INTERLEUKIN 2-INDUCED PROLIFERATION OF MURINE NATURAL KILLER CELLS IN VIVO

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Spontaneously arising NK cells and NK cells activated in vivo in response to IFNs are predominantly a population of CD3\(^-\), TCR\(^-\) cells (1-4) having the morphology of large granular lymphocytes (LGLs)\(^1\) (5, 6). They mediate lysis of a restricted range of target cells without apparent antigen specificity or restriction by histocompatibility molecules (7, 8). IL-2, originally isolated as a growth factor for T cells, has been shown to activate NK cells to lyse sensitive target cells more efficiently both in vivo and in vitro, and to support the proliferation of NK cells in vitro (2, 9-14). As of yet, however, IL-2 support of NK cell proliferation in vivo has not been established (15). Although previous reports have suggested that administration of IL-2 can support NK cell proliferation in vivo, these studies were carried out by prolonged and repeated exposure of mice to IL-2 (13, 14). Such protocols may have activated other cells, including T cells, to produce intermediary factors supporting NK cell proliferation. Furthermore, these studies were limited, as characterizations of the NK cells and NK cell expansion were based on functional analyses of cell-mediated lysis and appearance of cells having LGL morphology. Both of these characteristics are not specific to NK cells and can be shared by other cell types, including IL-2-activated T cells (16, 17). Thus, a physiological role for IL-2 in NK cell proliferation in vivo has not been documented.

Our laboratory has been studying the regulation of NK cell and T cell proliferation in vivo (15, 18-20). We have previously shown that both populations of cells undergo proliferation during viral infections. The kinetics of these two responses differ in that NK cells proliferate at early times post-infection with lymphocytic choriomeningitis virus (LCMV), whereas T cells proliferate at later times (15, 17, 18). The NK cell proliferation correlates with production of virus-induced IFNs, and administration of IFNs alone can induce the proliferation of NK cells (19). The cells responding to IFN signals are CD3\(^-\) (4) and are readily elicited in athymic mice.

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† Abbreviations used in this paper: AGM1, asialo ganglio-\(\alpha\)-tetraosylceramide; LCMV, lymphocytic choriomeningitis virus; LGL, large granular lymphocytes; PI, propidium iodide; poly I:C, polyinosinic-polycytidylid acid; RbC, rabbit H-2 complement.

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In contrast to NK cell proliferation, T cell proliferation correlates with the production of IL-2 (18). It was striking to note, in these studies of LCMV infection, that peak IL-2 production was apparent only after NK cell proliferation had subsided (18).

The experiments reported here were undertaken to directly examine the responsiveness of murine NK cells to IL-2 in vivo. To carry out these studies, we asked if rIL-2 could induce NK cell proliferation, within a 24-h period, in athymic as well as euthymic mice. In addition, induction of the gene for the p55 α chain molecule of the IL-2-R was examined in populations of cells responding to various in vivo activation/proliferation signals, including IL-2, IFN, and infection with LCMV. We report here that: (a) high doses of IL-2 in vivo can induce the proliferation of NK cells within a 24-h period; (b) administration of IL-2 in vivo results in the accumulation of transcripts for the p55 α chain gene in euthymic mice but not in athymic mice; (c) the kinetics of p55 α chain transcription during LCMV infection correlates with T cell proliferation as opposed to NK cell proliferation; and (d) murine NK cells are not induced to transcribe detectable levels of the p55 α chain gene, even when they are induced to proliferate in response to IL-2 in vivo. The results demonstrate that IL-2 can be a growth factor for NK cells in vivo, but suggest that the role of this factor in supporting murine NK cell proliferation in vivo is limited.

Materials and Methods

Mice. C3H/HeJ (H-2b) and C57BL/6 (H-2b) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. Specific pathogen-free athymic nude mice and euthymic nude/+ littermates (BALB/c AnBOM) were bred in our isolated facility. All food, bedding, and cage materials were presterilized. Experiments were done with 4-20-wk-old mice.

Treatment of Mice. Mice infected with the Armstrong strain of LCMV were injected intraperitoneally with 2 x 10⁴ plaque-forming units of virus (15, 17). Mice were killed at 0-9 d post-infection. When stated, mice were treated intraperitoneally with 100 μg of the chemical IFN inducer polyinosinic-polycytidylic acid (poly I:C; Sigma Chemical Co., St. Louis, MO). Mice receiving human rIL-2 or highly purified mouse IFN-β were injected intravenously 18-24 h before harvest. The rIL-2 (des-ala-ser₁₂₅), a generous gift of Cetus Corp., Emeryville, CA, had a sp act of 8 x 10⁶ BRMP U/mg protein. All reported IL-2 U are BRMP U. 8 x 10⁶ BRMP U is equal to 1.8 x 10⁷ IU. The IFN-β, purchased from Lee Biomolecular, San Diego, CA, had a sp act of 2 x 10⁶ international reference units/mg protein. Concentrations administered to mice were as stated.

