CYCLOSPORIN A INHIBITION OF INTERLEUKIN 2 GENE EXPRESSION, BUT NOT NATURAL KILLER CELL PROLIFERATION, AFTER INTERFERON INDUCTION IN VIVO

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NK cell cytolytic activity is enhanced after exposure to IFN and/or IFN inducers in vitro (1, 2) and in vivo (3–6). In vitro, IFN induces NK cell cytotoxicity on a per cell basis (1, 2). In vivo, IFN not only enhances the level of killing mediated by individual cells, but also induces the blastogenesis and proliferation of NK cells (3–6). NK cell blastogenesis is observed during viral infections at times coinciding with the production of antiviral IFNs (3–5), after treatment with the IFN-inducer, polyinosinic-polycytidylic acid [poly(I:C)][1] (5, 6), and after in vivo administration of exogenous IFN (6). We have extensively characterized NK cell blastogenesis and proliferation, and have documented the following changes in NK cells induced by exposure to IFN in vivo: (*a*) increased size (3, 4, 6) and decreased density (7); (*b*) increased sensitivity to the cell cycle–specific toxin, hydroxyurea (4); (*c*) entry into S and G2/M phases of the cell cycle (8, 9); (*d*) enhanced incorporation of the DNA precursor [^{3}H]thymidine by cells directly mediating lysis of NK target cells (3, 6); and (*e*) increased total numbers of NK cells per spleen (9). Taken together, these results conclusively demonstrate the proliferation of NK cells in response to IFNs in vivo. It has not been possible, however, to directly support NK cell expansion in vitro with IFN, suggesting that IFN does not act directly as a growth factor for NK cells. Thus, IFN-induced NK cell expansion appears to involve an as yet unidentified endogenous mediator whose production, activation, and/or effectiveness in vivo is linked to IFN.

The T cell growth factor IL-2 can directly induce NK cell activation and proliferation in vitro (10–12) and in vivo (8), and is a candidate to mediate endogenous NK cell responses. IL-2 is a product of mature, activated T cells, and T cells are presumed to be the major physiological source of the factor. During virus infection, IL-2 is produced in vivo with kinetics corresponding to those of T cell activation

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[1] *Abbreviations used in this paper:* CsA, cyclosporin A; LCMV, lymphocytic choriomeningitis virus; LGL, large granular lymphocyte; poly(I:C), polyinosinic-polycytidylic acid; SCID, severe combined immunodeficiency.

and expansion (13). Both $CD4^+$ and $CD8^+$ T lymphocytes contribute to endogenous IL-2 production during lymphocytic choriomeningitis virus (LCMV) infection (13, 14). Under certain conditions of in vitro stimulation, additional cell types may produce IL-2. Splenic B cells (15), B cell lines (15, 16), and cells with large granular lymphocyte (LGL) morphology (17) have all been reported to make IL-2 in vitro. The significance of alternative sources of IL-2 has yet to be evaluated in vivo. During virus infection (3) and after treatment with poly(I:C) (9), NK cell activation and proliferation are observed in athymic mice lacking mature T cells. Thus, if IL-2 is required for IFN-induced NK cell responses, cells other than T cells would have to be sources of the factor in vivo.

In this report, we investigate the role of IL-2 during NK cell division in response to poly(I:C)-induced IFN in vivo. For these studies, the proliferation of NK cells was examined in T cell-deficient athymic *nu/nu* mice. Blast NK cells were enriched in the B cell-depleted low density mononuclear cell population isolated from the spleens of mice treated with poly(I:C). These cells were able to recognize IL-2 and proliferate in response to the factor, but responsiveness required 1,000-fold higher levels of IL-2 than was required by blast T cells elicted in vivo. To evaluate in vivo induction of IL-2 under conditions of NK cell proliferation, IL-2 gene transcription was analyzed by in situ hybridization in cells isolated from mice treated with the IFN inducer. Poly(I:C) stimulated IL-2 gene expression in a small population of leukocytes isolated from euthymic and athymic animals, as well as in cells isolated from mice with severe combined immunodeficiency (SCID). Cyclosporin A (CsA), an inhibitor of IL-2 transcription and production (18, 19), was used to examine the requirement for IL-2 transcription during NK cell proliferation. Although CsA abrogated poly(I:C)-induced IL-2 gene expression in athymic mice, it did not block NK cell activation or proliferation. These results indicate that IL-2 may be induced in response to IFNs, but that the factor is not a major mediator of NK cell expansion during the acute proliferative response to IFNs in vivo. Moreover, these results demonstrate that NK cell proliferation and the factor(s) supporting it in vivo are CsA insensitive.

Materials and Methods

Mice. Specific pathogen-free athymic *nu/nu* mice (BALB/cAnBOM) and their *nu/+* littermates were bred in strict isolation in our facilities at Brown University. Young mice were used (6–10 wk of age) to avoid age-associated accumulation of T cells in the periphery (20). SCID (C.B-17, scid/scid) mice were a generous gift of Dr. Joan Stein-Streilein, University of Miami School of Medicine, Miami, FL. The C3H/HeNTacfBR mice were purchased from Taconic Laboratory Animals and Services, Germantown, NY.

In Vivo Treatment Protocols. Mice were treated with 100 μg of poly(I:C) (Sigma Chemical Co., St. Louis, MO) by intraperitoneal injection 1.5 d before death (5, 8). Activated T cells were prepared from C3H mice on day 7 post-infection with 10^4 PFU of Armstrong strain LCMV, as described previously (13).

In Vivo Depletion of AGM1-positive Cells. To deplete NK cells in vivo, mice were given a single intraperitoneal injection of antibody to AGM1 (21) (Wako Chemicals, Inc., Dallas, TX) 1.5 d before poly(I:C) (3 d before death), as described previously (5, 7). These conditions have been shown to specifically deplete NK cells in vivo (5, 22).

CsA Treatment. CsA (Oral solution, 100 mg/ml in olive oil-labrafil base; Batch 093L5; kindly provided by Dr. David Winter, Sandoz Pharmaceuticals, Hanover, NJ) was diluted in olive oil (Sigma Chemical Co.). Mice were given three dosages of 40 mg/kg each by intraperitoneal injection at 12-h intervals. The first dose was given 2 h before administration

of poly(I:C). Three dosages of 40 mg/kg CsA have been shown to profoundly inhibit CTL activity, T cell proliferation, and IL-2 production during infection of mice with LCMV (14). Control animals for these experiments received an equal volume of olive oil in place of CsA.

Preparation of Cells for Analysis. Mice were killed by cervical dislocation, and their splenocytes were harvested. RBC were lysed by ammonium chloride treatment. Low density leukocytes were isolated at the interface of a 38–54% discontinuous Percoll density gradient (5). In some cases, the splenocyte populations were depleted of B cells and polymorphonuclear leukocytes (PMNs) by treatment with the mAb J11d (23) and C', as described (5). Viable cells were collected by centrifugation through 38% Percoll. This was followed by a 38–54% discontinuous Percoll density gradient to isolate low density, $J11d^-$ cells.

