Radiation Sensitivity of Resting and Activated Nonspecific Cytotoxic Cells of T Lineage and NK Lineage

By Daniela Zarcone, Arabella B. Tilden, Vanessa G. Lane, and Carlo E. Grossi

Natural killer (NK) cell-mediated killing of tumor cells is a radiation-sensitive function that in most subjects is completely abrogated by treatment of the effector cells with 3,000 cGy. The radiation sensitivity of LAK (lymphokine-activated killer) cells and their precursors, the bulk of which are NK cells, is undetermined. In this study, functional cytotoxicity assays and electron microscopy were used to determine the effect of radiation on the cytotoxic function of NK cells. LAK cells (generated by three-day culture of peripheral blood lymphocytes with IL-2), and LAK cell precursors (lymphocytes irradiated prior to culture with IL-2). For comparison, we analyzed the radiation sensitivity of lectin-dependent cell-mediated cytotoxicity (LDCC), which is primarily a function of CD3+CD8+ granular lymphocytes. We also analyzed the radiation sensitivity of nonspecific cytotoxicity mediated by mitogen-activated T cells (AK activity). Following 3,000 cGy irradiation, NK cells retained their ability to bind to tumor cell targets but, as shown by both morphologic and functional analyses, they did not undergo activation after conjugate formation, and were unable to release the content of their granules. In order to evaluate LDCC, lymphocytes were depleted of CD16+ cells and tested in a cytotoxicity assay in the presence of Con A. The radiation sensitivity curve was comparable to that of NK cell-mediated cytotoxicity. IL-2-treated lymphocytes (LAK cells) were relatively radiation-resistant as compared with untreated NK cells, and their cytotoxic function was not abrogated until treatment with >10,000 cGy. Cells receiving such radiation doses displayed cytoplasmic blebbing and damage of their cytoskeletal structures, with disruption of centrioles and microtubules, and disarray of the intermediate filaments. As was shown with NK cells, irradiated LAK cells formed conjugates with tumor targets but failed to degranulate. The radiation sensitivity of nonspecific cytotoxicity mediated by mitogen-activated T cells was identical to that of LAK effector cells. Doses up to 2,000 cGy did not prevent generation of LAK cells from blood lymphocytes, but 3,000 cGy did so. Blast transformation similar to that observed in IL-2-stimulated controls occurred when lymphocytes irradiated with 3,000 cGy were cultured with IL-2. These transformed cells were not cytotoxic and displayed a normal cytoskeletal apparatus but did not bear electron-dense granules. The similar pattern of radiation sensitivity observed for NK cells and lectin-dependent cytotoxic T cells, and for IL-2-activated NK cells (LAK) and mitogen-activated T cells (AK) indicated that the genetic mechanisms responsible for the control of radiation sensitivity were similar for cells of different lineages.

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four days in RPMI-FCS in the presence of 100 IU of recombinant IL-2. The recombinant IL-2 was generously provided by Biogen, Inc (Boston, MA). To determine the effect of radiation on LAK cell precursors, aliquots of lymphocytes were irradiated prior to culture with IL-2. The cytotoxic activity of irradiated cells and control cells was determined following a three-day culture. For the generation of AK cells, lymphocytes were depleted of CD16 cells as described above and cultured for three days with 1.5 μg/mL PHA-P.

Radiation procedures. Aliquots of the different effector cell populations were irradiated at room temperature in a 177Cesium source irradiator (Gammacell 1000, Atomic Energy of Canada, Ltd). An aliquot of cells was maintained at room temperature without receiving irradiation. After irradiation, cells were placed on ice and were used in the cytotoxicity assay within one hour.

Immunofluorescence binding assay. The ability of control and irradiated cells to form conjugates with tumor cell targets was determined using a previously described immunofluorescence binding assay. Prior to the binding assay, cells were stained with Leu 1a (Becton Dickinson) anti-CD16 antibody labeled with fluorescein isothiocyanate, admixed with K562 at a 5:1 ratio, centrifuged at 500 rpm, incubated in a 37°C H2O bath for five minutes, gently resuspended and wet mounted. We determined the percentage of CD16+ cells forming conjugates with K562 cells and the percentage of target binding cells which expressed the CD16 antigen.

Cytotoxicity assays. Target cells were the standard NK-sensitive K562 cell line or the NK-resistant, LAK-sensitive, LDCC-sensitive HL60-R cell line. The characteristics of the latter cell target have been described in detail elsewhere. Briefly, HL60-R cells are NK resistant because of their inability to form conjugates with NK cells. Target cells were labelled with 51Cr by incubation with 200 μCi of Na51CrO4 (Amersham Corp. Arlington Heights, IL) for one hour at 37°C as described.

