Alteration of Lymphocyte Microtubule Assembly, Cytotoxicity, and Activation by the Anticancer Drug Taxol


Section of Natural Immunity, Departments of Surgical Oncology [L. T. C., E. L., K. R. C., A. M.] and Gynecologic Oncology [L. T. C., A. M., M. M., J. T. W.], The University of Texas M. D. Anderson Cancer Center, and Department of Pediatrics, Children's Nutrition Research Center, Baylor College of Medicine [J. H.], Houston, Texas 77030

ABSTRACT

We studied the effect of the anticancer drug taxol on the cytotoxic mechanism of major histocompatibility complex nonrestricted lymphocytes and their activation with interleukin 2. Unseparated lymphocytes or highly enriched natural killer or T-cells were treated with 0.2–10 μg/ml of taxol for various times and tested for cytotoxicity against the K562 cell line and the ovarian cell line, OV-2774. Taxol caused a dose- and time-dependent suppression of lymphocyte cytotoxicity. The most pronounced suppression was noted after treatment with 10 μg/ml of taxol for 6 h; a lower but significant decrease in cytotoxicity was observed after treatment with 2 and 5 μg/ml of taxol. In addition, taxol inhibited activation of lymphocytes with interleukin 2; however, the cytotoxicity of interleukin 2-activated lymphocytes was less sensitive to taxol treatment. Mechanism studies showed that taxol was not directly toxic to lymphocytes and did not alter their ability to form conjugates with target cells. Taxol treatment decreased a rate of kinetics of lysis and lymphocyte recycling ability. The immunofluorescence and electron microscopic analysis showed polymerization of microtubules in taxol-treated lymphocytes. These data demonstrate that taxol impairs lymphocyte cytotoxic function and activation and indicate the role of microtubules in these functions. Clinically, these findings suggest that activation of lymphocytes prior to taxol treatment may increase the therapeutic benefit of this drug.

INTRODUCTION

Taxol, a diterpenoid plant product isolated from the Western Yew, Taxus brevifolia, has been recognized as one of the important new chemotherapeutic agents (1–3). It has been shown to be effective in the treatment of several neoplasms, including melanoma and breast and ovarian carcinomas (1, 4). The action of taxol on cancer cells relates to the equilibrium of the microtubule system (5). However, unlike the other antimicrotubule agents utilized clinically (e.g., colchicine, vinblastine, and vincristine), taxol promotes assembly of microtubules from tubulin dimers and stabilizes them by preventing their depolymerization (5–7). Taxol contains a large oxetan ring, and it is the first compound with this ring to demonstrate cytotoxic action against tumor cells.

While taxol has been investigated quite actively for its effect on tumor cells, virtually no information is available on its effect on LY3 cytotoxic function, activation, and microtubule function and assembly. In light of the importance of LY-mediated cytotoxicity in anticancer defense (8–11), we examined the effect of taxol on the cytotoxicity, activation, and growth of NK and T-cells. We also studied the mechanism of cytotoxicity and changes in microtubule assembly in taxol-treated LY using immunofluorescence and electron microscopy.

MATERIALS AND METHODS

Preparation of Lymphocytes. Buffy coats with acid citrate dextrose, formula A (Fenwal Laboratory; Travenol, Deerfield, IL) as anticoagulant, were obtained from normal adults and separated on Ficoll-Hypaque (10). NK cells were enriched by separation on plastic and nylon wool columns, followed by the removal of T-cells, using sheep erythrocyte rosette technique (12). This fraction contained 72 ± 5% CD56+, CD3– NK cells, and 5 ± 0.4% CD56– , CD3+ T-cells as determined phenotypically and morphologically. T-cell-enriched fraction, obtained by Percoll separation (12), contained 93 ± 3% of CD3+ T-cells. Unseparated MNC were composed of 18 ± 2% CD56+, CD3– NK cells, and 74 ± 5% CD3+ T-cells.

Culture Medium and Growth Conditions. Control or taxol-treated LY (the dose of taxol is indicated in the “results”) were cultured at 106 cells/ml in RPMI 1640 (Whittaker Bioproducts, Walkersville, MD) supplemented with 10% heat-inactivated human AB serum (Pel Freez Freezer Systems, Brown Deer, WI), 10 μg 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (Flow Laboratories, McLean, VA), 2 μg glutamine (Gibco, Grand Island, NY), 50 μg/ml streptomycin, 500 units/ml penicillin, 50 μg/ml gentamicin, and 1000 units/ml of recombinant IL-2 (specific activity, 18 × 105 IU/mg; a generous gift of Cetus Corp., Emeryville, CA). The duration of culture is indicated in specific experiments.

