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INTERLEUKIN 2 AND STIMULATOR LYMPHOBLASTOID  
CELLS WILL INDUCE HUMAN THYMOCYTES TO BIND  
AND KILL K562 TARGETS\*

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The cellular lineage of natural killer (NK) cells has been an area of considerable controversy. The fact that athymic mice have NK activity (1) has been taken as evidence that NK cells are of nonthymic origin. However, because human NK cells share a number of features with T cells, it has been hypothesized that NK activity is actually a function of allospecific cytotoxic T lymphocytes (2). One possible solution to this uncertainty of the lineage of NK cells is the suggestion by Minato et al. (3) that murine NK cells are heterogeneous and composed of diverse cells of different lineages. These subsets would be under different regulatory controls but would share the common characteristic of selective cytotoxicity against various targets. At least one of the subsets proposed by Minato et al. would presumably mature under the influence of the thymus and is responsive to the regulation by interleukin 2 (IL-2). Our experiments were constructed to test whether human thymocytes could serve as precursors for the development of NK activity. Our results indicate that thymocytes cannot only be activated to kill NK-sensitive target cells, but that such activated cells bear a resemblance to the standard NK cell found in the peripheral blood of humans.

#### Materials and Methods

*Thymocyte Preparation.* Human thymocytes obtained at the time of partial thymus resection during open heart surgery in children were prepared as previously described (4).

*Thymocyte Cultures and Cytotoxicity Assays.* Stimulator cells, an allogeneic lymphoblastoid B cell line, UCLA-SO-L14 (L14), were irradiated (5,500 rad) and washed twice with 20% human serum AB before being placed in the culture tubes. The ratio of responder-to-stimulator cells was 10:1. Lectin-free IL-2, obtained from Associated Biomedic Systems, Inc., Buffalo, NY, was used at a 1:6 dilution in RPMI 1640.

In all cases,  $5 \times 10^6$  thymocytes were cultured for 5 d in 1.5 ml RPMI supplemented with 20% human AB serum and  $0.5 \times 10^{-4}$  M mercaptoethanol. Each aliquot of cells was treated with 0.1 ml (30 mg/ml DNase) for 30 min and washed twice before use in the K562 killing assays. A slight modification of the single-cell cytotoxicity assay described by Grimm and Bonavida (6) was used. Effector cells were labeled with an equal volume of fluorescein isothiocyanate (FITC), then incubated at 37°C for 30 min and washed twice in RPMI 1640 before resuspension at  $5 \times 10^6$  cells/ml. Equal numbers ( $2.5 \times 10^5$ ) of fluoresceinated effectors and K562 cells were mixed in a final volume of 0.1 ml. The cells were incubated for 10 min at 37°C and centrifuged at 1,200 g. 100  $\mu$ l of a 37°C 1% agarose solution was added to each tube and gently mixed with the cells using a wide-bore capillary pipette. The solution was then spread on a 2% agarose-coated glass slide. When the agarose had solidified, the slides were

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placed in RPMI 1640 and incubated for 3 h at 37°C. The slides were then stained with trypan blue for 6 min and rinsed in RPMI 1640 for 10 min. The percentage of dead targets was determined by counting the number of trypan blue-stained cells conjugated to fluoresceinated thymocytes for every 100 thymocytes counted. A total of 200 thymocytes was counted. In addition to the single-cell assay, cytotoxicity was assessed by  $^{51}\text{Cr}$  release (7). Thymocyte proliferation was measured by [ $^3\text{H}$ ]thymidine incorporation (4).

*Enrichment and Depletion of Cell Populations.* Separation of thymocytes into subpopulations of Fc $\mu$ -positive and Fc $\mu$ -negative cells was done as previously described (5), with one major modification. The thymocytes were treated with 0.1 ml of a 30 mg/ml DNase (Sigma Chemical Co., St. Louis, MO) solution and incubated for 30 min at 37°C. The addition of DNase was necessary to prevent clumping of the thymocytes. The DNase-treated thymocytes were washed twice with media containing 20% human AB serum and incubated for 24 h to achieve maximum expression of Fc $\mu$  receptors on the positive cell fraction. The cells were separated into Fc $\mu$ -positive and Fc $\mu$ -negative fractions by rosette formation, as previously described (5). The rosetting reagent was ox erythrocytes treated with purified rabbit IgM anti-ox. Removal of Fc $\gamma$  receptor-bearing cells from cultured thymocytes was performed in a similar manner, by rosetting with ox erythrocytes treated with rabbit IgG.

