Natural Killer Cell Activity in Renal Transplant Recipients Receiving Cyclosporine

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Normal subjects (n = 11) had a mean circulating natural killer (NK) cell activity of 188 lytic units per 10^7 peripheral mononuclear blood leukocytes. This activity was significantly enhanced by in vitro incubation with 500 U of alpha-interferon (+207 lytic units). The mean NK activity of renal transplant recipients on azathioprine (n = 17) or on cyclosporine (n = 17) studied at various times after transplantation was significantly decreased, as was the ability of interferon to enhance NK activity. In the cyclosporine group, interferon could not enhance NK titers 1 to 6 weeks after transplantation when the patients were on the highest doses of cyclosporine (mean, 1,002 mg/day) or when they were viremic for cytomegalovirus. After 18 weeks, when the patients received 546 mg/day or when viremia was no longer detected, the ability of interferon to enhance NK activity was more normal. Cyclosporine and cytomegalovirus infection may have a greater effect on the action of interferon on NK activity than on the NK titer per se. This defect may diminish the reserve of NK cells and contribute to post-transplant immunosuppression.

Natural killer (NK) cells are lymphocytes which destroy foreign tumor cells and virus-infected cells without prior sensitization, and they constitute part of the standing cellular immune mechanism (8). They may also be stimulated within a few days of onset of an acute virus infection, presumably by the induction of interferon, and may be important in the termination of acute infections (20, 24).

Virus infections, particularly with the herpesvirus group consisting of cytomegalovirus (CMV) and herpes simplex, varicella-zoster, and Epstein-Barr viruses, are significantly increased after tissue or organ transplantation (9). An important reason is the use of cytotoxic immunosuppressants, which until recently almost always included azathioprine (AZ). Cyclosporine (CyA) is a new, nonmyelotoxic, noncytotoxic suppressant that selectively inhibits certain T-lymphocyte functions (2, 4). It is more effective than AZ in maintaining a heterotransplant (12). We found that bacterial infections were decreased in renal transplant recipients on CyA compared with patients on AZ 6 months after transplantation, but infections with the herpesvirus group were not (J. S. Dummer, A. Hardy, A. Poorsattar, and M. Ho, Transplantation, in press). The mechanism by which CyA facilitates viral infection is unclear.

During 1981 and 1982, both AZ and CyA in combination with prednisone were used by our surgeons after renal transplantation. This provided us with the opportunity to study the NK cell responses in both groups.

In addition, as CMV infection is common within 6 months of transplantation (10) and may be an important factor affecting the NK response in such patients, evidence for such infection was obtained and its effects were evaluated. To determine perturbations in the NK system, measurements of cytotoxic activity of the peripheral leukocytes may be inadequate. We have previously found that, despite normal circulating activity, the capacity of the NK activity of mononuclear leukocytes to be enhanced by interferon may be depressed (3, 11). Also, we noted in mice that CyA can block the ability of interferon to enhance NK activity (6) without affecting NK activity itself. Hence, the interferon enhancement test was added in addition to assays of NK activity in the present studies.

MATERIALS AND METHODS

Human subjects. Eleven normal healthy volunteers served as controls. They consisted of healthy faculty and students, five males and six females. Their ages ranged from 22 to 47 years, with a mean of 36.4 ± 2.5 years.

Renal transplant recipients seen for routine follow-up were studied for NK activity. There were 17 patients on CyA and 17 patients on AZ. There were 9 males and 8 females in the first group and 10 males and
7 females in the second group. Their respective mean ages were 39.3 ± 2.9 and 39.1 ± 2.8 years. Serum creatinine levels were, respectively, 2.3 ± 0.2 and 1.7 ± 0.2 mg/dl (normal range, 0.5 to 1.4 mg/dl). The mean daily prednisone doses in these two groups were comparable, 18.3 ± 1.4 and 16.2 ± 1.7 mg. The two groups differed in time from transplant surgery. In the CyA group it was 11.5 ± 1 weeks (range, 1 week to 11 months), and in the AZ group it was 79.5 ± 16.2 weeks (range, 2 weeks to 4 years).

Patients on CyA were usually started on 17.5 mg/kg or about 1,000 to 1,200 mg/day. After 1 to 2 months, the doses were reduced to 10 mg/kg per day or less. Prednisone was also given at a dose of 200 mg on the day of operation. Daily dose reductions of 40 mg/day were then made over the first 4 postoperative days. Most patients were maintained on 15 to 25 mg/day after the first few weeks.