51-Chromium Release Cytotoxicity Assay. The NK-sensitive target cell line, YAC-1, was labeled with sodium ^{51}Cr (ICN, Irvine, CA), and incubated with effector cells for 5 h at 37°C in microtiter plates, as described (7). Spontaneous lysis was determined by incubating medium with the target cells. Maximum ^{51}Cr release was determined by adding 1% NP-40 to target cells. Percentage of lysis was calculated as: 100× (cpm test sample − cpm spontaneous lysis)/(cpm maximum release − cpm spontaneous lysis). A lytic unit (LU) was calculated as the number of effector cells required to mediate 20% target cell lysis.

Single Cell Cytotoxicity Assay. The $J11d^-$ low density splenocytes, prepared as described above, were labeled with the DNA precursor, [^{3}H]thymidine (60–90 Ci/mmol; ICN) for 1 h at 37°C. The labeled effector cells were used in a single cell-killing assay with autoradiography, as previously described (3, 4). Briefly, the effector cells were mixed with K562 target cells at an E/T cell ratio of 1:1, pelleted, and incubated for 30 min at room temperature to allow E/T cell conjugate formation. K562 target cells, previously shown to be sensitive to lysis by murine NK cells (3, 6), were used because their large size made them easily distinguishable from the smaller size effector cells. The conjugates were resuspended in 0.5% agarose (Sea-Plaque, low gelling temperature; FMC Corp., Rockland, ME) and spread in a thin layer over agarose-coated microscope slides. The slides were incubated at 37°C for 4 h to allow for cytotoxicity, stained with 0.1% trypan blue (Gibco Laboratories, Grand Island, NY) to visualize dead cells, extensively washed, and fixed in 0.1% formaldehyde. Slides were coated in nuclear track emulsion (type NTB-2; Kodak, Rochester, NY), exposed for 2 d, and developed. 100 conjugates, composed of a single effector and single target cell, were scored per slide. Percent cytotoxicity was quantitated by determining the fraction of E/T cell conjugates containing a dead target cell. The percentage of killing mediated by blast effector cells was determined as: 100× (number of conjugates containing a dead target cell bound to a blast effector cell)/(total number of conjugates containing a dead target cell).

In Situ Hybridization. The plasmid pmIL2-20 (24), containing 530 nucleotides of mouse IL-2 cDNA, was obtained through Dr. Gayle Woloschak of the Argonne National Laboratory, Argonne, IL, and used for in situ hybridization, as described (13). Northern blot analysis confirmed that the probe hybridized to the 12S mouse IL-2 transcript. The probe was labeled with ^{35}S dCTP (New England Nuclear, Boston, MA) by random hexanucleotide priming (kit from Boehringer-Mannheim Biochemicals, Indianapolis, IN) to a sp act of $>10^8$ cpm/μg, and added to a mixture of salmon sperm DNA and brewer's yeast tRNA. The nucleotide mixture was dehydrated, and denatured in 100% formamide at 90°C. The cells to be probed were spun onto microscope slides using a cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, PA), fixed in 4% paraformaldehyde, dehydrated, and stored in 70% ethanol. They were rehydrated in PBS before hybridization, then treated with 50% formamide at 70°C for 5 min. Hybridization was performed at 37°C for 3 h. The cells were washed extensively in formamide and SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0) at 4°C. The slides were dried, dipped in nuclear track emulsion (type NTB-2; Kodak), exposed at 4°C for 7–10 d, and developed. After autoradiography, the cells were stained with Wright's-Giemsa. The specificity of the technique was demonstrated by the following criteria: (*a*) a high degree of specific hybridization of the IL-2 probe to Con A–induced T cell blasts, shown to be IL-2 transcription positive by Northern blot analysis; (*b*) lack of hybridization of cells positive for IL-2 transcription to vector control DNA (pGEM-1, digested to the same size and labeled to the same specific activity as the IL-2 probe); and (*c*) lack of hybridization of a probe directed against the β constant region of the TCR gene to *nu/nu* spleen cells, positive for hybridization to the IL-2 probe.

Lymphocyte Proliferation Assay. Splenocytes were plated in microtiter wells (10^5 cells/well) in the absence of additional growth factors or mitogens. [^{3}H]Thymidine (1 μCi/well; ICN) was immediately added. After a 6-h incubation at 37°C, the cells were harvested using a Mini-MASH II (Whittaker M. A. Bioproducts, Walkersville, MD). Incorporation of [^{3}H]thymidine was assayed by liquid scintillation counting.

Single Cell [^{3}H]Thymidine Incorporation. Spleen cells were incubated at 10^6/ml in medium containing 2.5 μCi/ml [^{3}H]thymidine (ICN). After a 1-h incubation at 37°C, the cells were washed four times, and spun onto microscope slides using a cytocentrifuge. Cells were fixed in 4% paraformaldehyde for 15 min at room temperature, rinsed in 70% ethanol, and air dried. The slides were dipped in nuclear track emulsion (type NTB-2; Kodak), exposed at 4°C for 24 h, developed, and stained with Wright's-Giemsa.

IL-2 Responsiveness Assay. Cells were incubated at 10^5/well in microtiter plates with the indicated concentration of human rIL-2 (a gift of Cetus Corp., Emeryville, CA). Concentrations of IL-2 are reported as Cetus units (the specific activity of the rIL-2 was 3×10^6 Cetus units per mg protein, where 3 Cetus units = 18 IU). Cells were incubated for 24 h, with [^{3}H]thymidine (1 μCi/well; ICN) added for the final 6 h of incubation. Cells were harvested from plates using a Mini-MASH II, and incorporation of the DNA precursor was determined by liquid scintillation counting.

Determination of Lymphocyte Morphology. Cells were spun onto microscope slides using a cytocentrifuge (30,000–50,000 cells per slide). Centrifugation was performed at 400 rpm for 5 min. Cells were stained with Wright's-Giemsa, and morphology was examined.

Results

NK Cell Activation and Proliferation in Response to Poly(I:C). These experiments were undertaken to evaluate the role of IL-2 in mediating IFN-induced NK cell responses in vivo. The IFN inducer poly(I:C) stimulates NK cell activation and expansion in both athymic and euthymic mice (5, 6). To examine NK cell responses in the absence of T cells and T cell interactions, athymic (*nu/nu*) mice were treated with poly(I:C). As reported previously (5, 6), poly(I:C) treatment resulted in enhanced NK cell cytotoxic activity against the NK-sensitive target cell line, YAC-1 (Table I; Fig. 1 *A*). An average of 24 LU/spleen were produced. As these experiments required quantitation of NK cell numbers and analysis of NK cell division, it was necessary to establish protocols for enrichment of the NK cell subset. Density separations and subset depletions were used to isolate the activated NK cell population. Cells mediating YAC-1 lysis were enriched among low density splenocytes isolated from poly(I:C)-treated animals (Table I). The low density fraction represented 20–25% of the total spleen cell population isolated from poly(I:C)-treated, as well as control, athymic mice. Depletion of B cells and PMNs with the mAb, J11d, and C′ resulted in a further enrichment of cells mediating lysis in the low density fraction (Table I). The J11d$^-$ low density population corresponded to 2–14% of the total spleen leukocytes from control athymic mice, and 3–5% of the total from poly(I:C)-treated animals.