## Results

*Activation of Thymocytes Capable of Cytotoxicity against K562 Target Cells.* Thymocytes were cultured in the presence of either (a) IL-2, (b) irradiated stimulator L14 cells, (c) IL-2 + stimulator cells, or (d) media alone. After 5 d of culture, the thymocytes were tested for their ability to bind and kill K562 target cells. Our results demonstrate that thymocytes can be induced to kill K562 targets, as tested by both the single-cell assay (Table I) and the  $^{51}\text{Cr}$ -release assay (Fig. 1). These results also show that only the addition of both IL-2 and stimulator cells will markedly activate thymocytes to kill the K562 targets. Although IL-2 or stimulator cells alone induced some cytotoxicity, it was much less than when the two were combined. As can be seen in Table I, the total proportion of cytotoxic cells obtained after culture with either IL-2 or stimulator cells was <3%, while the combination of both factors resulted in >6% cytotoxic cells and >18% K562 conjugate-forming cells. Thymocytes cultured alone were incapable of cytotoxicity and contained only ~2% target-binding cells. Results from the Cr-release assay (Fig. 1) are similar, with the greatest cytotoxicity generated by the combination of stimulator cells plus IL-2, somewhat less cytotoxicity by co-culture with stimulator cells alone, a low level of cytotoxicity generated by IL-2 plus thymocytes, and no cytotoxicity evident when thymocytes were cultured in the absence of any stimulator. The results of proliferation assays are also shown in Fig. 1. The results are even more striking, as only the combination of stimulator cells and IL-2 provide a strong proliferative stimulus to the thymocytes. Cold target competition studies with either K562 or the stimulator cells resulted in complete inhibition of anti-K562 cytotoxicity by cold K562 and no inhibition by the stimulator lymphoblasts.

*The Expression of Fc $\gamma$  Receptors on Killer Cells.* Table I also shows a coincident increase in cells bearing Fc $\gamma$  receptors as cytotoxic activity increases. Therefore, we tested the cultured thymocytes for Fc $\gamma$  receptor expression. Table II shows that activated thymocytes with Fc $\gamma$  receptors on their surface were responsible for most (but not all) of the killing and binding activity. When the Fc $\gamma$  receptor-bearing cells were removed by EA $\gamma$  rosetting and gradient separation, the cytotoxic activity of the activated thymocyte population decreased to minor levels. Morphologically, cells binding to and killing K562 were predominately large, granulated cells of lymphoid appearance.

TABLE I  
*Induction of Natural Cytotoxic Cells from Thymocytes\**

| Effector cells‡                      | Days in culture | Percent binders | Percent killers§ | Percent    NK | Percent Fcμ¶ | Percent Fcγ** |
|--------------------------------------|-----------------|-----------------|------------------|---------------|--------------|---------------|
| Thymocytes                           | 0               | 1.8 ± 0.9       | 6.2 ± 6.2        | 0.1 ± 0.1     | 11.0 ± 1.0   | 1.0 ± 0.7     |
| Thymocytes                           | 5               | 2.3 ± 0.9       | 6.6 ± 4.1        | 0.2 ± 0.1     | 11.3 ± 0.6   | 0.8 ± 0.3     |
| Thymocytes + IL-2                    | 5               | 3.5 ± 0.6       | 47.0 ± 5.3       | 1.6 ± 0.4     | 11.0 ± 0.9   | 1.9 ± 0.4     |
| Thymocytes + L14 <sub>x</sub>        | 5               | 9.8 ± 0.9       | 28.5 ± 2.4       | 2.7 ± 0.3     | 9.9 ± 1.0    | 3.9 ± 0.4     |
| Thymocytes + IL-2 + L14 <sub>x</sub> | 5               | 18.6 ± 1.3      | 33.7 ± 2.4       | 6.2 ± 0.5     | 8.9 ± 0.5    | 7.3 ± 0.5     |

\* As measured by the single-cell assay against K562 targets.