Patients on AZ were begun at a dose of 2 to 3 mg/kg per day at the time of surgery. Maintenance doses of 1 to 2 mg/kg per day were targeted. They also received prednisone initially at 200 mg/day, and maintenance doses of 10 to 30 mg/day were reached after 2 to 3 months.

**Effector cells.** From heparinized venous blood obtained from subjects, peripheral blood mononuclear leukocytes (PBML) were separated by centrifugation on Ficoll-Hypaque gradients (LSM; Litton Bionetics, Kensington Md.). The cells were washed three times with Hanks balanced salt solution.

**Interferon.** Human leukocyte interferon (alpha-interferon) was supplied by K. Cantell of Helsinki, Finland. It was "P-IF" prepared from blood leukocytes induced with Sendai virus (16). The lyophilized material was reconstituted to contain 107 U/ml. The specific activity of this material was about 106 U per mg of protein.

**Medium.** RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% newborn bovine serum, 250 U of penicillin per ml, 250 µg of streptomycin per ml, and 0.002 g of NaHCO3 per ml was used for assays and for cell culture (complete medium). Medium without serum was used for washing target cells.

**Viral cultures.** PBML and neutrophils from blood taken for the NK studies were cultured on human foreskin cells (originated from primary tissue obtained from Magee Women's Hospital, Pittsburgh, Pa.; passages 9 to 30) in medium 199 (GIBCO Laboratories) supplemented with 2 mM glutamine, 250 U of penicillin per ml, 250 µg of streptomycin per ml, 0.002 g of NaHCO3 per ml, and 10% heat-inactivated (56°C for 30 min) fetal calf serum (MA Bioproducts, Bethesda, Md.) at 37°C in a 5% CO2-humidified atmosphere. Cultures were observed for characteristic CMV-mediated cytopathic effect for 3 to 5 weeks; negative cultures were passed two additional times at 2- to 3-week intervals (21).

**NK activity assay.** K-562 cells, derived from a patient with chronic myelogenous leukemia in blast crisis (1, 14), were used as target cells. They were propagated from a sample given by G. V. Quinnan, Bureau of Biologics, Bethesda, Md., and were negative for mycoplasma contamination by culture on standard mycoplasma media and by stain with DNA-binding dye. Immediately before use in the assay, 106 target cells were labeled with 50 µCi of 51Cr (sodium chromate; New England Nuclear, Boston, Mass.) in a volume of 0.2 ml. These cells were incubated for 1 h at 37°C, washed three times with medium, and suspended to a concentration of 5 × 105 cells per ml in complete medium.

A 100-µl portion containing 5 × 104 labeled target cells was added to each well of a 96-well round-bottomed plate (Linbro Scientific, Hamden, Conn.). Effector cells at each dilution were added to triplicate wells, 100 µl per well. Four serial twofold dilutions of effector cells were made beginning with the highest effector/target ratio of 50:1. Control wells containing either target cells and medium or target cells and 0.5% Triton X-100 (Packard Instrument Co., Downers, Grove, Ill.) were included to obtain values for spontaneous 51Cr release and total lysis. After 16 to 18 h of incubation at 37°C in a humidified 5% CO2 incubator, the supernatant fluid in each well was harvested with a supernatant collection system (Titertek; Flow Laboratories, McLean, Va.). Samples were counted in a Packard 5110 gamma counter (Packard Instrument Co.).

**Cytotoxicity from NK cell activity was expressed as percent specific release of 51Cr and calculated for each well as follows:** percent specific release = [(51Cr release from well with effector cells – spontaneous release)/(total release – spontaneous release)] × 100.

The percent specific release ± standard error was calculated. The standard error was usually <5%.

The titer of NK activity of a particular sample is expressed in number of 30% lytic units (LU) in 107 effector cells. The number of cells required to produce 30% lysis was estimated graphically after plotting percent specific lysis against number of effector cells in replicate wells on probability paper (5, 18).

**Test for NK enhancement with interferon.** To determine the effect of in vitro incubation with interferon on natural cytotoxicity of PBML, 500 U of interferon were added to 2.5 × 105 to 5 × 106 PBML in a total volume of 0.6 ml in complete medium. Cells incubated with complete medium alone served as untreated controls. Treated and untreated cells were placed in a capped tube and incubated in a 37°C water bath for 2.5 h. They were then washed three times, counted, and resuspended to an appropriate concentration of viable cells in complete medium before assay of cytotoxicity. The difference in NK titer (ΔLU) between the samples incubated with interferon and the control represented enhancement. Negative values denote depression of NK activity by interferon.