NK cell blastogenesis and proliferation after poly(I:C) treatment were assayed by analysis of LGL numbers, and by incorporation of the DNA precursor, [^{3}H]thymidine. The percentage of cells with the LGL morphology, characteristic of NK, was dramatically increased in the J11d$^-$ low density cell fraction after poly(I:C) treatment (Table I; Fig. 1 *B*). The total number of LGLs in this fraction more than doubled as a result of this treatment, from 3.4×10^5 in control animals to 1.5×10^6 in mice treated with poly(I:C). Lymphocytes undergoing DNA synthesis were also greatly enriched in the J11d$^-$ low density population (Table I; Fig. 1 *C*). These

TABLE I

Enrichment of IFN-activated Lymphocytes in the B Cell-depleted Low Density Fraction Isolated from Athymic Mice

Cell fraction	Untreated mice			Poly(I:C)-treated mice*		
	YAC-1 lysis (E/T = 25:1)‡	LGL§	[^{3}H]Thymidine uptake‖	YAC-1 lysis (E/T = 25:1)‡	LGL§	[^{3}H]Thymidine uptake‖
	%	%	*cpm*	%	%	*cpm*
Unseparated	0.7	1.9 ± 0.9	490 ± 306	19.5	4.0 ± 1.2	526 ± 84
Low density¶	0	2.8 ± 1.2	342 ± 245	21.8	8.2 ± 2.1	692 ± 99
J11d$^-$ low density**	0.1	5.3 ± 4.4	650 ± 559	39.5	72.8 ± 7.6	10,300 ± 2,066

* Mice were treated with 100 μg poly(I:C) 1.5 d before death.

‡ Cytotoxicity was measured in a 5-h ^{51}Cr release assay.

§ Data are the mean ± SD of four to six determinations.

‖ Incorporation of [^{3}H]thymidine by freshly isolated cells was measured after a 6-h pulse. Data are the mean ± SD of 5–10 values.

¶ Low density spleen leukocytes from athymic mice were isolated at the interface of 38–54% Percoll density gradients.

** J11d$^-$ low density cells were depleted of B cells and PMNs by treatment with J11d and C'. Viable cells were then run over a 38–54% Percoll density gradient to isolate the low density fraction, as described in Materials and Methods.

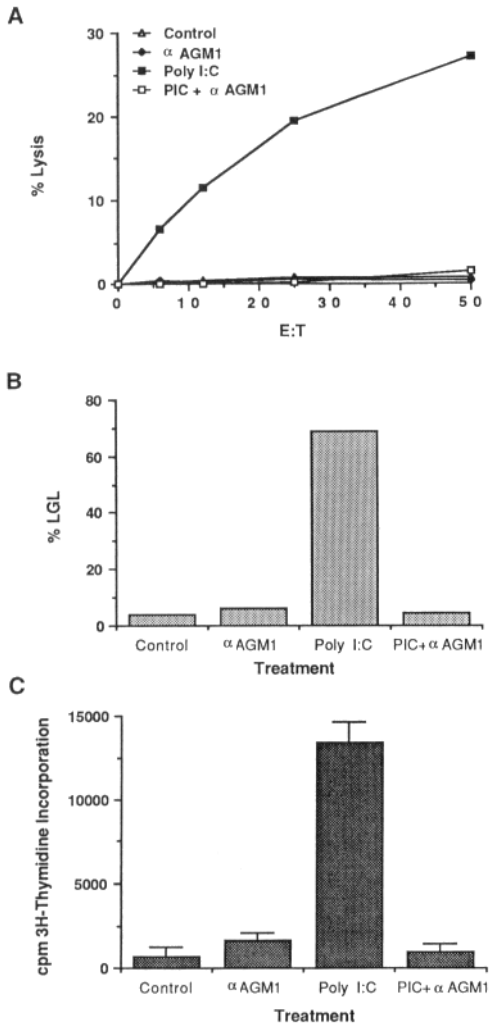

FIGURE 1. Effects of in vivo anti-AGM1 treatment on poly(I:C)-induced responses. Athymic (*nu/nu*) mice were treated with antiserum to AGM1 3 d before death. Poly(I:C) was administered 1.5 d later. Spleen leukocytes were harvested and fractionated as described in Materials and Methods. Responses were compared with those of untreated control mice, mice given antibody only, and mice given poly(I:C) only. (*A*) Cytotoxicity mediated by total plastic nonadherent cells in a 5-h ^{51}Cr release assay against YAC-1 target cells. (*B*) Percentages of cells with LGL morphology in the J11d$^-$ low density fraction. A total of 200 cells were scored per sample. (*C*) Incorporation of [^{3}H]thymidine by freshly isolated J11d$^-$ low density cells in a 6-h pulse. Data are the mean +/− SD for 5-10 replicates per sample.

results demonstrate that NK cells and dividing cells are enriched in low density J11d$^-$ populations after treatment with the IFN inducer.

The dividing cells were characterized as the NK cells by in vivo administration of antibody against the NK cell marker, AGM1. This treatment eliminated poly(I:C)-induced NK cytotoxicity (Fig. 1 *A*), cells with LGL morphology (Fig. 1 *B*), and cells incorporating [^{3}H]thymidine (Fig. 1 *C*). In additional experiments, the [^{3}H]thymidine-incorporating cells were shown to directly mediate lysis of NK-sensitive target cells in a single cell-killing assay with autoradiography (Table II). Of the 40% cytotoxicity mediated by poly(I:C)-activated NK cells, 54% was due to effector cells undergoing division. Taken together, these findings establish that the dividing J11d$^-$ low density cells are NK cells, and support our previous work demonstrating NK cell expansion after treatment with IFN inducers in vivo (3–6).

TABLE II

Cytotoxic Activity of Blast Effector Cells in a Single Cell Assay

Effector cells*	Dead target cells conjugated to effector cells (total)	Dead target cells conjugated to blast effector cells	Killing mediated by blast effector cells‡
		%	
Control	6.3 ± 4.0	0.3 ± 0.6	5.7
Poly(I:C)§	39.7 ± 4.7	21.3 ± 5.0	53.8

A 4-h single cell cytotoxicity assay with autoradiography was performed as described in Materials and Methods, using effector cells labelled with the DNA precursor, [^{3}H]thymidine, and K562 target cells. 100 E/T cell conjugates were scored per assay. Dead targets were visualized by staining with trypan blue. Blast effector cells, which had incorporated [^{3}H]thymidine, were visualized by autoradiography with nuclear track emulsion.

* J11d$^-$ low density spleen leukocytes, prepared from athymic mice as described in Materials and Methods, were used as effector cells.