Antibodies. The mAbs B23.1, directed against monocyte/macrophages (21), and J11d, directed against polymorphonuclear leukocytes and most B cells (22), were used with rabbit H-2 complement (RbC; Pel-Freez Biologicals, Rogers, AR) during the purification of in vivo activated lymphocytes as previously described (4, 20). The mouse anti-NK1.1 mAb, PK136 (23), was used to stain the NK cell subset in C57BL/6 mice. The PK136 antibody is of the IgG2b isotype. Staining was carried out with fluorescein-conjugated goat antibody, Fab', specific for mouse IgG Fc fragment (Jackson Immunoresearch Labs., West Grove, PA). The following mAbs, as well as PK136, were used with RbC' to phenotype the activated killer cells: 3.155, anti-Lyt-2.2 (24); GK1.5.6, anti-L3T4 (25); SH34, anti-α-ganglioside (anti-AGMI) (26); and 29B, anti-CD3 (27). The MARS 18.5 (28) was used to enhance complement fixation when required.

Isolation of In Vivo Elicited Cell Populations. Spleen cells were isolated from mice after the various treatment protocols as stated. RBC were lysed by treatment with ammonium chloride. Leukocytes were separated on the basis of size with an elutriation system (JE-6B; Beckman Instruments, Inc., Falo Alto, CA) as previously described (4, 15). Briefly, cells were loaded into the centrifugal rotor, which was spinning at 3,200 rpm. The flow rates for elutriation
fractions 1-6 were 15, 22, 28, 32, 38, and 46 ml/min, respectively. Using these conditions for separations, we have previously demonstrated that fractions 4-6 are enriched by three to ninefold for lymphocytes incorporating the DNA precursor [3H]thymidine (15). Total non-B lymphocytes were isolated after depletion of macrophages, polymorphonuclear leukocytes, and B cells by antibody (B23.1 and J11d) and RbC treatments. Total viable non-B lymphocytes were recovered after treatments by centrifugation over a one-step, 38% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient.

Chromium Release Cytotoxicity Assays. Target cells labeled with 51Cr (New England Nuclear, Boston, MA) were incubated with spleen cells for 5 h at 37°C in microtiter plates as described (15). Spontaneous lysis was determined by incubating medium with targets for 5 h. Maximum 51Cr release was determined by adding 1% NP-40 to target cells. Percentage of lysis was calculated as: 100 × (cpm test sample lysis - cpm spontaneous lysis)/(cpm maximum release - cpm spontaneous lysis). NK cell activity was analyzed as cytotoxicity against the NK-sensitive target cell line, YAC-1. The NK-resistant cell line, P815, was examined to determine target cell range. CTL activity was determined as virus-specific lysis of histocompatible target cells, as described previously (17, 18).

Northern Blot Analysis. Cytoplasmic RNA was isolated from cells lysed by NP-40 in the presence of RNase inhibitors (4). Nuclei were removed by centrifugation and RNA was prepared from supernatant by proteinase K digestion, phenol extraction, and ethanol precipitation. Samples (10-20 µg) were denatured by heating at 55°C in 50% formamide and separated by electrophoresis in a 1.2% agarose formaldehyde gel. The RNA was transferred to Biodyne filters (ICN Biomedicals, Inc., Irvine, CA), and the filters were baked for 2 h at 80°C. A first prehybridization wash was carried out for 30 min in 5 × SSC (5 × 0.75 M sodium chloride and 0.075 M trisodium citrate), 5 × Denhardts, 50 mM sodium phosphate, pH 6.5, 0.1% SDS, and 250 µg/ml salmon sperm DNA. DNA probes were 32P labeled using the random hexanucleotide priming kit from Boehringer Mannheim Biochemicals (Indianapolis, IN). Hybridizations were carried out overnight. Filters were washed three times in 2 × SSC with 0.1% SDS at room temperature, followed by two washes under stringent conditions in 0.1 × SSC and 0.1% SDS at 55°C. Probes were derived from plasmids carrying cDNA or DNA inserts coding for the p55α chain of the mouse IL-2-R (29), obtained from Dr. Ron Germain of the NIH, Bethesda, MD; mouse IFN-γ, obtained from Dr. Ken-ichi Arai, DNAX Research Institute, Palo Alto, CA; and β-actin (30).

In Situ Hybridization. The DNA insert complementary to the murine IL-2-R α chain gene was isolated, labeled with 35S dCTP by random hexanucleotide priming to a sp act of >10⁶ cpm/µg, and denatured at 90°C. The cells to be probed were spun onto microscope slides using a cytocentrifuge (Shandon Inc., Pittsburgh, PA), fixed in 4% paraformaldehyde for 15 min at room temperature, then dehydrated and stored in 70% ethanol at 4°C until use. They were rehydrated before use in Mg²⁺-PBS, then treated with 50% formamide at 70°C for 15 min. Hybridizations were performed as previously described with minor modification (18). The slides were then washed extensively in formamide and SSC, dried, dipped in nuclear track emulsion (type NTB-2; Kodak, Rochester, NY), exposed at 4°C for 7-10 d, and developed. Plasmid controls were done similarly.

