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Modulation of Natural Killer Cell Activity in Mice After Interferon Induction: Depression of Activity and Depression of In Vitro Enhancement by Interferon

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Splenic natural killer (NK) cell activity of BALB/c and C3H mice was assayed after administration of the interferon inducers *Escherichia coli* endotoxin or Newcastle disease virus (NDV). As expected, the NK cell activity rose early in response to the interferon inducers. At 1 to 3 days after an injection of endotoxin, NK activity was hyperresponsive to interferon stimulation. At 5 to 9 days after injection of either endotoxin or NDV, splenic NK activity was depressed, and the spleen cells showed a relative refractoriness to in vitro interferon stimulation. It is postulated that this phenomenon may be related to hyporeactivity, the inability to reinduce interferon after an initial period of interferon production.

Interferon and interferon inducers are now established enhancing agents of natural killer (NK) cell activity in humans, animals, and in leukocyte cultures (6, 7, 9, 25). Viruses such as Newcastle disease virus (NDV), lymphocytic choriomeningitis virus, murine cytomegalovirus (MCMV), and Kunjin virus stimulate peak NK activity within a few days after infection in mice (2, 9, 19, 29). Synthetic polynucleotides (e.g., polyinosinate-polycytidylate), endotoxin, *Mycobacterium bovis* BCG, and *Corynebacterium parvum* are also effective in augmenting NK cell activity (6, 11, 19, 30, 31).

When interferon was given to a patient over a prolonged period, the ability of interferon to stimulate NK activity of leukocytes in vitro was diminished (7). We found that in vitro interferon actually depressed NK cell activity of human leukocytes from a patient who had received about 61 × 10^6 U of alpha interferon in 8.5 weeks (13). These findings suggested that large amounts of interferon might initiate modulating influences on NK cell activity beyond simple enhancement. Since significantly more interferon can be induced than conveniently injected (14) and the effect of interferon inducers on the NK cell system is of intrinsic interest, we decided to study the kinetics of changes in the activity of NK cells and their responses to interferon in vitro after an injection of a bacterial endotoxin or a nonreplicating virus.

MATERIALS AND METHODS

Mice. Male BALB/c AnNCrlBr and C3H/HeNCRlBr mice, 7 to 9 weeks old, were obtained from Charles River Breeding Laboratories, Inc. Male ICR mice (HLS:[ICR]Br) (25 to 30 g) were from Hilltop Laboratories.

Endotoxin. Lipopolysaccharide B from *Escherichia coli* O127:B8 (Bovine extraction) was obtained from Difco Laboratories. A stock solution containing 500 μg/ml in sterile 0.9% saline was stored at 4°C until use. BALB/c and C3H mice were injected with a single dose (0.2 ml) containing 100 μg of endotoxin into the lateral tail vein.

NDV and interferon. NDV strain CG was prepared by allantoic inoculation of 10-day-old embryonated chicken eggs. Allantoic fluid was collected after 24 h and, on chicken embryo fibroblast monolayers, had a titer of 2 × 10^8 PFU/ml.

ICR mice were injected with 4 × 10^7 PFU of NDV (0.2 ml) into the lateral tail vein of each mouse. After 6 h, the mice were exsanguinated, and the serum was stored at −70°C. No infectious NDV was present in such serum, which had a titer of 2,000 U/0.1 ml by microassay on secondary ICR mouse embryo fibroblasts (1) standardized by the National Institutes of Health mouse interferon standard (catalogue no. 001904511). BALB/c and C3H mice were injected with the same dose of NDV in the same manner.

Medium. For NK cell assays, we used RPMI 1640 (GIBCO Laboratories) supplemented with 10% newborn bovine serum and antibiotics.

Enhancement of NK cell activity by interferon. Spleens were removed from the mice to be tested, and a cell suspension was prepared by forcing each spleen through a sterile stainless steel mesh. After the cells were washed, the concentration was adjusted to 10^7 cells per ml. A 0.9-ml cell suspension was incubated with 0.1 ml containing about 2,000 U of interferon for 1 h at 37°C. Another volume of spleen cell suspension was incubated with 0.1 ml of medium as a control to determine the base-line NK cell activity without the effect of interferon. After incubation, the cells were washed three times with 5 ml of medium and adjusted

to a density of 5×10^6 mononuclear cells per ml for the NK cell cytotoxicity assay.