‡ L14<sub>x</sub> is an irradiated lymphoblastoid cell line.

§ Percent binders that kill.

|| Total cells capable of killing K562 targets (percent binders × percent binders that kill).

¶ Cells bearing Fcμ receptors.

\*\* Cells bearing Fcγ receptors.

*Characterization of Precursor Thymocytes by Fcμ Receptors.* We also addressed the question of which subpopulation of thymocytes gives rise to the Fcγ<sup>+</sup> cytotoxic cells by separating thymocytes into Fcμ<sup>+</sup> and Fcμ<sup>-</sup> fractions. Fig. 2 indicates that the cytotoxic Fcγ<sup>+</sup> cells arise only from the Fcμ<sup>+</sup> subpopulation of thymocytes. Fcμ-depleted cells were incapable of generating significant anti-K562 cytotoxicity, even after culture with both stimulator cells and IL-2. Because fresh thymocytes do not have Fcγ receptors on their surface (Table I), it seems that modulation of Fc receptor isotype is part of the maturation process of thymocytes capable of killing K562 targets.

### Discussion

The lineage of NK cells has been one of the persistent puzzles in tumor immunology. Human NK cells can express a low density of T cell-associated antigens (8), may form rosettes with sheep erythrocytes, although at low affinity (9), and often have been classified as a subpopulation of T cells. However, the fact that murine NK cells can develop as an autonomous function of the bone marrow (10) and are found in the spleens of congenitally athymic mice (1) suggests that thymic development of NK cells is not an absolute necessity. One possible solution to the apparent conflict might be the suggestion of Minato et al. (3) that NK cells are heterogeneous and could include at least one subpopulation of T lineage. These investigators suggested that such T-derived NK cells would be regulated by IL-2. To test the hypothesis that there is a human NK cell population derived from the thymus, we sought to generate NK activity from human thymocytes. Untreated, freshly harvested thymocytes did not exhibit natural cytotoxicity (Table I). However, our data indicate that human thymocytes can generate anti-K562 cytotoxic cells. To generate maximum cytotoxic activity, both an allogeneic stimulus and exogenous IL-2 were required (Table I and Fig. 1). Thymocytes cultured in the absence of any stimulator had few cytotoxic cells for K562 targets. Culture of the thymocytes with allogeneic stimulator cells or with IL-2 alone increased the proportion of cytotoxic cells but not nearly to the degree of the combination of both agents. We chose a lymphoblastoid cell line as the stimulator cell because these cells are effective for generation of both allospecific cytotoxic T lymphocytes and cytotoxicity against NK-sensitive targets (11). Our data suggest that the allogeneic lymphoblastoid cell line stimulated a subpopulation of thymocytes (probably the more mature thymocytes), and we assume that this resulted in increased