Taxol Treatment of Lymphocytes. Taxol was a generous gift from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). It was kept as a stock solution of 10–4 M in DMSO at a final concentration of 0.1%, and stored at −20°C. EC were incubated with 0.2–10 μg/ml of taxol at 37°C in 5% CO2 for 1–6 h. The EC were then washed and diluted to the required concentration. The viability (trypan blue exclusion test) and the cell recovery for both the control LY (untreated but incubated in media or DMSO for the same time) and taxol-treated LY were >95%.

Target Cell Preparation and Cytotoxicity Assay. The K562 cell line was maintained as a maintained cell line in RPMI 1640, and the adherent ovarian tumor cell line, OV-2774, was maintained in L-15 medium (Gibco). Both media were supplemented with 10% heat-inactivated fetal bovine serum (Whittaker Bioproducts, Walkersville, MD), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, antibiotics, and glutamine. For the cytotoxicity assay, OV-2774 were collected from the flasks by incubating with trypsin-EDTA for 6 min, followed by washing twice with PBS.

For the 51Cr release assay, 5.0 × 103 (K562) and 2.5 × 104 (OV-2774) target cells were incubated for 30 min with 100 μCi (K562) or 50 μCi (OV-2774) of sodium chromate (51Cr) at 37°C in a 5% CO2. The target cells were then washed three times in RPMI 1640 and adjusted to 106 cells/ml. Cytotoxicity was tested in a 51Cr assay (10, 12). The LY were incubated at different effectortarget cell ratios (see “results”) for 4 h (K562) or 22 h (OV-2774). Spontaneous 51Cr release was analyzed by incubating target cells in medium alone and ranged from 8–10% for K562 and 22–25% for OV-2774. Maximum release, determined after HCl (2 N) treatment ranged from 90–100%. The percentage of specific cytotoxicity was calculated as

\[
\text{% Cytotoxicity} = \frac{\text{cpm experimental} - \text{cpm spontaneous}}{\text{cpm maximal} - \text{cpm spontaneous}} \times 100
\]

Flow Cytometry Analysis. Cell phenotypes were analyzed by MoAbs against CD3, CD8 (Ortho Diagnostics, Raritan, NJ), CD56 (Coulter Immunology, Hialeah, FL), CD16, CD11a, CD11b, CD18, and CD54 (Becton-Dickinson, Mountain View, CA) surface structures. Fluorescein isothiocyanate-
SUPPRESSION OF LYMPHOCYTE ACTIVATION AND CYTOTOXICITY BY TAXOL

labeled IgG1 and phycoerythrin-labeled IgG2a were used as control isotope antibodies. EC (1.25 × 10⁶) were labeled for 30 min at 4°C with 5 µl of the appropriate MoAb; washed twice in Hanks' balanced salt solution, and 0.1% sodium azide; and resuspended in 2% paraformaldehyde. Five thousand cells were counted on a FACScan analyzer (Becton-Dickinson). Two-color analysis was done by simultaneous incubation with two MoAbs.

**Single Cell Conjugation Assay.** Single cell conjugation assay was performed as described previously (13, 14). Briefly, target cells (2 × 10⁶/ml) were incubated with EC at a 2:1 ratio in 0.2 ml S-RPMI for 5 min at 37°C, centrifuged (1000 rpm for 5 min), and resuspended in S-RPMI. The cell mixture was then suspended in agarose, spread onto agarose-coated glass slides, and incubated for 4 h in S-RPMI with 10% fetal calf serum at 37°C and 5% CO₂; the slides were stained with 0.1% trypan blue for 5 min and fixed in 0.5% formaldehyde phosphate-buffered saline solution. The percentage of TBC was determined by counting 200 EC; percentage C-TBC by counting the dead target cells in 100 conjugates; and the frequency of C-TBC (i.e., the EC that killed the tumor) was calculated by the equation %TBC × %C-TBC. Each of the parameters was analyzed in duplicate.

**Analysis of Maximum Rate of Lysis and Recycling.** Maximum rate of lysis (Vₘₐₓ) and MRC were analyzed by the Michaelis-Menten kinetics model as described earlier (13). EC (1 × 10⁶) were incubated with 0.25 × 10⁶ to 4 × 10⁷ target cells in a 4-h ⁵¹Cr assay. Vₘₐₓ was determined by the Lineweaver-Burk equation (13). MRC was determined by combining data from the ⁵¹Cr and the conjugate assay by dividing Vₘₐₓ by the total number of killer EC (i.e., percentage of active EC multiplied by the number of EC in the Vₘₐₓ). MRC estimates the number of target cells that an active EC can kill in a 4-h assay.