Effector cells (E) were admixed with target cells (T) at 40:1, 20:1, and 10:1 E:T ratios in the NK and AK assays, and at 20:1, 10:1, and 5:1 E:T ratios in the LAK assay. For the LDCC assay, effector and target cells were incubated at a 40:1 E:T ratio in the presence of 10 μg/mL Con A. In all assays the cell mixtures were incubated for four hours at 37°C in round-bottomed 96-well microtiters and centrifuged at 200 rpm for five minutes at the end of the incubation period. After centrifugation, 100 μL of supernatant were harvested from each well and counted in a gamma counter. Spontaneous release, maximum release, and percent specific 51Cr release were determined as previously described. In the irradiation experiments, data are expressed as percent specific 51Cr release and also as percent of remaining activity after irradiation. The latter value was calculated as:

% lysis by irradiated cells = % lysis by non-irradiated cells × 100.

NK cell activation by target cells. This experimental system has been described in detail. In summary, the effector cells and the target cells were preselected according to their density, using a modified Percoll gradient. Effector cells collected at the bottom of the gradient and target cells recovered at the interface were subsequently admixed and allowed to form conjugates. After four-hour incubation in a petri dish, the conjugates were dissociated with 10 mmol/L EDTA and vigorous vortexing. The effector and target cells were subsequently separated on the same Percoll gradient used for preselection. The residual cytotoxic activity of the recovered effector cells was then tested as described above and expressed as percent of remaining activity in comparison with nonirradiated effector cells.

Electron microscopy. Control and irradiated effector cells were processed for electron microscopy as described. In addition, we analyzed the ultrastructural features of control and irradiated cells following conjugate formation with K562 target cells. All of the cell preparations were fixed with 1.25% glutaraldehyde for one hour at room temperature, post-fixed with 1% osmium tetroxide and embedded in Spurr’s medium. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a Philips 301 electron microscope.

RESULTS
Radiation sensitivity of resting cytotoxic effector cells. In this section we report experiments analyzing the effect of irradiation on the cytotoxic function of NK cells and on their morphologic characteristics. These studies were performed on both untreated NK cells and on NK cells activated by exposure to tumor target cells following irradiation. For comparison we investigated the radiation sensitivity of T cells in an LDCC assay.

In the first series of experiments we treated lymphocytes with radiation doses ranging from 500 to 3,000 cGy. The results of these experiments confirmed previous observations showing that a 3,000 cGy dose abrogates the lytic function of NK cells (Table 1). We next investigated the conjugate-forming ability of irradiated CD16+ lymphocytes. While 3,000 cGy irradiation abrogated the NK activity of these cells, their ability to bind to K562 cells was unaffected (Table 2).

The effect of irradiation on the activation events that occur in the effector cell following its interaction with the tumor target was analyzed. In this postbinding phase, the effector cell shows distinctive changes in its shape and structure with uropod formation, redistribution of cytoskeletal structures and granules, and degranulation. This is paralleled by a decrease in the cytolytic ability of the effector cells when they are subsequently exposed to another target cell. Ultrastructural studies demonstrated that at irradiation doses up to 1,500 cGy the effector cells underwent the typical morphologic changes that occur in the postbinding phase (Fig 1A-C). In contrast, following a 3,000 cGy radiation dose, no uropod formation, redistribution of the granules and degranulation were observed (Fig 1D), even though normal conjugate formation could be shown by an immunofluorescence binding assay (Table 2). Effector cells exposed to tumor target cells under the same experimental conditions used for the morphologic analysis were studied for their residual cytolytic activity upon re-exposure to tumor cell targets. In these experiments, cells were irradiated with doses up to 1,500 cGy. Irradiated cells were activated by target cell

| Table 1. Effect of Irradiation on NK Activity |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Irradiation Dose (cGy) | 40:1 | 20:1 | 10:1 | 0 | 39.0 ± 13.5 | 25.8 ± 9.6 | 17.1 ± 6.9 |
| 500 | 41.8 ± 11.3 | 31.1 ± 10.0 | 19.8 ± 6.1 |
| 1,000 | 39.6 ± 11.8 | 26.3 ± 10.0 | 7.0 ± 6.4 |
| 2,000 | 11.1 ± 3.6 | 6.6 ± 7.4 | 1.0 ± 0.5 |
| 3,000 | 1.5 ± 0.2 | 1.0 ± 0.5 | 1.0 ± 0.5 |

Effector cells were PBMC depleted of monocytes; target cells were 51Cr-labeled K562 cells.

