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Glycine Inhibits Growth of T Lymphocytes by an IL-2-Independent Mechanism

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Previously, it was shown that glycine prevented increases in intracellular calcium ([Ca\(^{2+}\)]\(i\)) in Kupffer cells. Since Kupffer cells and T lymphocytes are derived from the same pluripotent stem cell, it was hypothesized that glycine would prevent increases in [Ca\(^{2+}\)]\(i\), in lymphocytes and inhibit cell proliferation. Lymphocyte proliferation was measured in one-way MLC with spleen cells from DA and Lewis rats and in enriched T lymphocyte preparations stimulated by immobilized anti-CD3 Ab. Glycine caused a dose-dependent decrease in cell proliferation to about 40% of control. Con A caused a dose-dependent increase in [Ca\(^{2+}\)]\(i\), in Jurkat cells which was blunted maximally with 0.6 mM glycine. The effect of glycine was dependent on extracellular chloride and reversed by strychnine, an antagonist of the glycine-gated chloride channel. Similar results were obtained with rat T lymphocytes stimulated by anti-CD3 Ab. Surprisingly, glycine had no effect on IL-2 production in the mixed lymphocyte culture; therefore, the effect of glycine on IL-2-dependent proliferation was tested. Glycine and rapamycin caused dose-dependent decreases in IL-2-stimulated proliferation of T lymphocytes and inhibit cell proliferation. Lymphocyte proliferation was measured in one-way MLC with spleen cells from DA and Lewis rats and in enriched T lymphocyte preparations stimulated by immobilized anti-CD3 Ab. Glycine caused a dose-dependent decrease in cell proliferation to about 40% of control. Con A caused a dose-dependent increase in [Ca\(^{2+}\)]\(i\), in Jurkat cells which was blunted maximally with 0.6 mM glycine. The effect of glycine was dependent on extracellular chloride and reversed by strychnine, an antagonist of the glycine-gated chloride channel. Similar results were obtained with rat T lymphocytes stimulated by anti-CD3 Ab. Surprisingly, glycine had no effect on IL-2 production in the mixed lymphocyte culture; therefore, the effect of glycine on IL-2-dependent proliferation was tested. Glycine and rapamycin caused dose-dependent decreases in IL-2-stimulated growth of C6lL-2 cells to about 60% and 40%, respectively, of control. Moreover, glycine also inhibited the IL-2-stimulated growth of rat splenic lymphocytes. It is concluded that glycine blunts proliferation in an IL-2-independent manner. This is consistent with the hypothesis that glycine activates a glycine-gated chloride channel and hyperpolarizes the cell membrane-blunting increases in [Ca\(^{2+}\)]\(i\), that are required for transcription of factors necessary for cell proliferation. The Journal of Immunology, 2000, 164: 176–182.

Since glycine prevents increases in [Ca\(^{2+}\)]\(i\), in other types of white blood cells and calcium is important in activation of T lymphocytes, it was hypothesized that glycine would prevent T lymphocyte proliferation and be immunosuppressive by a mechanism involving activation of a glycine-gated chloride channel. To test this hypothesis, the effect of glycine on proliferation of T lymphocytes after anti-CD3 stimulation and in the one-way mixed lymphocyte culture (MLC) system was examined. Also, the effect of glycine on increases in [Ca\(^{2+}\)]\(i\), was determined in a human lymphoblast cell line as well as on anti-CD3-dependent proliferation of primary rat T lymphocytes. Preliminary accounts of these data have appeared elsewhere (14).

Materials and Methods

Animals and materials

Female DA and Lewis rats (175–200 g) were obtained from Harlan Laboratories (Indianapolis, IN). All animals received humane treatment in compliance with institutional and National Institutes of Health guidelines. Rats were anesthetized with methoxyflurane before all surgical procedures. RPMI 1640, glycine-free RPMI 1640, and MEM were made from powdered media from Life Technologies (Rockville, MD) prepared at the Lineberger Comprehensive Cancer Center Tissue Culture Facility (Chapel Hill, NC). Glycine was purchased from Fisher (Springfield, NJ). Cyclosporin A was purchased from Novartis Pharmaceuticals (East Hanover, NJ) and [\(^{3}H\)]thymidine was procured from Amersham (Arlington Heights, IL). Fura 2/acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR) and Pluronic F127 from BASF Bioresearch (Wyandotte, MI). All other chemicals were tissue culture grade and were purchased from Sigma Chemical (St. Louis, MO). Jurkat cells and C6lL-2 cells were acquired from the American Type Culture Collection (Manassas, VA). ELISA kits to measure IL-2 were purchased from BioSource International (Camarillo, CA). IL-2 was purchased from Sigma and rat anti-CD3 Ab (1F4 mAb) from Serotec (Oxford, U.K.).