**Statistics.** Standard errors are given after all values for NK activity titers and other data. Student's and the paired t tests were used for calculation of significance.

**RESULTS**

Comparison of NK cell response in three groups. The mean NK cell activity as well as enhancement by interferon of normal subjects and renal transplant recipients on CyA or AZ are presented in Table 1. The mean NK activity and enhancement of either transplant group were significantly depressed compared with normal subjects. The NK activity of the AZ group was also significantly lower than that of the CyA
group. The level of enhancement of the AZ group was higher than that of the CyA group, particularly if percent increase of NK activity over base line was compared (138 versus 20%). However, in terms of difference in LU, the method of comparison used in this report, the two groups were not significantly different.

Data on NK cell activities were available in 13 patients before transplantation and immunosuppression. The mean activity was $211 \pm 83$ LU. Mean in vitro enhancement by interferon in six patients was $104 \pm 52$ LU. The cells from one patient did not respond. These data are not significantly different from those for normal donors.

**Enhancement of NK activity after incubation with interferon in the CyA group.** Figure 1 describes the change of NK activity after incubation with interferon in relation to time of transplantation. To be noted is that there was no enhancement of NK activity after incubation with interferon 1 to 6 weeks after transplantation. Eleven of 23 samples were actually depressed by interferon. In the two later periods (7 to 18 and $>18$ weeks), gradual increases in enhancement were noted. Even so, the elevation in titer was not significantly greater after 18 weeks.

Table 2 presents a summary of the relationship of the dose of CyA and prednisone to NK activity and enhancement after incubation with interferon. The base-line levels of NK responses were not significantly different in all three periods. The amount of CyA and prednisone given was highest 1 to 6 weeks after transplantation. This may account for the lack of response to interferon noted during the early post-transplant period compared with later times. As the doses of immunosuppressants became lower after 7 weeks, the PBML regained their responsiveness to interferon.

**Enhancement of NK activity after incubation with interferon in the AZ group.** In contrast to the CyA group, studies on a small number of AZ-treated patients 1 to 12 weeks after transplantation showed that there was enhancement of NK activity by interferon (Table 3). Neither the base-line NK levels nor the enhancement response was significantly changed after 12 weeks. It should be noted that the patients received higher doses of prednisone during the first 12 weeks, the mean dose being comparable to what the CyA group received 1 to 6 weeks after transplantation (Table 2). The mean daily dose of AZ remained about the same. Hence, we do not believe that the lack of response to interferon in the CyA group 1 to 6 weeks after transplantation was due to prednisone.

**Effect of CMV infection on NK activity in CyA-treated transplant recipients.** CMV infection is the most consistent virus infection occurring after transplantation. Infection usually occurs in

<table>
<thead>
<tr>
<th>Group</th>
<th>Subjects</th>
<th>No. of subjects</th>
<th>No. of samples</th>
<th>NK titer (LU ± SE)*</th>
<th>IFN enhancement of NK (ΔLU ± SE)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal</td>
<td>11</td>
<td>18</td>
<td>$188 \pm 31$</td>
<td>$+207 \pm 72$</td>
</tr>
<tr>
<td>2</td>
<td>AZ (renal)</td>
<td>17</td>
<td>21</td>
<td>$34 \pm 10$</td>
<td>$+47 \pm 17$</td>
</tr>
<tr>
<td>3</td>
<td>CyA (renal)</td>
<td>17</td>
<td>67</td>
<td>$84 \pm 15$</td>
<td>$+17 \pm 6$</td>
</tr>
</tbody>
</table>

* Group 1 was significantly different from group 2 or 3 ($P < 0.01$). Group 2 and 3 were also significantly different ($P < 0.01$).
* Within each group, NK activity was significantly enhanced by interferon (IFN) ($P < 0.05$). The level of enhancement between group 1 and group 2 or 3 was significantly different, but not that between groups 2 and 3.