**Immunofluorescence Study.** Taxol-treated NK cells (5 × 10⁵ cells/ml) were plated on 18-mm square glass coverslips (American Scientific; McGraw Park, IL) in 35-mm Petri dishes, fixed for 20 min with 3% formaldehyde, extracted with 0.5% Triton-X for 5 min, and incubated with 50 µl (1:15 dilution in PBS with 1% BSA) sheep anti-tubulin (Accurate Chemical & Scientific, Westbury, NY) for 30 minutes. The coverslips were washed in PBS, incubated with 50 µl (1:50 dilution in PBS with 1% BSA) of the fluorescein-conjugated rabbit anti-sheep antibody (Jackson Immunochromee Research, West Grove, PA) for 30 min, washed in PBS, and mounted with fluoromount-G (Southern Biotechnology, Birmingham, AL) on clean microscope slides. Cells were examined on a Zeiss Axiophot fluorescence microscope using a 63X oil immersion objective

**Electron Microscopy.** NK cells (5 × 10⁵ cells/ml) were incubated with 10 µg/ml taxol at 37°C for 6 h. Control and taxol-treated cells were pelleted and resuspended in 2.5% glutaraldehyde in PBS for 1 h at room temperature. The cells were then washed twice in PBS, postfixed for 30 min in 1% osmium tetroxide in 0.1 M cacodylate buffer at pH 7.2, dehydrated in ascending concentrations of ethanol, and embedded in Spurr’s resin. Thin (80-nm) sections were taken on an RMC MT7 ultramicrotome using a diamond knife stained with uranyl acetate and lead citrate and viewed with a Phillips CM12 electron microscope at 60 kV.

**Statistical Analysis.** The data were analyzed statistically using a paired Student t test and analysis of variance.

**RESULTS**

**Suppression of Lymphocyte Cytotoxicity by Taxol.** In the first series of experiments, we investigated the effect of taxol on the cytotoxicity of MNC. The MNC from five donors were pretreated with 0.2-10 µg/ml of taxol for 6 h, and their cytotoxicity was tested against K562 (NK-sensitive) and OV-2774 (relatively NK-resistant) cell lines. As illustrated in Fig. 1, taxol treatment resulted in a dose-dependent suppression of MNC cytotoxicity against both targets. The MNC suppression was most prominent after treatment with 10 µg/ml of taxol. Less, although significant, suppression was observed after treatment with 5 and 2 µg/ml of taxol; the dose of 0.2 µg/ml was ineffective. Since taxol was diluted in DMSO, we also investigated the effect of DMSO against K562 on LY cytotoxicity. The cytotoxicity of LY from 6 donors treated with DMSO (19.3 ± 4.7, 36.1 ± 9.1, and 48.6 ± 10.8 at 25, 50, and 100 effector:target ratios, respectively) did not differ from control LY (21.2 ± 4.4, 35.9 ± 7.3, and 51.4 ± 8.3 tested at the same ratios) but differed from taxol-treated LY (10.7 ±
3.2, 16.7 ± 4.7, and 23 ± 7.0; \( P < 0.05 \). Similar results were obtained when the cytotoxicity of these groups of LY was tested against OV-2774.

To determine which LY subset among MNC was sensitive to taxol treatment, we analyzed the cytotoxic potential of taxol-treated NK cells and MHC-NR T-cells and compared to an untreated population. These studies showed that the cytotoxicity of both NK and T-cells against K562 and OV-2774 targets was impaired after taxol treatment (Fig. 1) and indicated that taxol exerts suppressive effect on different LY subsets.

We next examined the kinetics of taxol-mediated suppression. MNC were incubated either in medium alone or with 10 \( \mu \)g/ml of taxol (the most suppressive dose) for 1, 3, and 6 h and examined for lysis of K562 and OV-2774 targets. It can be seen from Fig. 2 that the suppression of cytotoxicity was most apparent after treatment of MNC with taxol for 6 h, although lower cytotoxicity values were observed also after 1- and 3-h treatment.

**Mechanism of Taxol-mediated Suppression of Cytotoxicity.**

Phenotypic analysis of untreated and taxol-treated MNC and enriched NK cells did not show any differences in distribution of LY subsets (Fig. 3). This together with the observation that taxol-treated and control groups showed the same viability and recovery (>90%) indicated that taxol was not directly toxic to LY. Moreover, the taxol-treated LY did not display a change in the expression of several adhesion molecules (CD11a, CD11b, CD18, and CD54; data not shown), which may play a role in the cytotoxic process.