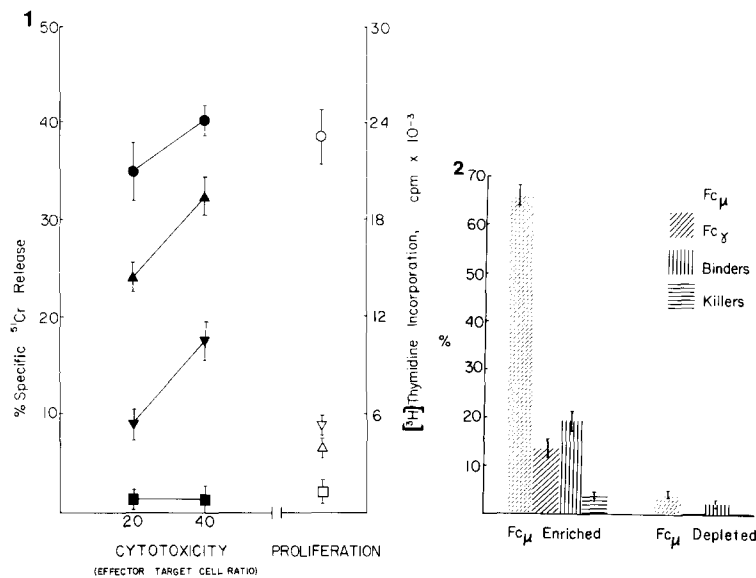


FIG. 1. Induction of cytotoxic cells from thymocytes, as measured by <sup>51</sup>Cr release. Left vertical axis indicates percent cytotoxicity (closed symbols); right vertical axis indicates proliferation as measured by [<sup>3</sup>H]thymidine incorporation (open symbols). Cell culture components are indicated in the figure. Thy + S + IL-2 (●, ○); Thy + S (▲, △); Thy + IL-2 (▼, ▽); Thy alone (■, □).

FIG. 2. Induction of cytotoxic cells from thymocytes separated on the basis of Fc<sub>μ</sub> receptor. Thymocytes were precultured for 24 h and separated by EA<sub>μ</sub> rosette formation. The rosetting and nonrosetting cells were then cultured for 5 d with allogeneic stimulator cells plus IL-2. Cells were recovered and tested for Fc<sub>μ</sub> and EA<sub>γ</sub> receptors by EA<sub>μ</sub> and EA<sub>γ</sub> rosette tests and for the proportion of K562 binders and total lytic cells by the single-cell assay.

expression of receptors for IL-2. Thus, the addition of IL-2 would provoke greater proliferation of this stimulated subpopulation and expansion of the cytotoxic cells. Presumably the thymocytes themselves produce little IL-2 and would be unable to cause the activated cells to proliferate in the absence of exogenous IL-2. Though the above scheme assumes that the active component is indeed IL-2, one cannot be definite on this point because the active component in the IL-2-containing media added to these cultures could involve some other substances such as interferon or IL-3. Further studies are planned to determine whether IL-2 is the required material, but at present it is clear that some component in the media of PHA-stimulated lymphocytes is required for the full generation of anti-K562 cytotoxicity from thymocytes. Whatever the culture requirements, the most important point is that NK-like cytotoxicity can be generated from thymocytes.

The cytotoxic cells generated in this fashion bear a number of similarities to peripheral blood NK cells. Not only are they cytotoxic to K562, but in preliminary experiments we found that they were cytotoxic to other NK-sensitive targets such as MOLT-4 but not an NK-resistant melanoma cell line (M14). They bear some morphological resemblance to the large, granular lymphocytes described by Saksela and Timonen (12), and they are certainly larger and more granulated than typical thymocytes. We feel that the most important evidence relating these cells to NK is the fact that they bear Fc receptors for IgG (Table II), whereas cells bearing such receptors are virtually absent from the thymocytes before culture or after culture in the absence of stimulator (Table I).

TABLE II  
*Depletion of Natural Cytotoxic Cells by EA $\gamma$  Rosettes\**

| Effector cells $\ddagger$        | Unfractionated  |                 |                     | Fc $\gamma$ depleted |               |            |
|----------------------------------|-----------------|-----------------|---------------------|----------------------|---------------|------------|
|                                  | Percent binders | Percent NK $\S$ | Percent Fc $\gamma$ | Percent binders      | Percent NK    | Percent Fc |
| Thymocytes                       | 3.5 $\pm$ 0.5   | 0.2 $\pm$ 0.5   | 0.5 $\pm$ 0.5       | 3.0 $\pm$ 0.0        | 0.2 $\pm$ 0.0 | 0.0        |
| Thymocytes + IL-2                | 5.5 $\pm$ 0.5   | 2.5 $\pm$ 0.5   | 2.5 $\pm$ 0.5       | 4.0 $\pm$ 0.5        | 1.8 $\pm$ 0.0 | 0.0        |
| Thymocytes + L14 $\times$        | 6.0 $\pm$ 1.0   | 1.6 $\pm$ 0.8   | 1.5 $\pm$ 1.5       | 5.5 $\pm$ 0.5        | 1.4 $\pm$ 0.1 | 0.0        |
| Thymocytes + IL-2 + L14 $\times$ | 22.6 $\pm$ 1.3  | 5.0 $\pm$ 0.4   | 10.9 $\pm$ 0.3      | 6.6 $\pm$ 0.6        | 1.7 $\pm$ 0.2 | 0.0        |

\* After 5 d in culture and as measured by the single-cell assay against K562 targets.