**FIG. 1.** Effect of interferon on NK cell activity in relation to time after transplantation. The horizontal line after 0 represents no change in NK titer after incubation in vitro with interferon. Positive values represent enhancement and negative values represent depression of NK activity by interferon. Mean change in titer and standard error are drawn for the three time periods.
TABLE 2. Relationship of CyA and prednisone to NK activity and its enhancement by interferon (IFN)

<table>
<thead>
<tr>
<th>Time after transplant (wk)</th>
<th>No. of subjects</th>
<th>No. of samples</th>
<th>CyA (mg/day, mean ± SE)</th>
<th>Prednisone (mg/day, mean ± SE)</th>
<th>NK titer (LU, mean ± SE)</th>
<th>IFN enhancement (ΔLU, mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-6</td>
<td>13</td>
<td>23</td>
<td>1.002 ± 0.46</td>
<td>25 ± 4</td>
<td>57 ± 9</td>
<td>+1 ± 6</td>
</tr>
<tr>
<td>7-18</td>
<td>12</td>
<td>31</td>
<td>700 ± 35</td>
<td>15 ± 1</td>
<td>81 ± 19</td>
<td>+18 ± 10</td>
</tr>
<tr>
<td>&gt;18</td>
<td>10</td>
<td>13</td>
<td>546 ± 66</td>
<td>15 ± 1</td>
<td>136 ± 63</td>
<td>+46 ± 17</td>
</tr>
</tbody>
</table>

a There was no significant difference in NK activity among the three groups.

b There was no significant enhancement 1 to 6 and 7 to 18 weeks after transplantation, but the enhancement after 18 weeks was significant compared with base-line NK response (P < 0.05). Enhancement values of the first and third groups were also significantly different (P < 0.05).

either AZ- or CyA-immunosuppressed patients within the second and third months after surgery (Dummer et al., in press). To determine the effect of CMV infection on the NK system in the CyA group, 54 blood samples which had no evidence of CMV were analyzed. Of this group, 22 samples were from patients receiving >850 mg of CyA per day (group A) and 32 were from patients receiving <850 mg/day (group B). The NK titers in groups A and B were 64 ± 12 and 93 ± 27 LU, not significantly different. However, the enhancement titers of the two groups in ΔLU were +6 ± 6.5 and +38 ± 8, which were significantly different (P < 0.05). This suggests that, irrespective of viremia, higher doses of CyA are associated with depressed enhancement by interferon as described above.

The second approach was to analyze results from patients who had CMV viremia. In total, 56 blood samples from 14 patients on CyA submitted for NK assays were also cultured for CMV. Three to nine samples were taken from each patient 1 to 24 weeks after transplantation. Ten patients had viremia at some time, and four remained consistently negative. Of the latter, two showed infection by positive throat culture or by serological rise. Only two patients were negative by both criteria. Symptomatic infections in the 14 patients were as follows: one patient had CMV pneumonia, one had genital herpes and urinary tract infection, one had undiagnosed pneumonia, one patient had oral herpes, and four others had urinary tract infection.

To evaluate the effect of active CMV infection, measures of NK activity were grouped according to whether they were done before, during, or after clearance of viremia (Table 4). The doses of CyA and prednisone are also included for reference. The NK titers and interferon enhancement titers are low in the group before viremia, but this is explainable on the basis of higher CyA doses. It is striking, however, that the enhancement titers during viremia were significantly lower (−14 ± 22 LU) than after viremia, even though the CyA and prednisone doses the patients received were similar. This suggests that the presence of viremia, apart from CyA, is associated with depressed enhancement of NK activity by interferon. There were not enough patients who remained free of CMV infection during the entire follow-up for analysis.

DISCUSSION

Normal subjects usually have significant circulating NK cell activity, which can be further enhanced by in vitro incubation with interferon (8). We present serial data on these two parameters in renal transplant recipients receiving CyA. As a whole, renal transplant recipients treated with CyA had both depressed NK cell activity and poor response to stimulation by interferon. The enhanced response after incubation with interferon was more suppressed shortly after transplantation, when the patients were receiving more CyA or when the patient was viremic.

TABLE 3. Relationship of AZ and prednisone to NK activity and its enhancement by interferon (IFN)

<table>
<thead>
<tr>
<th>Time after transplant (wk)</th>
<th>No. of subjects</th>
<th>No. of samples</th>
<th>AZ (mg/day, mean ± SE)</th>
<th>Prednisone (mg/day, mean ± SE)</th>
<th>NK titer (LU, mean ± SE)</th>
<th>IFN enhancement (ΔLU, mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-12</td>
<td>3</td>
<td>4</td>
<td>112 ± 22</td>
<td>25 ± 6</td>
<td>59 ± 26</td>
<td>+47 ± 16</td>
</tr>
<tr>
<td>&gt;12</td>
<td>15</td>
<td>17</td>
<td>112 ± 11</td>
<td>14 ± 1</td>
<td>28 ± 10</td>
<td>+47 ± 21</td>
</tr>
</tbody>
</table>

a Base-line NK values were not significantly different between the two groups.

b The enhancement (12 weeks after transplantation) was significant (P < 0.05).
This parameter became more normal with elapse of time as the dose of immunosuppressive agent decreased, or when viremia cleared.