The next series of experiments were designed at dissecting which step of the MNC and NK cell cytotoxic mechanism (i.e., tumor-binding, lysis, kinetics of lysis, or recycling) was impaired by taxol. Data from a single cell assay showed that the ability of MNC and NK cells to bind to K562 or OV-2774 target cells was not altered by taxol (Table 1). Similarly, the percentage of C-TBC and their frequency were not different in taxol-treated and control groups. This difference between taxol-induced suppression of cytotoxicity detected in the \( { }^{51} \)Cr assay (the system where NK cells are free to recycle, i.e., to kill repeatedly) but not in a single cell assay (not allowing dissociation of tumor-LY conjugates and consequently LY recycling due to suspension of the conjugates in agarose) indicated that taxol inhibited an ability of LY to recycle. This postulation was confirmed experimentally using a combination of a single cell and a \( { }^{51} \)Cr assay and calculating \( V_{\text{max}} \) and MRC. Table 1 shows that \( V_{\text{max}} \) and MRC of taxol-treated MNC and NK cells were significantly lower against both target cells than that of the control group, demonstrating taxol interference with LY kinetics of lysis and recycling.

**Effect of Taxol on Lymphocyte Microtubule Assembly.**

Using the immunofluorescence technique and anti-tubulin antibodies, we analyzed the microtubule organization in normal and taxol-treated NK cells. While untreated NK cells had an array of microtubules that radiated diffusely from the microtubule organizing center around the nucleus, the taxol-treated NK cells exhibited prominent changes in microtubule assembly manifested by microtubule “bundling” (Fig. 4). The tubulin bundles were thicker and significantly brighter than the microtubules of untreated LY. This effect was more prominent in NK cells treated with 10 \( \mu \)g/ml of taxol, where the normal pattern was replaced by prominent short bundles of microtubules that did not pass around the nucleus (data not shown). The changed pattern of microtubules was also obvious on taxol treated NK cells by electron microscopy (Fig. 5). Based on the electron microscopy studies, other cell organelles did not appear to be affected by taxol treatment. These results suggest that the fully functional microtubules are required for optimal NK cell cytotoxicity.

**Taxol Interferes with IL-2 Activation of Lymphocytes.**

We also investigated the effect of taxol on activation of NK cells and MHC-NR T-cells with IL-2. Specifically, NK and T-cell populations were either untreated or treated with 10 \( \mu \)g/ml of taxol and then incubated with 1000 units/ml of IL-2 for 7 days. At that time, both LY groups were tested for cytotoxicity. Fig. 6 shows that taxol interfered severely with IL-2 activation; while IL-2 activated NK cells and T-cells not treated with taxol manifested substantial increase in cytotoxicity against K562 and OV-2774 target cells, virtually no lytic activity against these targets was displayed by taxol-treated, IL-2-activated populations.

It was of interest and clinical relevance to determine whether cytotoxicity of IL-2-preactivated LY was also inhibited by taxol treat-
SUPPRESSION OF LYMPHOCYTE ACTIVATION AND CYTOTOXICITY BY TAXOL

Table 1 Effect of taxol on tumor-binding, cytotoxicity, and recycling of MNC and NK cells

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Taxol-treated</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNC%TBC</td>
<td>23.0 ± 2.0 NS</td>
<td>22.0 ± 1.1 NS</td>
<td>NS</td>
</tr>
<tr>
<td>%C-TBC</td>
<td>13.6 ± 5.0</td>
<td>12.0 ± 5.6</td>
<td>NS</td>
</tr>
<tr>
<td>C-TBC frequency</td>
<td>3.2 ± 1.2</td>
<td>2.5 ± 1.1</td>
<td>NS</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>2.7 ± 5.0</td>
<td>0.7 ± 2.1</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>MRC</td>
<td>1.1 ± 0.05</td>
<td>0.4 ± 0.3</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NK cells</th>
<th>Control</th>
<th>Taxol-treated</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>%TBC</td>
<td>20.0 ± 2.0</td>
<td>21.0 ± 1.3</td>
<td>NS</td>
</tr>
<tr>
<td>%C-TBC</td>
<td>15.0 ± 5.0</td>
<td>16.0 ± 5.6</td>
<td>NS</td>
</tr>
<tr>
<td>C-TBC frequency</td>
<td>3.0 ± 1.2</td>
<td>3.4 ± 1.1</td>
<td>NS</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>9.4 ± 1.5</td>
<td>4.9 ± 1.2</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>MRC</td>
<td>3.2 ± 0.4</td>
<td>1.4 ± 0.3</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

* Mean ± SE, NS, not significant.