NK cell cytotoxicity assay. YAC-1 cells were used as targets in these assays and were grown from a sample provided by A. Koros. The target cells (10^6) were labeled with 50 μCi of ^{51}Cr (New England Nuclear Corp.) in a volume of 0.2 ml for 1 h at 37°C immediately before the assay. These cells were washed with medium three times and suspended at a concentration of 5×10^4 cells per ml.

Four twofold serial dilutions were made of each effector cell suspension, starting with the highest effector-to-target (E:T) ratio of 100:1. Into each well of a 96-well U-bottomed microassay plate (Linbro Scientific; no. 76-013-05) was added 100 μl of the labeled target cell suspension. Each dilution of effector cells was added in triplicate to the wells, 100 μl of cell suspension per well. Medium or 0.5% Triton X-100 (Packard Instruments Co.) was added to target cells to determine spontaneous and total release. The plates were incubated at 37°C in 5% CO_2 for 6 h. After incubation, the supernatant in each well was harvested with a supernatant collection system (Titertek supernatant collection system; Flow Laboratories), and the samples were counted in a Packard 5110 gamma counter (Packard Instruments Co.).

Specific release of ^{51}Cr from the target cells was calculated as follows: % specific lysis = (^{51}Cr release by effector cells − spontaneous release)/(total release in Triton X-100 wells − spontaneous release). The reported values for specific lysis were obtained from the 100:1 E:T ratio. Mean percent specific release was calculated from the replicate mice in each test group along with the standard error. The effect of interferon on NK cell activity is expressed as the arithmetic difference of percent specific lysis before and after stimulation. Student's *t*-test was used to determine the significance of the difference of the means.

Assay for possible suppressors of NK cell activity. A modification of an assay used to detect *C. parvum*-induced suppressor cells (18) was used to look for NK suppressor cells in endotoxin-treated mice. Normal spleen cells from three BALB/c mice were obtained as described above. A portion of each cell sample was treated in vitro with interferon. After being washed, treated and untreated cells (effectors) were added to separate labeled target cells in a ratio of 50:1 in triplicate. To test for the presence of suppressor cells, spleen cells from a BALB/c mouse 7 days after an intravenous injection of 100 μg of endotoxin were added in the same number as effectors from the normal spleens. The test was repeated for two additional endotoxin-injected mice. As a control, washed, normal heat-killed spleen cells were added to the effector-target mixture instead of the endotoxin-treated spleen cells. Standard NK assays without suppressors were also done on normal endotoxin-treated cells with and without interferon stimulation as well as on the heat-killed cells.

RESULTS

Endotoxin treatment of BALB/c mice. NK cell activity was assayed by using spleen cells from endotoxin-treated and control BALB/c mice at 3 h and on days 1, 3, 5, 7, and 9 after the injection of endotoxin or saline. Four endotoxin-treated and two control mice were sacrificed at each time period. The NK cell activities of the control mice were averaged to determine the mean control group activity (Fig. 1). At 3 h after the injection of 100 μg of endotoxin, there was a significant rise of base-line spleen NK cell activity, 10.9 ± 1.3% specific lysis, compared with the mean control level (5.2 ± 0.8%, $P < 0.005$). The base-line activity continued to rise for 1 day after endotoxin injection. From then on, it decreased through days 3, 5, and 7, when the specific lysis values were, respectively, 9.7 ± 1.2, 0.5 ± 0.3, and −1.2 ± 0.4%. The latter two values were significantly depressed compared with controls ($P < 0.005$). After reaching its nadir on day 7, the base-line NK cell activity increased slightly to 1.4 ± 0.5% specific lysis. However, this was still significantly below the control NK cell activity.

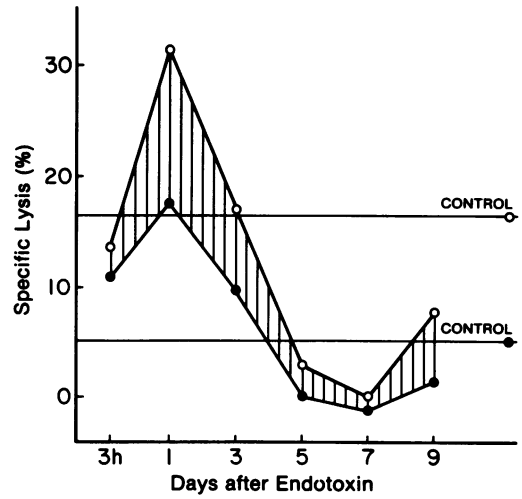

FIG. 1. Response of NK cell activity to a single injection of endotoxin in BALB/c mice on day zero. Horizontal lines indicate the mean values obtained from the control group. Symbols: (●) NK cell activity without in vitro stimulation with interferon; (○) NK cell activity after in vitro interferon stimulation.