†Data shown are the mean ± SD from five experiments using effector cells from different donors.

In cGy.
Table 2. Effect of Irradiation on the Conjugate-Forming Ability of CD16+ Cells (NK Cells)

<table>
<thead>
<tr>
<th>Cells</th>
<th>0 cGy</th>
<th>3,000 cGy</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD16+ CF*</td>
<td>91.3 ± 3.2</td>
<td>88.6 ± 5.9</td>
</tr>
<tr>
<td>CD16+ TB†</td>
<td>38.0 ± 12.4</td>
<td>35.3 ± 13.6</td>
</tr>
</tbody>
</table>

As determined by an immunofluorescence binding assay (see Materials and Methods).

*The percentage of CD16+ cells forming conjugates with K562 cells.
†The percentage of target binding cells expressing the CD16 antigen.

exposure more efficiently than nonirradiated cells, as shown by their lower residual cytotoxic activity (Table 3).

In another series of experiments we tested lymphocytes depleted of NK (CD16+) cells for their cytotoxic activity in the presence of Con A (LDCC). These cells were irradiated at the same doses used for NK cell assays. As can be seen in Table 4, the NK cell depletion was effective as shown by the lack of K562 target cell killing in the absence of Con A. In these experiments we also used the HL60-R cell target, which is more susceptible to LDCC in comparison to K562 cells. Data in Table 4 show that this T cell-mediated cytotoxic function had the same radiation sensitivity as NK

Table 3. Effect of Irradiation on NK Cell Activation by K562 Target Cells

<table>
<thead>
<tr>
<th>Irradiation Dose</th>
<th>Effector Cell</th>
<th>E:T</th>
<th>Untreated</th>
<th>Target Cell-Activated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 cGy</td>
<td>10:1</td>
<td>11.3†</td>
<td>4.6 (40)†</td>
<td></td>
</tr>
<tr>
<td>500 cGy</td>
<td>20:1</td>
<td>14.1</td>
<td>5.8 (41)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40:1</td>
<td>21.1</td>
<td>8.6 (40)</td>
<td></td>
</tr>
<tr>
<td>1,500 cGy</td>
<td>10:1</td>
<td>10.2</td>
<td>2.3 (22)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20:1</td>
<td>20.1</td>
<td>5.3 (26)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40:1</td>
<td>17.0</td>
<td>2.0 (11)</td>
<td></td>
</tr>
</tbody>
</table>

Lymphocytes irradiated with the doses indicated were either untreated or exposed to K562 cells, subsequently recovered, and tested for residual cytotoxic activity against K562 target cells.

†In cGy.

†Data shown are % specific 51Cr release from one representative experiment.

†Values in parentheses are % remaining activity after pre-exposure to target cells.

Fig 1. Ultrastructural features of resting and K562-activated NK cells. (A) A resting NK cell that showed typical granular lymphocyte features. (B) An NK cell treated with 500 cGy and subsequently exposed to K562 cell target. Activation was shown by uropod formation and almost complete degranulation. (C) A detail of the cell shown in B; centrioles and reorganization of microtubules were of note. (D) An NK cell treated with 3,000 cGy and subsequently exposed to K562 cell target. The lack of uropod formation and granule redistribution was evident. A few long microvilli or plicae suggested frustrated activation. Magnification was as follows: (A) ×9,000; (B) ×7,500; (C) ×19,000; and (D) ×5,600.
Preparations so obtained contained cytotoxicity. Further depleted of CD16 cells by antibody and complement-mediated blebbing and disintegration of centrioles and microtubules (Fig 2C and D).

In order to evaluate the effect of irradiation on non-specific cytotoxicity mediated by activated T cells (AK), lymphocytes were depleted of CD16+ cells, cultured for three days with PHA-P (1.5 μg/mL), and subsequently tested for cytotoxicity against K562 target cells. The same range of radiation doses was used for AK cells as was used for LAK cells. As shown in Table 6, a gradual reduction in the cytotoxic activity of AK cells was observed from doses of 5,000 cGy up to 15,000 cGy.