Isolation of lymphocytes

Cells were isolated as described by Linden et al. (15), with minor modifications. Briefly, the abdomen of the rat was shaved and washed with ethanol, a mid-line incision was made under aseptic conditions and the...
spleen and/or mesenteric lymph nodes were quickly removed. Fragments were placed on sterile nylon mesh over a 50-ml conical tube, covered with MEM, and gently teased with sterile forceps. Cells were rinsed through the nylon mesh and the volume of the cells increased to 50 ml. Cells were washed by centrifugation at 300 × g for 10 min, and the pellet was resuspended in 10 ml of MEM. Spleen cells were lysed with ammonium chloride. The volume was increased to 50 ml with media and centrifuged at 300 × g for 10 min. The pellet was resuspended in glycine-free RPMI 1640 containing 10% FCS, 10^{-5} M 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (subsequently referred to as MLC medium). Viability of cells determined by trypan blue exclusion was >90%. Furthermore, viability of lymphocytes cultured with added glycine was assessed after 48 h and/or under conditions after anti-CD3 Ab stimulation (described below). No significant change in viability was observed in the range 0–10 mM glycine (data not shown).

One-way MLC

Lymphocytes were counted using a hemocytometer, and viability was determined by trypan blue exclusion. One-way MLC were established essentially as described by Nemanlter et al. (16). Cells were suspended at 1 × 10^7 cells/ml in MLC media. Splenocytes from DA rats were irradiated with 2500 rad and served as stimulator cells. Splenocytes from Lewis rats served as responders (proliferating cells). DA and Lewis cells were mixed 2:1, and MLC were established by adding 200 μl in each well of a 96-well round-bottom plate. Cells were treated with 0–10 mM glycine at the beginning of culture and on day 4. Preliminary experiments had resulted in 30% MLC proliferation if glycine was added on day 0 or 4 only. Lymphoparin A (1–100 ng/ml) was added on day 0. Proliferation was determined from [3H]thymidine incorporation during 16 h of pulse labeling with 1 μCi/well after 5 days of culture (15, 16). Cells were lysed with double-distilled water, and DNA was harvested onto glass wool filters. Incorporation of radioactive thymidine was determined by counting [3H] using a scintillation counter. All experiments were completed in duplicate. To evaluate the effect of IL-2, DA and Lewis cells were mixed 1:1 and aliquots (200 μl, 4 × 10^5 cells/ml) were added to 96-well round-bottom plates. Plates were preincubated for 10 min. The supernatant was collected and stored at −80°C until measurement of IL-2 by standard ELISA techniques. All experiments were completed in triplicate.

Maintenance of CTLL-2 cultures

CTLL-2 cells, an immortal IL-2-dependent murine cytotoxic T lymphocyte cell line, were grown in RPMI 1640 media containing 2 mM l-glutamine, 10% FCS, 20 U/ml human recombinant IL-2, 100 U/ml penicillin, and 100 μg/ml streptomycin and split 1:1000 every week. Cultures of 1 × 10^6 cells/ml were deprived of IL-2 for 12 h before experiments to synchronize the cell cycle in G0/G1 (20), washed twice by centrifugation for 10 min at 300 × g, and resuspended in glycine-free RPMI 1640 media prepared as described above. Aliquots (200 μl) at 10^7 cells/well were plated in 96-well plates and treated with glycine (0–10 mM) or rapamycin (0–100 ng/ml) and 10 U/ml of IL-2. After 18 h, 1 μCi of [3H]thymidine was added to measure cell proliferation. Cells were harvested 6 h later on glass wool filters and lysed with distilled water, and incorporation of radioactivity was determined by counting [3H] using a scintillation counter. All experiments were completed in duplicate.