Mononuclear cells collected at these times were also incubated with interferon to determine the response of NK cell activities to its stimulation. Cells obtained 3 h after endotoxin were stimulated only by +2.3 ± 2.3% above the base-line specific lysis level, which was significantly below the mean normal enhancement (+11.5 ± 1.8%, $P < 0.01$). On days 1 and 3 after endotoxin treatment, the increases in specific lysis after in vitro interferon stimulation were +14.4 ± 5.4% and +6.6 ± 5.3%, respectively. These values were not significantly different from the mean enhancement of NK cell activity in the control group. However, at 5 and 7 days after endotoxin

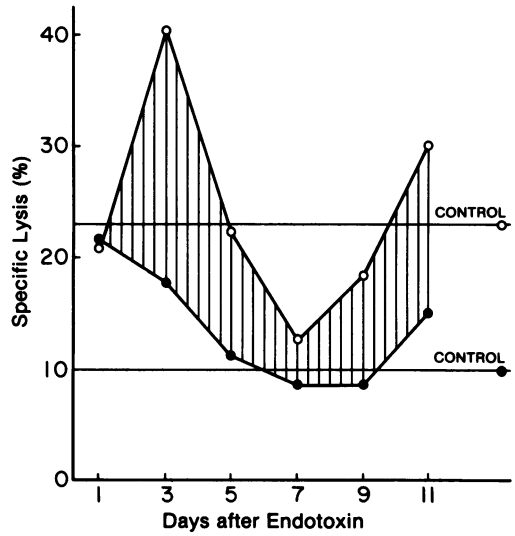

FIG. 2. Response of NK cell activity to a single injection of endotoxin in C3H mice on day zero. Horizontal lines indicate the mean values obtained from the control group. Symbols: (●) NK cell activity without in vitro stimulation with interferon; (○) NK cell activity after in vitro interferon stimulation.

injection, the mean enhancement of NK cell activity after in vitro interferon stimulation was +1.9 ± 0.3% and +1.1 ± 0.9%, which are specific lysis values significantly below those of the control group to interferon ($P < 0.005$). This refractoriness to interferon stimulation was partially overcome by day 9, when an enhancement of +6.5 ± 2.6% over the base-line activity was recorded.

Endotoxin treatment of C3H mice. Identical numbers of C3H mice were injected and assayed as in the previous experiment with BALB/c mice. Assays were done on days 1, 3, 5, 7, 9, and 11 after the injection of endotoxin. At day 1 after injection, the mean NK cell specific lysis of spleen mononuclear cells was 21.5 ± 8.7%, which subsequently decreased on days 3, 5, and 7 to 17.6 ± 3.8%, 11.1 ± 4.9%, and 8.5 ± 2.6%, respectively (Fig. 2). The mean specific lysis remained low, at 8.7 ± 0.2% through day 9, although this value was not significantly lower than the mean control activity (9.8 ± 1.4%, $P > 0.25$). The same trend of initial elevation of NK cell activity followed by a depression at around day 7 was thus noted as for BALB/c mice, although the precise timing varied.

The spleen cells were also tested after in vitro incubation with interferon. On day 1, after the injection of endotoxin, interferon affected NK cell activity by only −0.6 ± 5.9%. This shows that NK cells became refractory to in vitro stimulation ($P < 0.05$), compared with the stimulation of NK cell activity of the control cells (+14.3 ± 1.9%). By day 3 however, there was an excessive response of NK cell activity to interferon (+22.9 ± 2.3% specific lysis, significantly elevated compared with controls, $P < 0.025$). This hyperesponsiveness was no longer observed by day 5 (+11.4 ± 3.9%). As for BALB/c mice, the interferon-boosted NK cell activity on day 7, however, was only increased by +4.2 ± 2.6%, reflecting a state of NK cell hyporesponsiveness ($P < 0.01$). By days 9 and 11, NK cell activity was increased by interferon to +9.7 ± 3.4% and +15.2 ± 0.45%, respectively, which was not significantly different from the response of the controls ($P < 0.5$).