‡ Calculated as 100 × (number of conjugates containing dead targets and blast effector cells)/(number of total conjugates containing dead target cells), for 100 total conjugates scored.

§ Mice were treated with 100 μg poly(I:C) intraperitoneally 1.5 d before death.

IL-2 Responsiveness of NK Cells in Poly(I:C)-treated Mice. To determine whether the IFN-activated cells from nude mice were able to recognize and respond to IL-2, incorporation of [^{3}H]thymidine was assayed after exposure to IL-2 in vitro. IL-2 responsiveness was found, but only among the low density J11d$^-$ subset enriched for NK cells. The concentration dependence of IL-2-supported proliferation was examined. The J11d$^-$ low density cells isolated from control mice did not proliferate in response to IL-2 concentrations of up to 2.5 × 10^5 U/ml. When these cells were exposed to levels >5 × 10^5 U/ml, however, proliferation was observed (Fig. 2). Similar results were found using cells prepared from either euthymic or athymic animals. The J11d$^-$ low density cells isolated from poly(I:C)-treated athymic mice responded to lower levels of the factor, requiring ~2.5–5 × 10^3 U/ml rIL-2 for half-maximal proliferation (Fig. 2). The responding cell type was characterized as NK based on its sensitivity to in vivo anti-AGM1 treatment (Fig. 3). We have previously shown that dividing spleen T cells, activated during LCMV infection, are induced to respond to IL-2 (14). The J11d$^-$ low density spleen cells isolated from euthymic

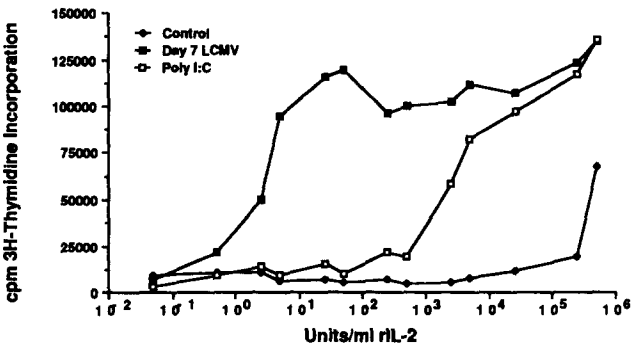

FIGURE 2. Responsiveness of poly(I:C)-elicited blast cells to IL-2. The J11d$^-$ low density cells were isolated, and incubated for 24 h with the indicated concentrations of rIL-2 (Cetus Corp.) The proliferative response of the cells was determined by addition of [^{3}H]thymidine for the final 6 h of incubation. Cells were derived from athymic (*nu/nu*) mice treated with poly(I:C) (□), untreated euthymic (C3H) mice (◆), and C3H mice 7 d post-infection with LCMV (■).

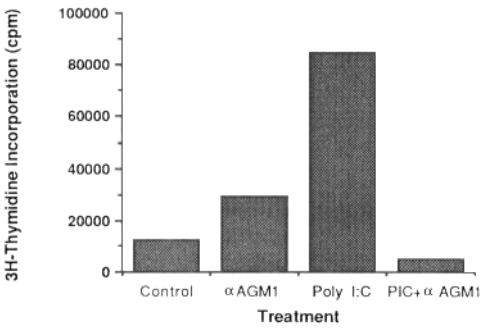

FIGURE 3. Administration of anti-AGM1 in vivo eliminates IL-2 responsiveness in poly(I:C)-treated mice. Athymic (*nu/nu*) mice were treated with antiserum to AGM1 3 d before death, and given poly(I:C) 1.5 d later. Spleen leukocytes were harvested, and the J11d$^-$ low density fraction prepared as described in Materials and Methods. Cells were incubated with 5×10^3 U/ml rIL-2 (Cetus Corp.) for 24 h. Proliferation of the cells in response to IL-2 was determined by addition of [^{3}H]thymidine for the final 6 h of incubation. In the absence of exogenous IL-2, [^{3}H]thymidine incorporation ranged from 460 to 3,187 cpm for the various samples.

mice at the peak of the T cell response, day 7 post-infection with LCMV, were prepared for comparison. Activated T cells required ~2.5–5 U/ml rIL-2 for half-maximal proliferation (Fig. 2). Thus, IFN-induced NK cells are responsive to IL-2, but require 1,000-fold higher concentrations of the factor than those required for the proliferation of in vivo activated T cells.

Induction of IL-2 Gene Transcription by Poly(I:C). To determine if IFN-induced NK cell blastogenesis is accompanied by induction of IL-2, in situ hybridizations were performed to examine the activation of IL-2 gene expression. Hybridizations were carried out on paraformaldehyde-fixed cells using a cDNA probe to the mouse IL-2 gene. Low but significant percentages of cells isolated from poly(I:C)-treated, but not from untreated, athymic mice expressed the IL-2 gene (Table III). As we have previously demonstrated IL-2 gene expression in spleen T cells of euthymic mice during infection with LCMV, poly(I:C)-mediated induction of IL-2 expression was also examined in euthymic (*nu/+*) mice. A low percentage of expressing cells were also found in spleens of *nu/+* mice treated with the IFN inducer (Table III). Thus, IFN induction in vivo, under conditions that promote NK cell proliferation, is ac-

TABLE III

Percentage of IL-2 Transcription-positive Cells by In Situ Hybridization

Effector cells	Cell fraction	Phenotype		
		nu/nu	*nu/+*	SCID
			%	
Control	Unseparated*	2.6 ± 2.4	3.2 ± 1.9	1.4 ± 1.9
	Low density‡	3.5 ± 3.3	4.4 ± 3.6	3.0 ± 5.2
Poly(I:C)§	Unseparated*	11.9 ± 6.1	5.9 ± 3.9	14.5 ± 7.4
	Low density‡	20.4 ± 8.6	18.9 ± 7.6	33.8 ± 3.0

The percentage of cells positive for IL-2 gene expression was determined by in situ hybridization as described in Materials and Methods. In separate experiments, 7–10 silver grains per cell was taken as a positive result. Data are the mean ± SD of 4–16 determinations.

* Total spleen leukocytes were prepared as described in Materials and Methods.

‡ Low density spleen leukocytes were isolated at the interface of discontinuous 38–54% Percoll density gradients.

§ Mice were treated with poly(I:C) 1.5 d before death.

companied by the activation of IL-2 gene expression in both athymic and euthymic mice. These data demonstrate that the major responding cell type is not a T cell.

To further define the cellular basis of the IL-2 response to poly(I:C), SCID mice were treated with the IFN inducer. Although SCID mice are deficient in both T and B lymphocytes, they do contain full NK cell activity (25, 26). In these experiments, poly(I:C) treatment dramatically elevated the lysis of NK-sensitive target cells by spleen leukocytes of SCID mice, from undetectable activity in control animals, to 19 LU/spleen in mice treated with poly(I:C). This increase in NK cell activity was accompanied by the induction of IL-2 gene expression. In situ hybridization demonstrated that the percentage of IL-2-expressing cells increased from 1.4% in untreated to 14.5% after poly(I:C) treatment of SCID mice (Table III).