Radiation sensitivity of LAK cell development. In order to test the radiation sensitivity of LAK cell development, lymphocytes were irradiated with 2,000 and 3,000 cGy and subsequently cultured with IL-2. A dose that impaired NK cell function (2,000 cGy) also hindered the ability of IL-2 to increase the cytolytic activity of these cells, i.e., to induce LAK activity (Table 7). A dose that abrogated NK cell function (3,000 cGy) also abolished the ability of these cells to respond to IL-2 with increased cytotoxicity. Surprisingly, the appearance of cells from IL-2 cultures after irradiation was similar to control cultures in that we observed cell clustering and blast transformation in both cases (not shown). This prompted an evaluation of the ultrastructure of these cells in order to help determine the reason for lack of cytotoxic activity with transformed cells. As shown in Fig 3A, blast cells derived from lymphocytes irradiated prior to IL-2 culture were largely devoid of electron-dense granules, although the cytoskeletal structures were intact (Fig 3B). It thus appears that irradiation prior to IL-2 culture prevents the process of granule formation that results in the higher cytotoxic ability of LAK cells vs their unstimulated precursors.

DISCUSSION

In agreement with previous reports, NK cell function was abrogated by a dose of 3,000 cGy in most individuals. We further examined the effect of this dose of radiation on the...
Fig 2. Ultrastructural features of K562-activated LAK cells. The irradiation preceded the exposure to the tumor cell targets. (A) An LAK cell treated with K562 target cells showed release of the granule content, likely preceded by fusion of granules. (B) A cytoplasmic area of an LAK cell following 3,000 cGy irradiation. The integrity of the centriole apparatus and microtubules was of note. (C) An LAK cell following 7,500 cGy irradiation and exposure to K562 target cells. There was no redistribution or fusion of the electron-dense granules. The cell surface was irregular due to the presence of cytoplasmic blebs, which possibly indicated cell damage. (D) Detail of an LAK cell following 7,500 cGy irradiation. Damage of the centrioles was clearly evident. Magnification was as follows: (A) ×4,500; (B) ×18,000; (C) ×6,500; and (D) ×24,000.

Table 6. Effect of Irradiation on Nonspecific Killing of K562 Target Cells Mediated by Mitogen-Activated T Cells (AK)

<table>
<thead>
<tr>
<th>Irradiation Dose* (cGy)</th>
<th>E:T 40:1</th>
<th>E:T 20:1</th>
<th>E:T 10:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>38.0 ± 3.7†</td>
<td>29.9 ± 6.1</td>
<td>21.0 ± 5.0</td>
</tr>
<tr>
<td>5,000</td>
<td>22.4 ± 10.8</td>
<td>15.5 ± 8.4</td>
<td>11.0 ± 5.8</td>
</tr>
<tr>
<td>7,500</td>
<td>17.1 ± 7.5</td>
<td>11.4 ± 5.2</td>
<td>8.8 ± 3.9</td>
</tr>
<tr>
<td>10,000</td>
<td>12.3 ± 2.6</td>
<td>8.7 ± 0.3</td>
<td>5.1 ± 0.6</td>
</tr>
<tr>
<td>15,000</td>
<td>6.6 ± 1.6</td>
<td>3.9 ± 1.5</td>
<td>3.4 ± 1.8</td>
</tr>
</tbody>
</table>

*In cGy.
†Data shown are the mean % specific ⁵¹Cr release ± one SD from three experiments using effector cells from different donors.

Conjugate-forming ability and on the ultrastructural features of NK cells, following their binding to K562 target cells. Although conjugate formation was normal, irradiated lymphocytes failed to undergo the morphologic changes that normally occur in the post-binding phase. Thus, irradiated cells did not change their shape nor did they form uropods following interaction with target cells; they also did not redistribute their cytoskeletal structures (centrioles, microtubules, intermediate filaments, and submembranous actin filaments) and their electron-dense granules. Furthermore, a release of granule content was not observed. Additionally, irradiated effector cells exposed to target cells showed multiple projections of the cell membrane without the typical directed orientation to the cell target. This lack of polarization suggested a frustrated attempt at effec-
Fig 3. Ultrastructural features of cells cultured with IL-2 after 3,000 cGy irradiation. (A) A large blast cell that showed that irradiation completely prevented formation of granules (see Fig 4A and C for comparison to the typical granule content). (B) A cytoplasmic area which showed integrity of centrioles and microtubules. Magnification was as follows: (A) ×6,500; and (B) ×15,000.