NDV stimulation of BALB/c mice. NDV was injected intravenously into 10 BALB/c mice. One group of four was assayed at 3 days postinjection, and the remaining six mice were assayed on day 6. Six age- and sex-matched control mice were assayed along with the test groups. The mean base-line NK cell activity of BALB/c mice 3 days after a single injection of NDV was 15.5 ± 3.7% specific lysis (Table 1). This value was significantly higher than the mean base-line control level of 5.0 ± 0.7% ($P < 0.025$). By day 6, however, the base-line activity had dropped to 5.3 ± 0.5% and was approximately equal to the activity level of the control group. The NK cell activity of spleen mononuclear cells responded

TABLE 1. Effect of intravenous NDV injection on base-line NK activity levels and on NK cell boosting by treatment with interferon in vitro in C3H and BALB/c mice

Mouse strain	Interferon treatment in vitro	% Specific lysis ± SE at day after NDV injection:				Control
		3	5	6	9	
BALB/c	Without	15.5 ± 3.7	ND[a]	5.3 ± 0.5	ND	5.0 ± 0.7
	With[b]	+10.8 ± 3.9	ND	+1.2 ± 1.6	ND	+7.4 ± 1.9
C3H	Without	55.9 ± 1.2	34.9 ± 0.8	ND	20.1 ± 2.4	26.2 ± 5.2
	With[b]	+7.9 ± 4.6	+11.4 ± 5.8	ND	+3.9 ± 1.5	+15.2 ± 1.1

[a] ND, Not done.
[b] Values indicate the increase above the base-line level in NK cell activity after in vitro incubation with interferon.

to in vitro incubation with interferon 3 days after the injection of NDV by an increased activity of +10.8 ± 3.9%, which was not significantly different from the enhancement observed in the control group (+7.4 ± 1.9%). However, by day 6, the response to in vitro interferon stimulation was only +1.2 ± 1.6%, which was significantly depressed when compared with the control group ($P < 0.05$).

NDV stimulation of C3H mice. Assays for NK cell activity in C3H mice were done with pairs of mice on days 3, 6, and 9 postinjection along with pairs of control mice in each assay. As with the BALB/c mice, the C3H mice also showed a significant increase in base-line NK cell activity to 55.9 ± 1.2% specific lysis after a single injection of NDV ($P < 0.05$). This elevation in base-line activity decreased at day 5 to 34.9 ± 0.8% and decreased yet further to 20.1 ± 2.4% by day 9, which was approximately equal to the control group activity of 26.2 ± 5.2%. The effect of interferon on NK cell activity 3 days after NDV injection was one of enhancement (+7.9 ± 4.6%), which was not significantly different from the effect of interferon on control cells (+15.2 ± 1.1%). However, a high degree of refractoriness to interferon stimulation was seen 9 days after the NDV injection, with an increase of only +3.9 ± 1.5% ($P < 0.005$).

Suppressor cell activity. Spleen cells obtained from three BALB/c mice 7 days after an injection of 100 μg of endotoxin were examined for cells which could suppress the NK cell activity of normal spleen cells with and without in vitro interferon stimulation. Cells of the three endotoxin-treated mice were tested separately in triplicate.

The NK cell activities of the normal effector cells alone, without and with interferon stimulation in vitro, were found to be 4.1 ± 0.7% and 9.5 ± 0.9% specific lysis, respectively. When heat-killed spleen cells were added to the effector-target mixture, the activity was 2.9 ± 0.8% without in vitro interferon stimulation and 9.4 ± 0.9% after in vitro interferon stimulation. After the addition of spleen cells from the endotoxin-injected mice to the effector-target mixture, NK cell activities of 4.8 ± 1.5% without interferon stimulation and 8.0 ± 0.7% after interferon stimulation were observed.

The NK cell activities obtained with the effector cells alone or with the addition of the endotoxin-treated cells did not differ significantly, either with interferon stimulation of the effector cells ($P > 0.05$) or without ($P > 0.1$). There was also no significant difference between the NK cell activity of the effector cells with heat-killed cells added or with the endotoxin-treated cells added, either with interferon stimulation ($P > 0.05$) or without ($P > 0.1$).

The NK activity of the spleen cells from mice 7 days after endotoxin injection that were used was shown to be depressed: 0.6 ± 0.1% without interferon treatment and 3.9 ± 0.5% after interferon stimulation at an E:T ratio of 100:1. The heat-killed cells were without NK cell activity. Thus, we were unable to demonstrate any suppressor cell activity in the spleens of endotoxin-injected mice.