To enrich the IL-2-transcribing cell, low density populations containing activated cells were prepared from control and poly(I:C)-treated mice. Greater than 18% of the low density cells isolated from poly(I:C)-treated athymic, euthymic, or SCID mice were positive for IL-2 gene expression (Table III; Fig. 4). The spleen leukocytes of SCID mice contained a large number of cells with the doughnut-shaped nuclear morphology characteristic of granulocyte precursors. Although these cells were abundant in the low density fraction (Fig. 4, *c* and *d*), the granulocyte precursors were distinctly negative for IL-2 gene expression when isolated from either control or poly(I:C)-treated mice (Fig. 4, *c* and *d*). These findings demonstrate the induction of IL-2 gene expression in a population of low density spleen cells after exposure to IFN, and indicate that the transcription-positive cell type is not a T cell, B cell, or granulocyte.

CsA Effects on Poly(I:C)-induced IL-2 Gene Transcription. IL-2 production is inhibited in vitro (18, 19) and in vivo (14) at the level of gene transcription by the immunosuppressive drug CsA. To determine whether the IL-2 gene expression induced by poly(I:C) treatment is CsA sensitive, this drug was administered to poly(I:C)-treated athymic mice. The percentages of IL-2 transcript-positive cells in the low density population were reduced by 85–100% as a result of CsA treatment (Fig. 5). Antiviral IFN is resistant to CsA (27), and poly(I:C)-induced IFN levels in the serum were not affected by drug treatment.

CsA Effects on NK Cell Activity. To determine the consequences of loss of IL-2 transcription on NK cell-mediated lysis, spleen leukocytes of athymic mice treated with poly(I:C) in the presence or absence of CsA were used in a cytotoxicity assay against YAC-1 target cells. An apparent increase in cytotoxicity after CsA treatment was observed (Fig. 6 *a*). Cells of poly(I:C)-treated mice mediated 13.5% lysis at an E/T of 25:1, as compared with 21% for cells isolated from mice given CsA in addition to poly(I:C). The enhanced activity proved to be artificial, however, as spleen leukocyte yields were decreased by 42% in CsA-treated animals. When the number of LU per spleen was calculated, no effect of CsA on the level of NK cell activity was evident; poly(I:C)-treated animals had 17 LU/spleen, as compared with 15 LU/spleen for mice given poly(I:C) and CsA. Thus, the level of NK cell activity per spleen was unchanged by CsA treatment. These results demonstrate that IFN-induced NK cell activity is independent of IL-2, and of other CsA-sensitive agents.

CsA Effects on NK Cell Blastogenesis and Proliferation. To evaluate the effects of CsA on poly(I:C)-induced NK cell expansion, LGL numbers and [^{3}H]thymidine incorporation by the NK cell population of CsA-treated nude mice were analyzed. CsA treatment did not diminish the recovery of LGLs in the NK cell-enriched J11d$^-$ low

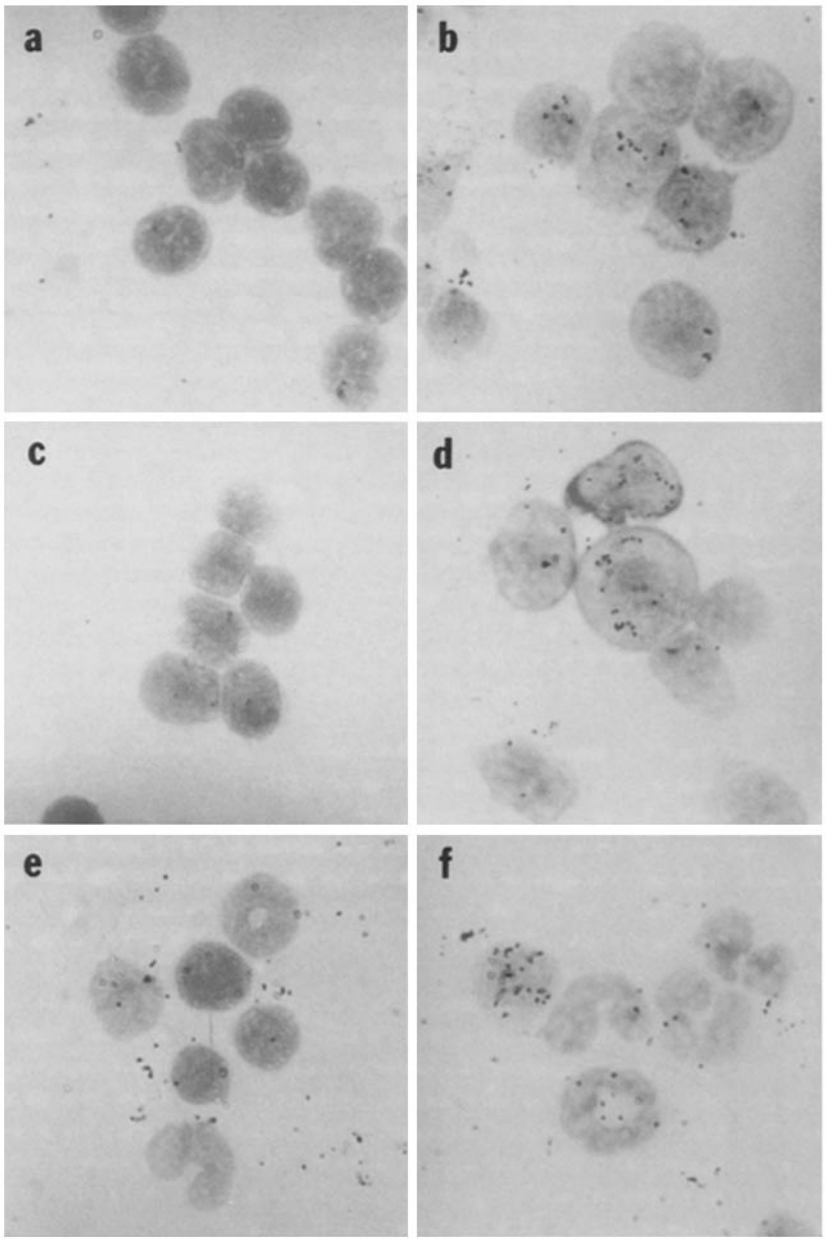

FIGURE 4. In situ hybridization for IL-2 gene expression in low density spleen cells isolated from poly(I:C)-treated mice. In situ hybridization was performed on cells isolated from untreated and poly(I:C)-treated mice, using a ^{35}S-labeled cDNA probe to the mouse IL-2 gene. Positive cells contained seven or more grains. Percentages of positive cells in each fraction are reported in Table III. Cells were derived from: *a*, euthymic (*nu/+*) mice, control; *b*, euthymic (*nu/+*) mice, poly(I:C) treated; *c*, athymic (*nu/nu*) mice, control; *d*, athymic (*nu/nu*) mice, poly(I:C) treated; *e*, SCID mice, control; *f*, SCID mice, poly(I:C) treated.