Cell Cycle Analysis. Cell cycle analyses were done by quantitative staining of DNA with propidium iodide (PI) (4). Briefly, cells were fixed in 70% ethanol, washed in PBS, and resuspended in 20 µg/µl PI (Sigma Chemical Co.) with 50 µg/ml ribonuclease A (Sigma Chemical Co.).

Flow Cytometric Analysis. A FACS 440 (Becton Dickinson & Co., Mountain View, CA) was used for analyses. Laser output was 300 mW at 488 nm. Green fluorescence was collected through a DP585/40-nm filter. For cell cycle analysis with PI stain, emitted light was collected through a 625/35-nm filter. The green fluorescence signals were amplified logarithmically. The PI signals were amplified with a linear amplifier. In certain experiments, cells were sorted on the basis of green fluorescence.

Cytochemical Staining. Cells were sedimented in a cytocentrifuge (Shandon Inc.), air dried, and stained with Wright's Giemsa. All buffers were at pH 7.2. Morphology was examined and percentages were determined on counts of 200-400 cells.
Results

Activation of NK Cells In Vivo After Administration of rIL-2. To determine the concentrations of rIL-2 required for induction and/or enhancement of NK cell activity during an 18-24-h period in vivo, mice were treated with rIL-2 intravenously, and cytotoxic activity mediated by total spleen cell populations was measured against NK-sensitive YAC-1 (target cells) (Table I). The nu/+ BALB/c mice maintained in our sterile colony had nondetectable endogenous NK cell activity. This result is consistent with earlier reports from other laboratories using germ-free animals. Treatment with low-dose rIL-2 (3 × 10^4 U) resulted in marginally detectable activity (Table I; 2% lysis at E/T ratios of 100). In contrast, treatment with high-dose rIL-2 (10^6 U) induced a >10-fold increase in killing of YAC-1 cells (Table I; 20% lysis at E/T ratios of 100). Cytotoxic activity was readily detectable in conventionally housed normal C3H and C57BL/6 mice, and this activity could also be induced in response to treatment with higher dosages of IL-2 (Table I). Experiments with spleen cells isolated from untreated athymic nu/nu BALB/c mice maintained in our sterile colony demonstrated that these cells had low but detectable cytotoxic activity, mediating 6-8% lysis of YAC-1 cells at E/T ratios of 100. Treatment of the nude mice with low-dose rIL-2 resulted in a threefold increase in YAC-1 target cell lysis. High-dose rIL-2 treatment induced a greater than fivefold increase in activity (Table I).

The killer cells induced by treatment with rIL-2 were characterized as NK cells

| Mice       | Treatment | Percent lysis of YAC-1 target cells\* | Percent LGL\* |
|------------|-----------|-------------------------------------|---------------|
|            |           | 100 | 33 | 11 |               |               |
| Nu/+ BALB/c control\^5 | 1 | <1 | <1 | 1 |               |               |
| 3 × 10^4 U rIL-2 | 2 | 1 | <1 | 2 |               |               |
| Nu/Nu BALB/c control\^5 | 8 | 4 | 3 | 2 |               |               |
| 3 × 10^6 U rIL-2 | 26 | 13 | 6 | 3 |               |               |
| Nu/+ BALB/c control | <1 | <1 | <1 | 2 |               |               |
| 10^6 U rIL-2 | 20 | 11 | 6 | 7 |               |               |
| Nu/Nu BALB/c control | 6 | 4 | 2 | 2 |               |               |
| 10^6 U rIL-2 | 34 | 22 | 11 | 7 |               |               |
| C3H Control | 18 | 11 | 7 | 3 |               |               |
| 3 × 10^5 U rIL-2 | 24 | 37 | 23 | 8 |               |               |
| C57BL/6 Control | 4\^1 | 3\^1 | 2\^** | 1 |               |               |
| 10^6 U rIL-2 | 18\^1 | 9\^1 | 9\^** | 5 |               |               |

Mice were treated at 20-24 h before harvest, as described in Materials and Methods. Spleen cells were examined.

\* Cytotoxicity was determined in a 4-8-h \(^{51}\)Cr release assay.

\^ Total spleen cell populations were examined to determine percent LGL.

\^ Controls in these experiments received excipient control that did not contain IL-2. In all other aspects, it was equivalent to the preparation of rIL-2.

\^ Lysis was measured at an E/T of 50.

\^ Lysis was measured at an E/T of 25.