Results

Effect of glycine and cyclosporin A on proliferation in the MLC

Since glycine has been shown to inhibit agonist-induced increases in [Ca^{2+}]_i in Kupffer cells, neutrophils, and alveolar macrophages (1, 21, 22), it was hypothesized that glycine would prevent T cell activation and growth via the same mechanism. To test this hypothesis, MLC were treated with glycine (Fig. 1A). Cultures were also treated with cyclosporin A (0–100 ng/ml) as positive controls (Fig. 1B). Proliferation was decreased by glycine in a dose-dependent manner and was diminished significantly at all concentrations of glycine above 0.6 mM. Maximal suppression of proliferation due to glycine was about 60%, and the IC50 value was 0.44 mM. As expected, cyclosporin A also prevented cell proliferation in the MLC in a dose-dependent manner with an IC50 value of 6.2 ng/ml. Growth was suppressed completely with cyclosporin A concentrations above 50 ng/ml. To test the effect of glycine on proliferation of T lymphocytes, enriched T lymphocyte preparations were stimulated with immobilized anti-CD3 Ab. Glycine inhibited proliferation of T lymphocytes in a dose-dependent manner (Fig. 2C).

Effect of glycine on Con A-mediated increases in [Ca^{2+}]_i in Jurkat cells and anti-CD3-mediated increases in rat T lymphocytes

To determine whether the glycine-mediated inhibition of proliferation in T lymphocytes was due to the prevention of agonist-induced increases in [Ca^{2+}]_i, the effect of glycine on increases in [Ca^{2+}]_i, was studied in Jurkat cells and rat T lymphocytes. Con A stimulates Jurkat cells by activating CD3, a component of the T cell receptor complex, leading to increases in [Ca^{2+}]_i. (23). Con A increased [Ca^{2+}]_i in Jurkat cells from values around 25 nM to nearly 300 nM and was maximal at a concentration of 30 μg/ml (data not shown).
The effect of glycine on Con A-mediated increases in [Ca$^{2+}$]$_i$ in single Jurkat cells is shown in Fig. 3A and summarized in Fig. 3B. The effect of glycine on Con A-induced increases in [Ca$^{2+}$]$_i$ was dose dependent and prevented the maximal increase in [Ca$^{2+}$]$_i$, by almost 60% at concentrations above 0.6 mM (IC$_{50}$, 0.3 mM). Similar results were observed in rat T lymphocytes (Fig. 2B). Jurkat cells exhibited a uniform response pattern after Con A activation (Fig. 3). In contrast, rat T lymphocytes stimulated with anti-CD3 showed several patterns of [Ca$^{2+}$]$_i$; >60% of traces followed the pattern of Fig. 2A in control or glycine-treated lymphocytes. The remainder had sustained [Ca$^{2+}$]$_i$ responses of various duration and amplitude.

FIGURE 1. A, Effect of glycine or cyclosporin A on [3H]thymidine incorporation in the MLC. Cells were cultured as described and in Materials and Methods. Glycine (0–3 mM) was added at the beginning of culture (day 0) and on day 4 (A) or cyclosporin A (0–100 ng/ml) was added on day 0 (B). Cells were pulse labeled with [3H]thymidine for 16 h on day 5 of culture. Data are means ± SEM, n = 4. * p < 0.05 by one-way ANOVA with Bonferroni’s post hoc test.

Mechanism of inhibition of agonist-mediated increases in [Ca$^{2+}$]$_i$ in T lymphocytes

In Kupffer cells, neutrophils, and alveolar macrophages, glycine blunts increases in [Ca$^{2+}$]$_i$, by opening a glycine-gated chloride channel (1, 21, 22) with properties similar to the glycine-gated chloride channel in the spinal cord (24). To test the hypothesis that glycine blunts increases in [Ca$^{2+}$]$_i$, by a mechanism dependent on chloride flux, extracellular chloride in the buffer was replaced with gluconate before addition of Con A or anti-CD3 Ab (Fig. 4 and Fig. 2B). As shown above (Fig. 3), glycine significantly blunted increases in [Ca$^{2+}$]$_i$, in Con A-stimulated cells (Fig. 2B). In chloride-free buffer, however, glycine failed to block agonist-induced increases in [Ca$^{2+}$]$_i$, (Fig. 4, bar 1–3). In the CNS, strychnine blocks glycine-gated chloride channels at micromolar concentrations (24). In this study, strychnine (1 mM) prevented the effect of glycine on Jurkat cells and T lymphocytes (Fig. 4, bar 5; Fig. 2B).