Concerning the effect of AZ, our data are consistent with the reported decreases in NK titers in patients receiving AZ, but there was still significant enhancement of NK activity by interferon in this group. Lipinski et al. (13) showed a depression of NK activity against K-562 cells in 15 renal transplant recipients on AZ. Moreau (17) found a progressive depression of NK activity between 3 and 60 months after transplantation, which persisted for about 5 years after transplantation. According to Guillou et al. (7), 48 to 72 months after transplantation NK cell responses may return to normal levels.

A few of our patients had symptomatic infections, but almost all had inapparent CMV infections. It is well known that a number of infections, particularly viral, affect the NK system (24). The NK activity of mice infected with murine CMV is transiently elevated 2 to 3 days after infection (20), but enhancement of NK activity by in vitro incubation with interferon is suppressed 6 to 9 days after acute infection (15). In a separate study, we found that the NK titer and its response to interferon in previously healthy subjects suffering from spontaneous CMV mononucleosis were the same as in normal subjects (22). It is possible that both changes noted in the animal studies may have been missed during CMV mononucleosis as patients were not seen earlier than 10 days after onset of symptoms. In renal transplant recipients receiving CyA, we were able to compare NK activity before, during, and after onset of CMV viremia. It was higher, although not statistically different, during viremia. Suppression of enhancement of NK activity by interferon was found during viremia, which appeared to be independent of the effect of the dose of CyA. These findings are consistent with what we observe in animals (15).

Other factors might contribute to the perturbation of the NK system in the complex renal transplant recipient. These concern the underlying disease of the patient and immunosuppressive drugs. It is admittedly difficult to ascribe any changes in their NK system to a single factor with complete confidence. Lipinski et al. (13) found that uremic patients on chronic hemodialysis had normal NK cell activity, and acute rejection episodes may elevate NK activity. Our patients were mostly ambulatory and had compensated renal function. The relative roles of immunosuppressants received after renal transplantation are difficult to untangle, but certain conclusions may be suggested. It has been widely reported that large doses of steroids can acutely depress basal NK activity (19). The acute stress of transplant surgery, perhaps with the aid of endogenous steroids, depressed the NK cell levels for 2 days (7). Steroids were given to patients on either AZ or CyA, and how much they contributed in producing the diminished base-line NK levels in both patient groups is not known.

Perhaps the most interesting finding of this work is that the PBML of patients on CyA, besides having lower NK activity, responded poorly in a dose-responsive manner to stimulation by interferon. Steroids cannot explain this hyporesponsiveness as cells from patients on AZ who responded better to interferon than did CyA-treated patients received comparable or greater doses of prednisone. This effect may therefore be specific for patients on CyA. The enhancement response may be biologically meaningful because it may measure the NK reserve, which is enhanceable by interferon during virus infections. The depressed NK activity and poorly enhanced response to interferon may help to explain the propensity of transplant patients on CyA to develop viral infections and lymphomas that have been associated with Epstein-Barr virus infection (23).

ACKNOWLEDGMENTS
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TABLE 4. Relationship of CMV viremia to NK activity and its enhancement by interferon (IFN) in CyA-treated renal transplant recipients

<table>
<thead>
<tr>
<th>Timing of sample</th>
<th>No. of samples</th>
<th>NK titer (LU, mean ± SE)*</th>
<th>IFN enhancement (ΔLU, mean ± SE)*</th>
<th>CyA (mg/day, mean ± SE)</th>
<th>Prednisone (mg/day, mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before viremia</td>
<td>9</td>
<td>40 ± 7</td>
<td>+6 ± 6</td>
<td>1,033 ± 55</td>
<td>29 ± 9</td>
</tr>
<tr>
<td>During viremia</td>
<td>12</td>
<td>98 ± 45</td>
<td>−14 ± 22</td>
<td>658 ± 77</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>After viremia</td>
<td>12</td>
<td>82 ± 29</td>
<td>58 ± 17</td>
<td>708 ± 43</td>
<td>16 ± 1</td>
</tr>
</tbody>
</table>

* Samples were obtained from 10 patients before, during, and after clearance of viremia.

* Base-line NK values were not significantly different among the three groups.

* Enhancement of NK activity was only significant (P < 0.05) in samples taken after viremia. The difference in enhancement was also significantly different during and after viremia (P < 0.05).
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LITERATURE CITED