\^ Lysis was measured at an E/T of 12.
by the following criteria. (a) They were readily elicited in the athymic nude mice. (b) The cell surface phenotype of the cytotoxic cells induced by IL-2 was that of NK cells (Table II); the killer cells elicited in C57BL/6 mice expressed the NK cell-specific alloantigen, NK1.1, and high asialo-ganglio-\(n\)-tetraosylceramide (AGM1), characteristic of NK cells. They did not express the T cell markers Lyt-2, L3T4, or CD3. (c) The activated cells demonstrated the target cell range of NK cells; lysis of the NK-sensitive target, YAC-1, was elevated to high percentages, whereas lysis of the NK cell resistant target, P815, was constantly <1% with all mice examined (data not shown). (d) IL-2 treatment resulted in an increase in the percentages of cells having the LGL morphology characteristic of NK cells (Table I).

**Blastogenesis and Proliferation of NK Cells After Treatment with rIL-2.** The observation that LGL percentages were increased after treatment with IL-2 suggested that the NK cells were expanding in response to IL-2 in vivo. As the LGL morphology can be induced after activation of NK precursor cells (31), and as the morphology is not absolutely unique to the NK cell subset (16, 17), the increase in LGL percentages is consistent with, but does not prove that, NK cells are dividing in response to IL-2 in vivo. We have previously shown that NK cells undergoing blastogenesis and proliferation can be isolated in fractions of blast size cells (15). To determine whether or not IL-2 induced blast size NK cells in vivo, spleen leukocytes were separated by elutriator centrifugation into six fractions. Fraction 1 contained the smallest size cells, residual erythrocytes, and the dead cells and cell debris. Fraction 6 con-

### Table II

| Antibody treatment with RbC | Percent reduction of YAC-1 target cell lysis |
|-----------------------------|---------------------------------------------|
|                             | Control cells | IFN-activated cells | IL-2-activated cells |
| Exp.                        |               |                   |                   |
| 1* Anti-NK1.1               | 41            | 59                | 45                |
| Anti-Lyt-2                  | <1            | 4                 | <1                |
| Anti-L3T4                   | <1            | 2                 | <1                |
| 2* Anti-NK1.1               | 5             | 100               | 100               |
| Anti-AGM1                   | -             | 100               | 100               |
| Anti-CD3                    | -             | <1                | <1                |

Experiments were carried out with C57BL/6 mice. Mice were control treated, treated with >5 \(\times\) 10^5 U of IFN, or treated with >5 \(\times\) 10^5 U of rIL-2. Antibody and RbC treatments of killer cells were as described in Materials and Methods. Cytotoxicity was measured in a 4–5-h \(^{3}\)Cr release assay against YAC-1 target cells at ratios of 50:1, 25:1, 12:1, and 6:1. Under the conditions used, anti-NK1.1 and anti-AGM1 resulted in <10% inhibition, anti-Lyt-2 resulted in >90% inhibition, and anti-CD3 resulted in a 27–48% inhibition of virus-specific cytotoxic T cell-mediated lysis on day 7 post-infection with LCMV.

* Experiment 1 was carried out with populations that had been enriched for NK cell activity by depletion of J11d- and B23.1+ cells.

1 Experiment 2 was carried out with total spleen cell populations. An additional E/T ratio of 100:1 was used.

5 It was not possible to determine percent reduction of control cells in this experiment, as the control NK cells mediated <1% of YAC-1 target cells at all of the ratios examined.
tained the largest size cells. Under the conditions used, fractions 2 and 3 were enriched for small to medium size lymphocytes in G0/G1 of the cell cycle, whereas fractions 4, 5, and 6 were enriched for large size cells and blast lymphocytes in S and G2/M phases of the cell cycle (4, 15). The cytolytic activity of the various size spleen cell populations isolated from control and IL-2-treated mice was determined against YAC-1 target cells.

In both athymic and euthymic mice, only populations of cells that mediated NK cell activity when isolated from untreated mice were enhanced after treatment with low-dose IL-2. Cells mediating lysis isolated from athymic nude mice were enriched in the medium size populations recovered in fraction 3, however, modest cytolytic activity was detected with cells recovered in each of the size classes. Treatment of the athymic mice with low-dose (3 × 10⁴ U) rIL-2 resulted in an enhancement of activity mediated by cells from each of the size classes, and the cytotoxic cells were still enriched in fraction 3 (Fig. 1 A). Euthymic mice had marginally detectable activity mediated only by cells recovered in fraction 3, and treatment of euthymic nu/+ mice with low-dose rIL-2 (Fig. 1 B) enhanced only the activity mediated by this population. The level of enhancement was approximately proportional in each of the lytic subsets. These results are consistent with enhancement of activity mediated by pre-existing NK cells. In contrast, high-dose rIL-2 (10⁶ U) induced proportion-

![Figure 1](image-url)
ally more activity mediated by blast size cells than by small to medium size cells. This was observed in both athymic nude BALB/c (Fig. 1C) and euthymic nu/+ BALB/c (Fig. 1D). High-dose rIL-2 also induced proportionally more activity in blast size cells isolated from C3H and C57BL/6 mice (data not shown). These results demonstrate that high-dose IL-2 induces blast size NK cells. We have previously demonstrated that IFNs and IFN inducers elicit NK cell blastogenesis in vivo. The induction of NK cell blastogenesis by 10⁵ U of IFN-β, during the same time period in nude mice, is presented in Fig. 1C for comparison.