...ment. Data depicted in Fig. 7 clearly show that the cytotoxicity of IL-2-preactivated LY was also decreased; however, significant levels of cytotoxicity (substantially higher than those displayed by unstimulated LY) were observed after taxol treatment. This indicates that cytotoxic activity or IL-2-preactivated LY is less sensitive to taxol treatment.

DISCUSSION

Because of the promising chemotherapeutic possibilities of taxol in the treatment of several cancers (1), our investigations were designed to study the effect of taxol on function and activation of MHC-NR LY. In spite of extensive studies concerned with the antitumor effects of taxol, no information is available on how this drug regulates LY function and activation. Only one group of investigators tested taxol for its effect on LY; the limitations of this study have been that LY were treated only for a short time (60–90 min) with a single taxol concentration (2 μg/ml) and the cytotoxicity was tested only against one target cell. However, in therapeutic trials, taxol is administered in higher doses, and the LY are exposed to this drug for a longer time interval. Based on these incomplete data, it was concluded that taxol had no or only a minor effect on NK cell activity; additionally, it was deduced that microtubules are not important for LY lytic mechanism (15). The latter conclusion contrasted with the reports of other investigators (using systems other than taxol) demonstrating the important role of microtubules in LY cytotoxicity (16–18).

Fig. 4. Immunofluorescence staining of control and taxol-treated NK cells with antibody to α-tubulin. (a) phase contrast of control NK cells. (b) and (c), immunofluorescence analysis of control NK cells; microtubules are thin, elongated, radiating from the centrosome, and surrounding the nucleus; (d–i), immunofluorescence analysis of different profiles of taxol pretreated NK cells; microtubules are thickened, shortened, and brightened, arising from the centrosome, but not surrounding the nucleus. (× 2500).
SUPPRESSION OF LYMPHOCYTE ACTIVATION AND CYTOTOXICITY BY TAXOL

In order to clarify these inconsistencies in the literature and to carefully evaluate the effect of this therapeutically important drug on LY cytotoxicity, the NK and T-cells were incubated with various concentrations of taxol for different times, and their cytotoxic function was analyzed. We also investigated the effect of this drug on the activation of LY with IL-2 as well as the sensitivity of IL-2-preactivated LY to taxol treatment. We observed a dose- and time-dependent inhibition of cytotoxicity of NK and T-cells against two different target cell lines. The highest degree of inhibition was seen after 6 h of treatment with 5 and 10 μg/ml of taxol. In agreement with the above investigators, treatment of LY with 2 μg/ml of taxol resulted in less inhibition (15).

Studies on the mechanism of inhibition demonstrated that taxol did not decrease the number of LY, since no change in viability, recovery or distribution of LY subsets, or frequency of cytotoxic LY was observed after taxol treatment. Instead, our data have shown a decrease...
in the rate of kinetics of lysis ($V_{\text{max}}$) and in the ability of LY to kill tumor targets repeatedly, i.e., to recycle. The LY binding to tumor cells was unaffected by taxol.

The electron microscopy and immunofluorescence studies using antitubulin antibodies showed that taxol treatment resulted in polymerization, aggregation, or crystallization of microtubules in NK cells. These data support the reports of others using antimicrotubular agents other than taxol, implicating the microtubules in NK cell cytotoxic function (16–18). Our studies also showed that taxol inhibited the activation of NK cells and MHC-NR T-cells with IL-2, suggesting the importance of microtubules in IL-2-dependent activation. In contrast, IL-2-preactivated NK cells and MHC-NR T-cells showed lower sensitivity to taxol treatment and maintained higher cytotoxic levels than the unstimulated populations. These data may be of clinical interest. They suggest that the incomplete therapeutic effect of taxol may be due to its adverse effect on LY function, activation, and proliferation. If these LY-related adverse effects could be reversed and the antitumor effectiveness of taxol could be preserved, therapeutic benefit of this drug may be facilitated. Alternatively, the administration of taxol after rather than prior to immunotherapeutic intervention may result in retention of substantial levels of cell-mediated cytotoxicity and increase therapeutic benefits of this drug.

ACKNOWLEDGMENTS

We acknowledge Dr. C. A. Savary for careful proofreading of this manuscript, Cetus Corporation for the generous gift of recombinant IL-2, and Joyce W. Garner for her assistance in preparation of this manuscript.

REFERENCES

Altering of Lymphocyte Microtubule Assembly, Cytotoxicity, and Activation by the Anticancer Drug Taxol


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/54/5/1286

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.