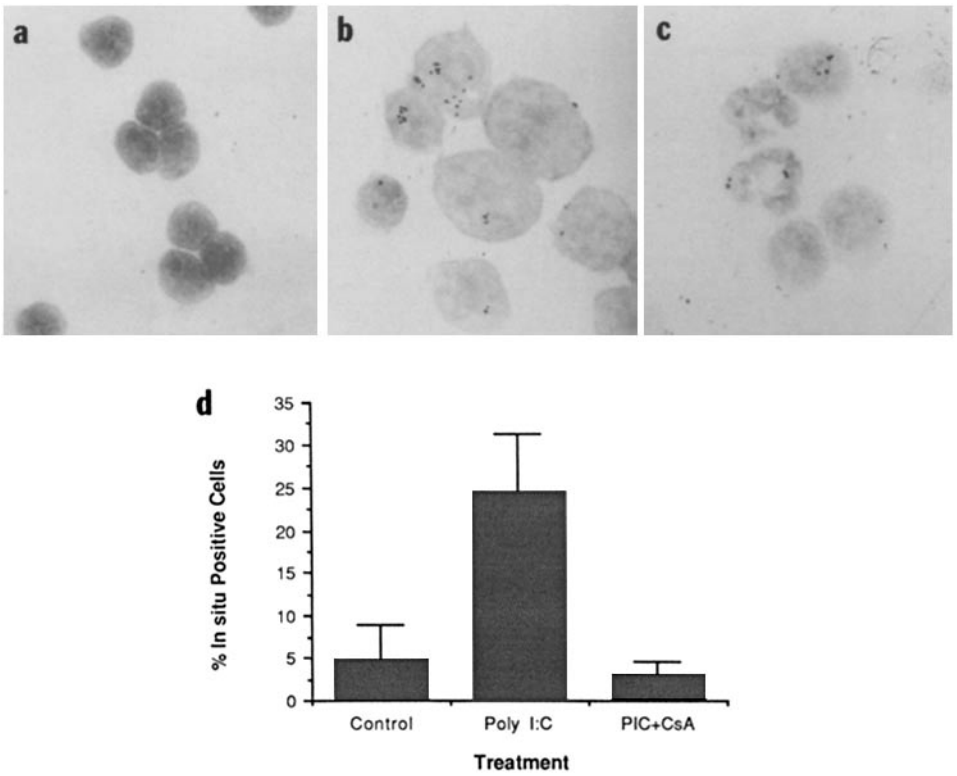

FIGURE 5. In situ hybridization to determine effects of CsA on poly(I:C)-induced IL-2 gene expression. In situ hybridization was performed as described in Materials and Methods, using low density spleen leukocytes isolated from athymic mice. (*a*) Control; (*b*) treated with 100 μg poly(I:C) 1.5 d before death; (*c*) treated with three dosages of 40 ng/kg CsA at 12-h intervals, in addition to poly(I:C). Control and poly(I:C)-treated mice were administered olive oil in place of CsA. (*d*) The percentage of cells positive for transcription was determined, using a value of seven or more grains per cell as a positive result. 100 cells were scored per experiment. Data are presented as mean ± SD for three to five separate experiments.

density fraction (Fig. 6 *b*). Greater than 70% of the cells in this fraction isolated from mice treated either with poly(I:C) alone, or with CsA in addition to poly(I:C), had LGL morphology. Mice treated with CsA alone had 1.5×10^5 LGLs per spleen, whereas CsA-treated animals given poly(I:C) had 5.7×10^5 LGLs per spleen. This represents a three- to fourfold increase in LGL number, indicating that expansion of LGLs was not inhibited by CsA.

To evaluate effects of CsA on NK cell blastogenesis and proliferation in response to poly(I:C), [^{3}H]thymidine incorporation by the J11d$^-$ low density population was assayed. Cells isolated from mice treated with CsA in addition to poly(I:C) incorporated as much or more [^{3}H]thymidine than did cells isolated from mice that had received poly(I:C) alone (Fig. 6 *c*). Furthermore, examination of [^{3}H]thymidine incorporation into individual cells by autoradiography revealed that the percentage of cells undergoing DNA synthesis was largely unaffected by the drug (Fig. 7). These

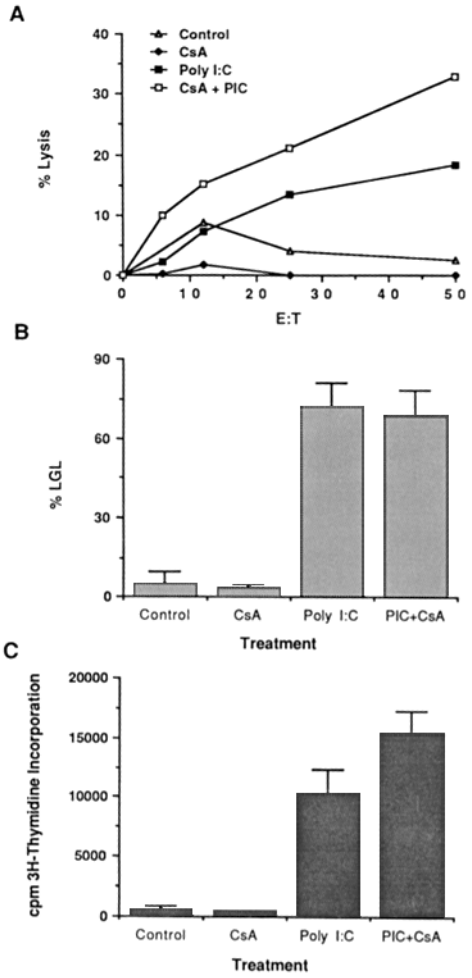

FIGURE 6. Effects of CsA on poly(I:C)-induced NK cell activity. Spleen leukocytes were prepared from athymic mice treated with poly(I:C) in the presence or absence of CsA, and fractionated as described in Materials and Methods. Control animals were administered olive oil only or CsA only. (*a*) Cytotoxicity mediated by total plastic nonadherent splenocytes in a 5-h ^{51}Cr release assay against YAC-1 target cells. (*b*) Percentages of cells with LGL morphology in the J11d$^-$ low density spleen cell fraction. A total of 200 cells were scored per sample. Data are presented as the mean +/− SD for five to eight individual experiments. (*c*) Incorporation of [^{3}H]thymidine by J11d$^-$ low density cells in a 6-h pulse. With the exception of the sample from mice treated with CsA only, data are shown as the mean +/− SD for 5–10 determinations.

results demonstrate that, although CsA drastically inhibits poly(I:C)-induced IL-2 gene transcription, it does not block NK cell blastogenesis or proliferation.