To determine if the NK cells were undergoing DNA synthesis and proliferation in addition to becoming blast size cells, experiments were undertaken using the NK1.1 alloantigen-positive C57BL/6 mice. As stated previously, the NK1.1 determinant is specifically expressed on CD3⁺ NK cells isolated from these mice (3). The numbers of NK1.1⁺ cells within populations of lymphocytes from C57BL/6 mice was determined using flow cytometry. Total numbers of NK1.1⁺ cells were increased twofold after high-dose treatment with IL-2 for 24 h.

Cell cycle analyses were carried out by labeling with the quantitative DNA stain, PI. DNA content within the NK cell subset was measured by two-color fluorescence analysis using fluorescein-conjugated NK1.1 (green) and PI (red). The increase in NK1.1⁺ cells was accompanied by a preferential increase in the cycling of the NK1.1⁺ cell subsets after treatment with IL-2. These experiments were first carried out by examining the NK1.1⁺ cells within the total spleen cell population (data not shown). As the NK1.1⁺ cell populations within the subsets were very small, <5-10%, the experiments were also carried out with populations enriched in NK1.1⁺ cells, >10-20%, after depletion of B cells, macrophages, and polymorphonuclear cells (PMNs). For these experiments, cells were prepared from control mice, mice that had been treated with 5 x 10⁵ U of rIL-2, and mice that had been treated with 5 x 10⁵ U of IFN-β. The treatment of mice with either high-dose rIL-2 or IFN-β resulted in a modest increase in the proportion of non-B lymphocytes in the S and G2/M phases of the cell cycle (Fig. 2). When the NK1.1⁺ cell subset was specifically examined, it was clear that the increase in cycling cells preferentially occurred in the NK cell subset; after treatment with either rIL-2 or IFN-β, almost 50% of the NK1.1⁺ cells were in S and G2/M phases of the cell cycle (Fig. 2). These results demonstrated the IL-2-induced blastogenesis and proliferation of NK cell subsets in vivo.

Induction of the IL-2-R p55α Chain in Response to IL-2 In Vivo. Responsiveness to IL-2 is regulated by the expression of the IL-2-R. Work from other laboratories has demonstrated that IL-2 induces the transcription of the gene for the α p55 molecule of the IL-2-R (32-34). To evaluate the induction of the IL-2-R p55 chain during NK cell proliferation, RNA was prepared from blast lymphocytes after treatments to induce blastogenesis in vivo. The blast cell populations were isolated to select for the minority of the total spleen cells that had responded to the in vivo treatments. Blast cells of interest were further enriched by antibody and C' depletion of B cells, macrophages, and PMNs. Viable cells were recovered by density gradient centrifugation. We have previously demonstrated that this isolation procedure selects for the dividing non-B lymphocytes and that these cells represent <10% of the total spleen cell populations (4).

For these experiments, RNA was prepared from a panel of dividing non-B lymphocytes that had been elicited in vivo, as well as from in vitro maintained cells
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**Figure 2.** Cell cycle analyses after treatments with IL-2 or IFN. Non-B lymphocytes were isolated from C57BL/6 control mice, mice that had been treated with $5 \times 10^5$ U rIL-2, and mice that had been treated with $5 \times 10^5$ U of IFN-β at 24 h before experiment. The cells were stained, as described in the Materials and Methods, with fluorescein-conjugated reagents to mark the NK1.1+ cell subset, and PI to quantitatively stain DNA content. Cell cycle analyses presented in the panels on the left were performed using the total non-B lymphocyte population. Cell cycle analyses presented on the right were performed by acquiring data gated for only the green, NK1.1+ cells. Low intensity cells in the G0-G1 phase of the cell cycle, thus having 1× DNA content, are found in the first peaks. High intensity peaks are cells with 2× DNA content are in the G2-M phases of the cell cycle. The broad distribution of cells in the center are in S phase with >1× but <2× DNA. Gaussian curve fit analyses were carried out to determine proportions of cells in each phase of the cell cycle. Shown in parentheses are the percentages of cells with >1× content of DNA, i.e., in S and G2-M phases.

