. Thus, 3 x 10^6 effector cells were added to the individual microwells that contained either the antibody-coated or the non-antibody-coated target cells (3 x 10^5 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 media.
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 \((10 \mu g/ml)\) or mannitol \((40 \text{mM})\) 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 \(\mu M\) \(H_2O_2\). 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 \(H_2O_2\) 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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**Table 2. The Effect of Additional Scavengers on Oxidant Injury to Lymphocyte Cytotoxicity**

| Antibody-dependent cellular cytotoxicity (ADCC)* | 70.9 ± 6.5 | 12.1 ± 15.4 | 17.2 ± 9.9 | 7.1 ± 10.1 |
| Non-antibody-dependent cytotoxicity (Non-ADCC)* | 25.3 ± 3.5 | 3.8 ± 3.1 | 3.4 ± 3.3 | 3.2 ± 3.8 |

*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 3. Percent Cytotoxicity Lymphocytes Exposed to Uric Acid***

<table>
<thead>
<tr>
<th></th>
<th>ADCC</th>
<th>Non-ADCC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Uric Acid</td>
</tr>
<tr>
<td>Donor 1</td>
<td>74.0</td>
<td>74.2</td>
</tr>
<tr>
<td>Donor 2</td>
<td>67.7</td>
<td>64.6</td>
</tr>
</tbody>
</table>

*Concentration uric acid 0.1 mM in assay as described in text.
mediator of the injury. The highly significant but incomplete protection afforded to the lymphocytes by catalase raised the possibility that other reactive compounds in addition to hydrogen peroxide may be involved in the observed functional impairment.

The generation of 'O₂ and hydroxyl radical by the xanthine oxidase enzyme system appears to require the interaction of O₂ and H₂O₂. 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 O₂ and any 'O₂ or hydroxyl radicals derived from it were probably not involved in the injury caused by this system. Mannitol in concentrations as high as 40 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 impaired 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 (O₂⁻) and hydroxyl radical (OH⁻). An existing enzymatic defense mechanism, superoxide dismutase, converts superoxide to hydrogen peroxide by the reaction: O₂⁻ + O₂ + 2H⁺ → H₂O₂ + O₂. 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

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