DISCUSSION

At 1 to 3 days after an intravenous injection of endotoxin or NDV, there is an enhancement of splenic NK cell activity consistent with the induction of interferon by these agents (9, 11). It is notable that this enhancement takes place significantly later than the time needed to induce peak amounts of serum interferon, which in the case of these two inducers is 2 to 6 h after administration (3, 15). This is in contrast to previously reported NK cell activity kinetics after intraperitoneal endotoxin injection, which show that the NK cell activity increases rapidly 3 h after injection and subsequently decreases on the following days (6, 11). This may be due in part to different routes of administration, i.e., intraperitoneal instead of intravenous injection.

The early period of refractoriness to in vitro interferon stimulation 3 h to 1 day after injection of endotoxin may be due to a decrease in the number of NK cells available for boosting or a direct decrease in the sensitivity of these cells to interferon. In both BALB/c and C3H mice, the maximum levels of NK cell activity observed after injection of endotoxin were comparable to those of the control groups after in vitro interferon stimulation. This may represent a maximum threshold for spleen cells of normal mice.

More difficult to explain is a hypereactivity to in vitro stimulation of interferon seen 3 days after an injection of endotoxin in C3H mice. This could reflect an increased number of active NK cells available for additional augmentation by interferon or an increase in the number of precursors available to become active NK cells. Alternatively, the hyperesponsiveness may indicate an increased sensitivity to interferon augmentation.

The change may not be due to interferon alone since it was not observed after an injection of NDV. It has been recently reported that interleukin 2, which can indirectly and possibly directly be stimulated by bacterial lipopolysaccharide (8), will augment NK cell activity either alone or at least additively with interferon in vitro (10, 16).

A common phenomenon which followed 5 to 9 days after an injection of either endotoxin or NDV was a period of depressed NK activity and

depressed responsiveness to interferon. There are several possible explanations for this, although none has yet been proven.

(i) There may have been a depletion of active NK cells and NK precursors. More work should be done to enumerate accurately these cell subtypes by functional or immunological markers. One objection to this theory is that repeated interferon administration in humans (7) and in mice (20) can maintain NK activity at an elevated level. Our own data (unpublished) indicate that repeated administration of NDV to mice can also produce elevated NK cell activity for at least 2 weeks.

(ii) Suppressors of NK cell activity may have been elicited by the interferon inducers. Suppression of NK cell activity by suppressor cells has been reported to follow the injection of agents such as *C. parvum* (18, 24), adriamycin (23), pyran copolymer (22), and *t*-carrageenan (5). Both T cells and macrophages have been implicated. Our preliminary assays for suppressor cell activity in the spleen cells of endotoxin-injected BALB/c mice were negative. We could not demonstrate suppressive effects on the NK cell activity of normal spleen cells or on interferon-enhanced NK cell activity.

Prostaglandins have been implicated in suppression of NK activity (4, 18) and have also been implicated as mediators of lipopolysaccharide action (21). Further studies will be needed to explore the role of prostaglandins in our system. Very likely, prostaglandins will not offer a simple explanation of the depression of NK cell activity since prostaglandins may act synergistically with interferon in boosting NK cell activity or antagonize its effect, depending on when the NK cells are exposed (27).

(iii) The third theory uses the suppression of endogenous interferon to explain the depression phenomenon. It is well known that a single injection of either endotoxin or virus makes an animal hyporeactive; i.e., it is unable to produce interferon in response to a homologous or a heterologous inducer for about 1 week (12). Örn et al. (20) recently reported that after the administration of anti-interferon serum to mice, a drop in the splenic NK cell activity is observed. Presumably, this drop is mediated by a lack of interferon. The drop in NK cell activity may reflect a need for a constant level of endogenous interferon for the development and maintenance of active NK cells in vivo. If we postulate that endogenous interferon must be constantly induced and that the hyporeactive animal is unable to induce it, then the phenomenon of depressed NK cell activity and stimulating ability may be explained. We note that NK cell activity in response to in vitro interferon stimulation returned to normal in endotoxin-injected mice in 9 to 11 days, which was a few days after hyporeactivity subsided.

Of course, we have as yet no direct evidence that there is endogenous interferon in the body or that the hyporeactive state applies to it. However, this hypothesis is useful because it helps explain a related phenomenon. It is known, for example, that virus infections in mice and in humans produce hyporeactivity to interferon induction (17, 26). Renal transplant patients with cytomegalovirus infection frequently have low NK cell activity, and their cells respond poorly to interferon (unpublished data).

In summary, although we cannot yet fully explain the complex reactions of NK cells to an interferon inducer, our findings suggest multiple factors affecting these cells, each of which may be profitably sorted out.

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