$\ddagger$  L14 $\times$  is an irradiated allogeneic lymphoblastoid line.

$\S$  Total cells capable of killing K562 targets (percent binders  $\times$  percent binders that kill).

|| Cells bearing Fc $\gamma$  receptors.

The lineage of these cells is interesting in that we were able to generate the activity only from the thymocyte population that bore the Fc receptor for IgM (Fc $\mu$ ). This is in agreement with the study of Piantelli et al. (13), showing that only mature thymocytes bearing the Fc $\mu$  receptor but without the peanut lectin receptor were able to respond to IL-2 activation. Thus, the precursor for these K562 cytotoxic cells appears to be a mature thymocyte. It is interesting to note that the cells responsible for this activity bear the Fc $\mu$  receptor but once activated bear the Fc $\gamma$  receptor. This is in agreement with the results from several laboratories (14, 15) showing that cells can switch from an Fc $\mu$  receptor to an Fc $\gamma$  receptor and vice versa. Because Jondal and Merrill (16) have shown that there is a small subpopulation of peripheral blood NK cells that bear the Fc $\mu$  receptor, it is interesting to speculate that the normal lineage of at least one subpopulation of NK cells involves a T cell subpopulation that changes from Fc $\mu$  to Fc $\gamma$  phenotype. Previous studies of mixed lymphocyte culture-activated cells performed in our laboratory have shown an Fc $\mu$ -bearing cytotoxic cell (5). It is important to note that our studies (5, 17) with peripheral blood cells activated in mixed lymphocyte cultures showed no requirement for exogenous IL-2, and the cytotoxic cells contained only a minor subpopulation of Fc $\gamma$ -bearing effectors. Thus, there are substantial differences between the in vitro activation of thymocytes and the in vitro activation of peripheral blood lymphocytes for NK-like activity. In the thymocytes, the effectors are predominantly Fc $\gamma$  bearing, whereas with the peripheral blood cells, the major increase in cytotoxic activity is associated with Fc $\mu$ -bearing cells. Taken together, these results suggest that there is a subpopulation of human T cells that can contribute to NK cytotoxicity. Among such cells, the Fc receptors, whether for IgM or IgG, can be quite useful as markers of differentiation. These results also suggest that the scheme developed by Minato et al. (3), which includes a subpopulation of T cells regulated by IL-2 among the NK cells, may be applicable to the lineage of some human NK cells. Alternatively, all NK cells may be of T lineage, but differences in activational events and degree of differentiation of the NK precursors could result in NK cells of varying phenotypes.

#### Summary

Human thymocytes cultured in the presence of IL-2 and an irradiated B cell line became cytotoxic to K562 target cells. Thymocytes cultured alone or with only IL-2 exhibited almost no killing, but thymocytes cultured in the presence of stimulator cells alone exhibited low levels of cytotoxic activity. Removal of Fc $\gamma$  receptor-bearing cells from the activated thymocyte population almost completely abolished the

binding and lytic activity. Separation of thymocytes into  $Fc\mu^+$  and  $Fc\mu^-$  cells before culturing with IL-2 and stimulator cells revealed that only the  $Fc\mu^+$  subpopulation developed into K562 killer cells. These findings indicate that modulation of  $Fc\mu$  to  $Fc\gamma$  receptors on the thymocyte cell surface is part of the maturation process of this particular subset of cytotoxic cells. Morphologically, most of the activated  $Fc\gamma^+$  K562-binding cells were large, granulated lymphocytes. Only very few of the round, nongranulated small thymocytes were bound to K562 target cells.

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