known to be positive for p55 gene transcription. Northern blot analysis was carried out with these samples (Fig. 3). Transcription of the p55 gene was readily detectable when IL-2 was present in euthymic mice in vivo. The multiple transcripts characteristic for the p55 gene (29) were observed with RNA isolated from C3H mice that had received high-dose rIL-2 (Fig. 3, lane 6). We have previously shown that IL-2
is induced in vivo when T cells are dividing on day 7 post-infection with LCMV (18), and p55 transcripts were also readily detectable with RNA isolated from these cells (Fig. 3, lane 4). Greater than 80% of the blast non-B lymphocytes on day 7 post-infection were T cells (4). The p55 chain transcripts were marginally detectable on day 3 post-infection with LCMV, the peak of NK cell proliferation (Fig. 3, lane 5). It was not possible to demonstrate transcripts for the IL-2-R p55 chain with RNA prepared from either euthe mic or athymic blast cell populations that had been elicited by poly I:C (Fig. 2, lanes 2 and 3). As the blast populations elicited in response to the IFN inducer are >75% CD3− NK cells (4), these results conclusively demonstrate that IFN-induced blast NK cells do not transcribe detectable levels of the IL-2-R p55 gene.

To determine the proportion of the cells that were positive for α chain transcription, in situ hybridization was carried out with the probe for the p55 gene (Table III). Control non-B blast lymphocytes were only 5% positive, and IFN-induced non-B blast lymphocytes were only 7% positive for α chain transcription by in situ hybridization. In contrast, the non-B blast lymphocytes prepared from euthe mic mice after treatment with high-dose rIL-2 were 41% positive (Table III). Although treatment of athymic mice with high-dose rIL-2 induced NK cell blastogenesis, only 12% of the non-B blast lymphocytes were positive for transcription of the p55 gene (Table III). These results show that the p55 gene is not induced in high numbers of NK cells from IL-2-treated athymic mice.

As 41% of the blast lymphocytes isolated from euthe mic mice after IL-2 treat-
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TABLE III
Proportion of Cells Positive for IL-2-R p55 Gene Transcription Evaluated by In Situ Hybridization

| Cells                                      | Percent Positive ± SD |
|-------------------------------------------|-----------------------|
| Blast size non-B lymphocytes isolated from: |                       |
| Control C3H mice                          | 4.9 ± 1.7*            |
| IFN-activated C57BL/6 mice                | 7.3 ± 3.1*            |
| IL-2-activated C3H mice                   | 40.6 ± 8.8*           |
| IL-2-activated nu/nu BALB/c mice           | 11.8 ± 5.7*           |
| Total non-B lymphocytes isolated from:     |                       |
| Control C57BL/6 mice                      | 6.5 ± 2.1*            |
| IL-2-activated C57BL/6 mice               | 11.8 ± 4.3            |
| NK1.1+ cells isolated from:               |                       |
| Control C57BL/6 mice                      | 4.8 ± 1.4             |
| IL-2-activated C57BL/6 mice               | 3.5 ± 3.5             |

Control mice, mice treated with high-dose rIL-2, and mice treated with high-dose IFN-8 were used for these experiments. In situ hybridizations were carried out as described in Materials and Methods. Percentages were determined by counting grains over 200 cells per slide. The number of grains per cell constituting a positive was determined in each experiment based on background controls and negative controls. Results presented represent the means of two to five slides done during one to four experiments.

* Cells in these experiments were also examined for hybridization to a probe complementary to the murine IL-2 gene. All of the populations had <10% of the cells hybridizing to this probe.

ment expressed the p55 gene, it was necessary to determine whether the positive cells were NK cells or other cells such as T cells. The NK1.1+ cells were isolated from control and IL-2-treated C57BL/6 mice by FACs. Less than 5% of the total NK1.1+ cells isolated from either control, untreated mice, or rIL-2-treated mice were positive for p55 chain transcripts (Table III). These percentages were lower than those observed in total non-B lymphocyte populations. Taken together, the data demonstrate that murine NK cells are not induced to transcribe detectable levels of the IL-2-R p55 α chain gene in response to IL-2 under these in vivo conditions.

Kinetics of IL-2-R p55 α Chain Gene Expression During Infection with LCMV. Transcripts for the p55 α chain gene were marginally detectable with RNA isolated from blast non-B lymphocytes on day 3 post-infection, but expressed at high levels on day 7 post-infection with LCMV (Fig. 3). To examine the kinetics of α chain transcription during infection more closely, total non-B lymphocytes were prepared for in situ hybridization on days 0, 3, 5, 7, and 9 post-infection of C3H mice with LCMV. This approach requires much fewer numbers of cells than does Northern blot analysis and makes possible the determination of percentages of positive cells within mixed populations. Transcripts for the IL-2-R p55 gene were expressed in significant percentages of cells, 12-18%, on days 5, 7, and 9 post-infection (Fig. 4). As NK cell proliferation peaks on day 3 and subsides by day 7 post-infection (15), these results demonstrate that the kinetics of the accumulation of IL-2-R p55 α chain gene transcripts are distinct from the kinetics of NK cell proliferation during infection.