Table 7. Effect of Irradiation on the Development of LAK Activity

<table>
<thead>
<tr>
<th>Irradiation Doses*</th>
<th>K562 Target Cells E:T 20:1</th>
<th>10:1</th>
<th>5:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>69.0 ± 7.0†</td>
<td>64.4 ± 7.5</td>
<td>53.4 ± 7.5</td>
</tr>
<tr>
<td>2,000</td>
<td>51.0 ± 12.0</td>
<td>36.0 ± 13.3</td>
<td>25.7 ± 14.0</td>
</tr>
<tr>
<td>3,000</td>
<td>16.5 ± 4.2</td>
<td>7.7 ± 2.2</td>
<td>4.2 ± 0.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HL60 Target Cells E:T 20:1</th>
<th>10:1</th>
<th>5:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>41.3 ± 9.5</td>
<td>35.0 ± 11.5</td>
<td>35.3 ± 11.9</td>
</tr>
<tr>
<td>13.6 ± 5.5</td>
<td>9.0 ± 6.0</td>
<td>5.6 ± 4.6</td>
</tr>
<tr>
<td>2.6 ± 1.5</td>
<td>1.6 ± 0.2</td>
<td>0.6 ± 0.5</td>
</tr>
</tbody>
</table>

Lymphocytes were irradiated at the doses indicated and subsequently cultured for 3-4 days with 100 IU IL2.

*In cGy.
†Data shown are the mean % specific ⁵¹Cr release ± one SD from three experiments using effector cells from different donors.

tor cell activation by the target cell. Although this is probably the expression of a functional impairment of the cytoskeleton, no ultrastructural evidence of cell damage was detected by electron microscopy in cells irradiated with 3,000 cGy.

The radiation sensitivity of other nonspecific cytotoxic effector cells (eg, T cells) is not known. Therefore, we investigated the radiation sensitivity of T cells that mediate LDCC. The results of these experiments show that the nonspecific cytotoxic functions of resting NK cells and T cells have identical susceptibility to the effect of irradiation.

LAK cells are usually defined as cytolytic effector cells that, after IL-2 activation, are capable of killing NK-resistant tumor cells. They are largely derived from NK cell precursors, although some T cell subsets may also give rise to LAK cells. In comparison to their progenitors, LAK cells display a much larger granule content and possibly have a mechanism for a facilitated degranulation. Thus, it appears that the higher cytotoxic ability of LAK cells and their wider range of susceptible target cells in comparison to NK cells could be simply viewed as a quantitative phenomenon as hypothesized by Itoh et al. This greater cytotoxic activity is likely the result of both increased availability of cytotoxic factors and a broader target recognition capability. Whatever mechanism is operative in LAK cell-mediated killing, these cytolytic effector cells have the morphologic features of activated cells. Accordingly, we showed that they displayed a radioresistant phenotype in comparison to that of resting NK cells. An impairment of LAK cell-mediated cytotoxicity was detected at radiation doses >5,000 cGy. These radiation doses prevented the morphologic changes that typically occur following the effector cell interaction with the target cell. Furthermore, these high radiation doses produced a direct damage of the LAK cells, as shown by cytoplasmic blebbing and disruption of the centriole-microtubule system, but not a loss of viability. This cell damage is presumably due to generation of oxygen-free radicals, which in turn produce chemical changes due to breakage of bonds in polymeric molecules (eg, tubulins), which are believed to occur after high-dose irradiation. A radiation dose response curve identical to that of LAK cells was obtained for the nonspecific cytotoxic activity of T cells stimulated by mitogen (AK activity). Therefore, similar to the cytotoxic activity of resting NK cells and resting T cells, the radiation sensitivity of cytolytic functions by activated NK cells (LAK) and activated T cells (AK) was identical.

Analyses of the effect of radiation on the development of LAK activity showed that doses that abolished the cytotoxic function of NK cells also impaired their ability to develop LAK activity. This impairment appeared to be independent of IL-2-induced blast transformation and could not be attributed to cell damage. It appeared to be the result of a radiation-induced inability of these cells to produce electron-dense granules that are necessary for the expression of cytolytic functions.

In conclusion, this study suggests that the effect of gamma irradiation on cytotoxic cell functions is not determined by
the lineage but rather by the state of activation of the effector cells. Since the radiation sensitivity of NK cell function is under genetic control, i.e., an X-linked co-dominant gene, it appears that the same control mechanism is operative in cytotoxic cells of other lineages and states of activation.

REFERENCES

Radiation sensitivity of resting and activated nonspecific cytotoxic cells of T lineage and NK lineage

D Zarcone, AB Tilden, VG Lane and CE Grossi