CsA Effects on IL-2 Responsiveness. To determine the effects of CsA on IL-2 responsiveness among the IFN-induced NK cells, cells were isolated from athymic mice treated with CsA in addition to poly(I:C). CsA, at concentrations that resulted in complete inhibition of IL-2 gene transcription, caused only a moderate reduction in IL-2 responsiveness (Fig. 8). These results suggest that IFN-induced NK cells remain responsive to IL-2 in the presence of CsA.

Discussion

Previous work from this laboratory has shown that IFN and IFN inducers elicit acute NK cell blastogenesis and proliferation in vivo. The IFN-elicited effector cells have been thoroughly characterized. They demonstrate elevated cytolytic activity against NK-sensitive, but not NK-resistant, target cells (5), have the morphology

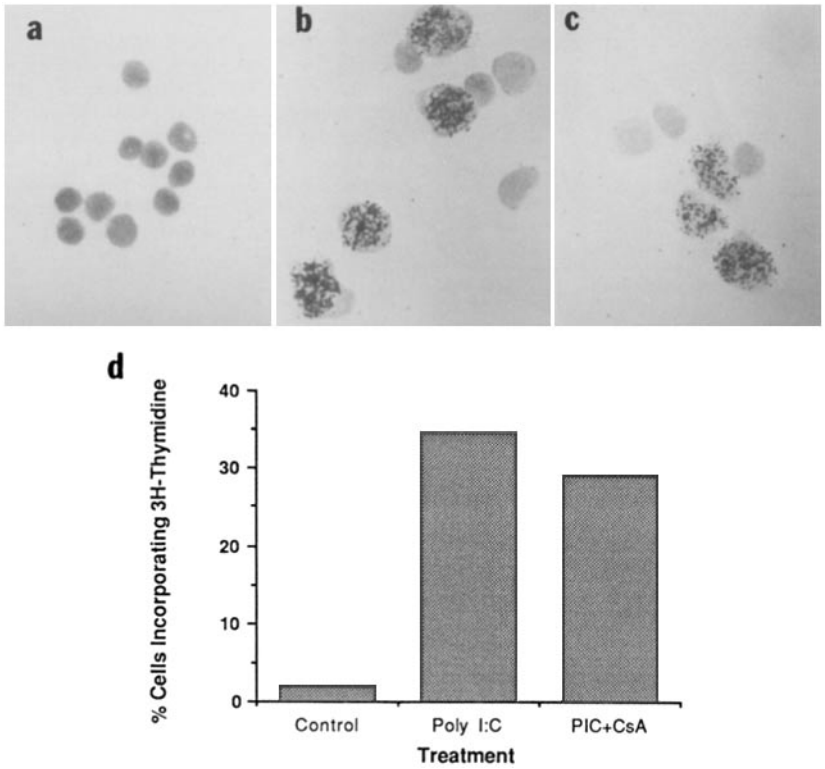

FIGURE 7. Single cell [^{3}H]thymidine incorporation in response to poly(I:C): lack of inhibition by CsA. The J11d$^-$ low density populations were isolated from athymic mice treated with poly(I:C) in the presence or absence of CsA, as described in Materials and Methods. Cells were incubated for 1 h with the DNA precursor, [^{3}H]thymidine, spun onto microscope slides, and exposed to emulsion. The emulsion was developed after 20 h, and cells were stained with Wright's-Giemsa. Cells were derived from: *A*, control mice; *B*, poly(I:C)-treated mice; *C*, mice treated with CsA in addition to poly(I:C); *D*, the percentage of cells incorporating [^{3}H]thymidine was quantitated, of 100 total cells scored per sample.

of LGLs (5), and express a high level of the NK cell surface determinants AGM1 (5) and NK 1.1 (8). Furthermore, these cells are negative for expression of TCR genes α, β, γ, T3δ, and T3ϵ (9). The activated NK cells are clearly dividing, as they are driven into the S and G2/M phases of cell cycle (8, 9), incorporate the DNA precursor, [^{3}H]thymidine (3, 6), and increase in number (8). In the studies reported here, blast NK cells were isolated in J11d$^-$ low density populations prepared from poly(I:C)-treated athymic mice. The dividing cells were characterized as NK cells based on the findings that they were eliminated by anti-AGM1 treatment in vivo, and that the [^{3}H]thymidine-incorporating cells directly mediated lysis of NK-sensitive target cells. The goal of the present studies was to assess the requirement for IL-2 during IFN-elicited NK cell proliferation. Studies presented here suggest that IL-2 may be induced after exposure to IFN in vivo, but show that the factor is not necessary to support NK cell proliferation.

We have previously examined the IL-2-dependent expansion of T lymphocytes

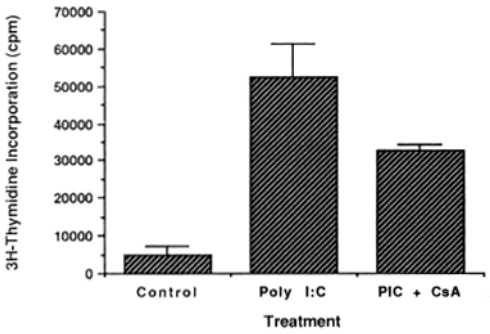

FIGURE 8. CsA effects on the IL-2 responsiveness of poly(I:C)-induced blast cells. J11d$^-$ low density populations were incubated with 5×10^3 U/ml rIL-2 for 24 h, as described in Materials and Methods. [^{3}H]thymidine (1 μCi/well) was added during the final 6 h of incubation. Incorporation of [^{3}H]thymidine by the cells was assayed by liquid scintillation counting.

during LCMV infection in vivo (14). In those studies, treatment with the immunosuppressive agent, CsA, blocked IL-2 transcription and production, as well as T cell activation and proliferation during infection (14). In contrast, NK cell cytolytic activity was elevated in the virus-infected mice treated with CsA at times when T cell activity and IL-2 transcription were inhibited. This result suggested that NK cell proliferation might be CsA insensitive and IL-2 independent. Although IL-2 is able to directly induce NK cell proliferation in vivo and in vitro (10–12), and is the only factor currently described to have these properties, its involvement in a normal in vivo NK cell–proliferative response has not been previously addressed. The present study directly examined the role of IL-2 in IFN-induced NK cell proliferation in a T cell–independent system. Although poly(I:C) treatment resulted in IL-2 gene transcription, IL-2 was not found to be an important mediator of NK cell proliferation. CsA treatment completely inhibited IL-2 transcription, but did not block NK cell expansion. These results demonstrate that acute NK cell proliferation is IL-2 independent and CsA insensitive.