Expression of IFN-γ. Northern blot analysis with a probe for the IFN-γ gene was carried out to demonstrate that the blast lymphocytes elicited in vivo had been in-
FIGURE 4. Kinetics of IL-2-R p55 α chain transcription during LCMV infection of euthymic mice. Non-B lymphocytes were prepared from C3H mice on days 0, 3, 5, 7, and 9 post-infection with LCMV. The cells were fixed and examined by in situ hybridization with 35S-labeled probe complementary for the IL-2-R p55 α chain gene. The conditions for hybridization are as described in Materials and Methods. Plotted are percentages of cells with high intensity of hybridization (>5–10 grains per cell) for the IL-2-R p55 chain probe. Results are means of 6–18 slides from three to nine separate experiments. Bars show SD of the means.

FIGURE 5. Northern blot analysis for expression of the IFN-γ gene. Cellular RNA was prepared from control cells and blast non-B lymphocytes elicited in vivo. The blot is as described in Fig. 3. Hybridization using 32P-labeled probe complementary to the IFN-γ gene is shown.

duced to express a gene associated with lymphocyte activation. All of the in vivo elicited blast lymphocyte populations were activated to transcribe the gene for IFN-γ (Fig. 5). These included the blast cells that did not express the IL-2-R p55 α chain gene, i.e., those elicited by IL-2 in athymic mice and elicited by poly I:C in both athymic and euthymic mice (Fig. 5, lanes 2, 3, and 5).

Discussion

The data presented in this paper conclusively demonstrate that IL-2 can induce the proliferation of NK cells in vivo. The cells are clearly dividing as they are undergoing blastogenesis, are driven into S and G2/M phases of the cell cycle, and increase in number. The results also show that murine T cells are activated to transcribe the IL-2-R p55 α chain gene in response to IL-2 in vivo. In contrast, blast NK cells, elicited under a variety of in vivo conditions, including treatment with IL-2 and/or IFNs, do not express detectable levels of the p55 gene. These results thoroughly characterize the responsiveness of murine NK cells to IL-2 in vivo.

The NK cells examined in this study were responding to IL-2 signals in the absence of detectable p55 α chain transcripts. The receptor for IL-2 is made up of
several molecules, including a p70/75 β chain, as well as the p55 α chain (35–37).
Independently, the p70/75 and the p55 chains have low to intermediate affinity for IL-2. Most resting cells do not express the p55 molecule on their surfaces. Exposure to IL-2 has been shown to induce transcription and production of the p55 chain (33, 34). The p70/75 and p55 molecules combine to form a high affinity receptor (35, 36). Although the p55 molecule can not function independently, the p70/75 β chain of the IL-2-R can bind to IL-2 and deliver proliferation signals in the absence of the p55 chain. It is possible, therefore, for NK cells to directly respond to administered IL-2 in the absence of a p55 chain.

We appreciate the fact that p55 α chain expression can be regulated by complex mechanisms at both the RNA and protein levels (38). Although negative results are limited by the detection methods used, three sensitive techniques were used in these studies to examine NK cell expression of the p55 chain. The expression of the p55 molecule could not be demonstrated by staining of in vivo elicited cells with mAbs directed against a p55 protein determinant (data not shown). Northern blot analysis showed that p55 transcripts were not detectable with RNA isolated from the total population of blast cells elicited under conditions that induce NK cell blastogenesis in vivo. The in situ hybridizations were carried out to determine whether small subsets of cells within the mixed proliferating populations and highly purified cells expressed the gene, and these studies demonstrated that the vast majority of NK cells were not induced to express the p55 gene. The results clearly show that, under the conditions examined, p55 gene expression is dramatically more apparent when murine T cells are activated than when murine NK cells are activated. These data help to explain the studies of Wagner and Nelson (39), demonstrating that, although repetitive administration of IL-2 activated NK cells in both euthymic and athymic mice, soluble IL-2-R p55 chain could only be demonstrated in the euthymic mice. Whether or not other stimuli or secondary stimuli would augment p55 expression with NK cells remains to be determined. Work done by other investigators has suggested that the p55 chain may contribute to the maturation of NK-like killer cells (40), and the chemotactic responsiveness of LGLs isolated from the peritoneum (41).

The results presented here are in contrast to those that have been reported with human NK cells. The p55 α chain is readily induced in vitro in human NK cells after exposure to IL-2 (33, 34), and/or after signally through the Fc receptor on the surface of NK cells (38). The difference between those experiments and the ones reported here could be species differences or the result of doing experiments in vitro vs. in vivo. The former is more likely, as expression of the p55 gene can not be detected in highly purified murine NK cells exposed to rIL-2 in vitro (Dr. Vinay Kumar, personal communication).

As transcription of the p55 gene is more apparent when murine T cells are activated than when murine NK cells are activated, the activated T cells may have a competitive advantage over NK cells in binding IL-2. Such an interpretation could explain the apparent lack of NK cell proliferation at peak times for IL-2 production during viral infections of euthymic mice (18). At these times, the NK cells would not successfully compete with T cells for endogenously produced IL-2. It is not clear whether or not endogenously produced IL-2 is an important growth factor for NK cells under any conditions. The recent demonstration of detectable p70/75 molecules on normal human peripheral NK cells, but not normal peripheral T cells (42),
suggests that at early times during an immune response, NK cells may have an advantage over T cells in binding the factor. As the binding by the p70/75 receptor is of lower affinity, however, conditions for high IL-2 production in the absence of T cell activation would have to exist. We are carrying out experiments using virus-infected CD8 cell-depleted mice to ask if endogenously produced IL-2 can drive NK cell proliferation. The virus-infected and CD8 cell-depleted mice have highly active CD4+ T cells producing IL-2, but lack the major proliferating CD8+ T cell subset. It appears that endogenously produced IL-2 can support the proliferation of NK cells in these animals, as the kinetics of NK cell proliferation in CD8 cell-depleted mice extends into periods of IL-2 production (Biron, C. A., K. Leite-Morris, and M. T. Kasaian, unpublished results).

Although IL-2 is clearly inducing NK cell proliferation in these experiments, it is not possible to definitively state that all of the observed effects are mediated by direct stimulation of NK cells. An 18–24-h period is required to observe DNA synthesis by resting cells in response to proliferaton signals. The experiments reported here were all carried out within a 24-h period to maximize direct consequences of IL-2 exposure and minimize the production of intermediary molecules. However, as transcription of IFN-γ was induced, and as we have previously shown that administration of IFN-γ can induce NK cell proliferation in vivo (19), the contribution made by IL-2-induced IFN-γ is difficult to evaluate. IFN product could not be detected in the serum of mice treated with IL-2 for 24 h (data not shown). Experiments are in progress with neutralizing mAbs directed against IFN-γ to directly examine the role of this factor during IL-2-driven NK cell proliferation in vivo.

In conclusion, the work presented in this paper clearly shows that NK cells can proliferate in response to IL-2 in vivo. The results suggest that activated T cells in mice have a competitive advantage for the growth factor, as they are induced to transcribe the IL-2-R p55 α chain gene, whereas the NK cells are not induced to express detectable levels of this gene. The data help to explain the previously reported kinetics of NK cell and T cell proliferation during viral infections.

Summary

The growth factor, IL-2, was administered to mice to evaluate the in vivo responsiveness of NK cells to this factor. The immediate effects of this factor on NK cells were determined by examining cytotoxic activity at 18–24 h after a single treatment with rIL-2. Although moderate doses of rIL-2 (3 × 10⁴ U) could be shown to activate existing cytotoxic cells on a per cell basis, higher doses (10⁶ U) were required to elicit blast size killer cells. The elicited killer cells were characterized as NK cells by the following criteria: (a) they were readily induced in athymic mice; (b) they mediated killing of NK-sensitive YAC-1 target cells but not NK-resistant P815 target cells; and (c) they expressed the NK cell determinants asialo ganglio- n-tetraosylceramide and NK1.1, but not the T cell determinants CD3, L3T4, or Lyt-2. High-dose IL-2 treatment induced not only the appearance of blast size NK cells, but also the expansion of this population. After treatments, the number of large granular lymphocytes and the number of NK1.1+ cells were increased at least twofold. Analysis of DNA content within the NK1.1+ cell subset demonstrated that IL-2 preferentially drove NK1.1+ cells into S and G2/M phases of the cell cycle.

The in vivo elicited blast lymphocytes were examined by Northern blot analysis
and in situ hybridization for expression of the IL-2-R p55 α chain gene. As previous work from this laboratory has demonstrated that NK cells proliferate in response to IFNs and IFN inducers in vivo, blast lymphocytes were also prepared after IFN treatments. The NK cells were not induced to express detectable levels of the α chain gene under any of the conditions examined. Blast T lymphocytes, isolated at times during viral infections when IL-2 production can be demonstrated in vitro, were induced to transcribe the α chain gene. Treatments of euthymic mice with high-dose IL-2 also induced transcription of the α chain gene in 41% of the non-B blast lymphocytes, but only background percentages of the NK1.1+ cells expressed the α chain gene. Transcription of the α chain gene was not induced in the NK cell-abundant athymic mice after IL-2 treatment. All of the in vivo elicited blast lymphocytes were induced to express IFN-γ. Taken together, these data definitively demonstrate that IL-2 can induce NK cell proliferation and expansion in vivo. They also show that exposure to IL-2 in vivo, either by administration or endogenous production of the factor, induces transcription of the IL-2-R α chain gene in populations of cells containing T cell subsets. The results suggest, however, that murine NK cells are not induced to express high levels of the α chain gene in response to IL-2 in vivo.

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