Although NK cell expansion occurred in the absence of IL-2 gene transcription in vivo, our results demonstrate that IFN-elicited blast NK cells are able to proliferate in response to exogenous IL-2 in vitro. This suggests expression of an IL-2 binding site on IFN-activated NK cells. The receptor for IL-2 consists of two subunits: the low affinity p55, α chain molecule, and the intermediate affinity p70/75, β subunit (28, 29). A high affinity binding site results from interaction of these two subunits, and is thought to mediate the response of T lymphocytes to IL-2. Although a proliferative signal is transduced most efficiently by the high affinity receptor, the p70/75 molecule is able to independently deliver a growth signal (30). Recent work from other laboratories suggests that NK cells may constitutively express the p70/75 molecule on the cell surface (31–34). The p55 chain is not sufficient to mediate a cellular response to IL-2. We have recently shown that murine NK cells elicited in vivo in response to either IFN or IL-2 do not express detectable levels of the p55 chain gene (8), and thus cannot form the high affinity receptor. The results reported here are consistent with lack of expression of a high affinity IL-2-R by in vivo activated NK cells. The IFN-elicited blast NK cells requird ~1,000-fold higher concentrations of IL-2 to achieve a half-maximal proliferative response than did blast lymphocytes of mice undergoing a T cell response to LCMV. This concentration dependence

is consistent with NK cell expression of a lower affinity receptor form than that expressed on T cells, and may reflect use of the p70/75 molecule.

The J11d$^-$ low density populations isolated from untreated control mice were also able to proliferate in response to exogenous IL-2 in vitro. However, this response required extremely high concentrations of IL-2 (>5 × 10^5 U/ml). The increased responsiveness of J11d$^-$ low density cells isolated from poly(I:C)-treated mice, as compared with that of control mice, may be explained by any of the following: (*a*) expansion of the IL-2-responsive cell type; (*b*) increased numbers of receptors per cell; and (*c*) expression of a higher affinity form of the receptor. Additional information concerning the kinetics of IL-2 binding by NK cells is required to distinguish between these possibilities.

It is not clear whether or not the IL-2 transcription observed in spleen cells of poly(I:C)-treated mice is accompanied by synthesis of functional IL-2. It was not possible to detect IL-2 production in response to poly(I:C), even in the low density lymphocyte fraction enriched for transcription-positive cells (data not shown). However, this may be due to technical limitations of the IL-2 production assay. The production of IL-2 is determined as the release of functional IL-2 into supernatants of overnight leukocyte cultures (13). As the IL-2-producing cells may also bind and use the factor, demonstration of production requires inhibition of IL-2 consumption by the producing cells. Inhibition has been achieved previously by addition of antibodies directed against the p55 chain of the IL-2-R (13). If the IL-2-producing cells express p70/75 only, and do not use the p55 chain, IL-2 utilization would not be prevented by anti-p55 antibodies. Experiments are in progress to characterize antibodies directed against p70/75, which may then be evaluated for their ability to inhibit IL-2 consumption in this system.

Despite the lack of demonstrable product, the in situ hybridization studies clearly revealed poly(I:C)-induced activation of IL-2 gene expression. This expression was CsA sensitive, and was found in spleen cells isolated from poly(I:C)-treated euthymic, athymic, and SCID mice. These findings suggest that a source of IL-2 exists in vivo other than the mature T cell. Although the positive cells have yet to be identified, preliminary studies suggest that IL-2-transcribing cells can be found in both J11d$^+$ and J11d$^-$ low density populations. The frequency of the IL-2-transcribing cell in the J11d$^-$ low density fraction appears to be higher in athymic mice as compared with euthymic mice. Experiments are in progress to definitively characterize the cell type responsible for IL-2 transcription in this system.

Taken together, the following observations suggest that although IL-2 gene expression is induced by poly(I:C), it is unlikely that IL-2 plays a critical role in mediating the NK cell response to IFN. (*a*) The percentage of unfractionated spleen leukocytes expressing the IL-2 gene in response to poly(I:C) is low. (*b*) CsA blocked IL-2 gene transcription, but not NK cell cytotoxicity or expansion, conclusively demonstrating that the IL-2 gene expression in response to poly(I:C) is not necessary to drive NK cell blastogenesis or proliferation. (*c*) although poly(I:C) induced IL-2 responsiveness among the J11d$^-$ low density population enriched for blast NK cells, this response required very high concentrations of the lymphokine (>2.5 × 10^3 U/ml). Such high levels of IL-2 are not likely to be physiological. Thus, if IL-2 is involved in the in vivo response to IFN, it may interact with a cell type other than NK.

It was observed that treatment of athymic mice with CsA alone induced a cytopenia in the spleen, indicating that one or more cell types in this system are influenced by CsA-sensitve agents. IL-2 is thought to influence the process of NK cell maturation from hematopoietic precursors (35–37). Our findings address only acute changes in NK cell number and function resulting from CsA treatment, rather than effects of long-term CsA administration. Potential CsA effects on NK cells or other cell types may be more apparent at the level of lymphocyte homeostatis, rather than IFN-induced activation.

In conclusion, this report has documented that IFN-induced NK cells are responsive to IL-2, and that IL-2 gene expression is activated in mice treated with an IFN inducer. The results demonstrate that IFN-induced NK cell proliferation is IL-2 independent, however, as CsA administration blocked IL-2 transcription, but did not affect NK cell activation or expansion in response to poly(I:C). These results demonstrate the IL-2 independence of NK cell activation in response to IFN, and establish the CsA insensitivity of the signals produced in vivo to mediate NK cell proliferation.

Summary

The IFN inducer, poly(I:C), elicits acute NK cell blastogenesis and proliferation in vivo. The role of IL-2 in mediating this proliferation was investigated in the studies presented here. Blast NK cells were isolated from poly(I:C)-treated, T cell-deficient athymic mice. Dividing cells, incorporating [^{3}H]thymidine, were enriched in the J11d$^-$ low density populations isolated from poly(I:C)-treated mice, and were characterized as NK by the following criteria: (*a*) they were eliminated by treatment with anti-AGM1 in vivo; and (*b*) they directly mediated lysis of NK-sensitive target cells in a single cell cytotoxicity assay with autoradiography. These poly(I:C)-induced blast NK cells were responsive to IL-2, but, when compared with in vivo activated T cells, responsiveness required 1,000-fold higher concentrations of the factor.

The technique of in situ hybridization was used to evaluate induction of IL-2 gene expression after poly(I:C) treatment in vivo. Treatment of euthymic, athymic, and severe combined immunodeficient mice with poly(I:C) activated IL-2 gene expression in a small percentage of spleen leukocytes. The transcription-positive cells were enriched in low density cell populations. These findings demonstrate that IL-2 transcription occurs after IFN induction in vivo, and suggest that an endogenous source of IL-2 exists other than the mature T cell. To assess the IL-2 dependence of in vivo NK cell expansion, poly(I:C)-treated athymic mice were given cyclosporin A (CsA), an agent that regulates IL-2 production at the level of gene transcription. The drug resulted in an 85–100% reduction in the percentages of cells transcribing IL-2. In contrast, CsA administration did not block IFN-enhanced NK cell cytolytic activity, expansion of large granular lymphocyte numbers, or NK cell proliferation. These findings demonstrate that although the proliferation of blast NK cells can be supported by IL-2, IL-2 is not an important mediator of IFN-induced NK cell expansion. Moreover, they establish that the acute proliferation of NK cells in response to IFNs is CsA insensitive.

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