On the other hand, strychnine mimics the effects of glycine in the kidney and the liver at high concentrations (25, 26). Indeed, high concentrations of strychnine (1 mM) blunted Con A-mediated increases in [Ca$^{2+}$]$_i$, significantly (Fig. 5). The inhibition in Con A-stimulated increases in [Ca$^{2+}$]$_i$, by strychnine was almost identical to that caused by similar concentrations of glycine (Fig. 3A).
Glycine acts independent of IL-2

Because glycine blunted increases in $[\text{Ca}^{2+}]_i$, it was hypothesized that it would inhibit production and secretion of IL-2 because transcription of the IL-2 gene is calcium dependent (6). To test this hypothesis, IL-2 was measured in the supernatant of MLC in the presence of glycine or cyclosporin A. Surprisingly, glycine (1 mM) had no effect on the production of IL-2 in MLC (Table I). As expected, cyclosporin A (50 ng/ml) inhibited production of IL-2 almost completely (27). These data further support the hypothesis that glycine does not prevent proliferation of lymphocytes by the same mechanism as cyclosporin A. To determine whether glycine affected IL-2-dependent proliferation, Ctl1-2 cells, an IL-2-dependent murine cytotoxic T lymphocyte cell line, were cultured in the presence of IL-2 and increasing concentrations of glycine or rapamycin, an immunosuppressive compound which prevents proliferation of T lymphocyte by blocking activation of p70 S6 kinase and inhibiting growth without altering IL-2 production (28). As shown in Fig. 6, glycine and rapamycin inhibited proliferation of Ctl1-2 cells in a dose-dependent manner. The effect of glycine was dose dependent with a maximal effect of nearly 40% at concentrations above 0.6 mM (IC$_{50}$, 0.41 mM). Rapamycin inhibited Ctl1-2 growth maximally at 60 nM (IC$_{50}$, about 2 nM). Moreover, IL-2 stimulated lymphocyte growth as expected; however, glycine inhibited the growth of T cells even in the presence of additional exogenous IL-2 (Fig. 7). Therefore, it is concluded that glycine inhibits T lymphocytes via an IL-2-independent mechanism.

FIGURE 3. Effect of glycine on Con A-stimulated increases in $[\text{Ca}^{2+}]_i$ in Jurkat cells. Jurkat cells were plated and loaded with fura 2 as described in Materials and Methods. Cells were incubated for 3 min in m-HBSS or m-HBSS containing glycine (0–1 mM). The buffer was replaced with the same solution containing 30 µg/ml Con A and peak $[\text{Ca}^{2+}]_i$ concentration was determined fluorometrically. A, Representative data of an experiment repeated four times. B, Dose-dependent effect of glycine. Data are means ± SEM from four independent experiments (8–10 cells in each experiment). *p < 0.05 by one-way ANOVA with Bonferroni’s post hoc test.

FIGURE 4. Effects of chloride-free buffer or strychnine on Con A-stimulated increases in $[\text{Ca}^{2+}]_i$ in Jurkat cells. Jurkat cells were plated and loaded with fura 2 as described in Materials and Methods. Cells were incubated for 3 min in m-HBSS or chloride-free buffer containing glycine (1 mM) or strychnine (1 µM). The buffer was replaced with the same solution containing 30 µg/ml Con A, and $[\text{Ca}^{2+}]_i$ was determined fluorometrically. Data are means ± SEM from four experiments. a, significantly different from basal; b, significantly different from cells treated with Con A alone; c, significantly different from Con A plus glycine. p < 0.05 by two-way ANOVA with Bonferroni’s post hoc test.

FIGURE 5. Effects of high concentrations of strychnine on Con A-stimulated increases in $[\text{Ca}^{2+}]_i$ in Jurkat cells. Jurkat cells were plated and loaded with fura 2 as described in Materials and Methods. Cells were incubated for 3 min in m-HBSS containing strychnine (1 mM). The buffer was replaced with the same solution containing 30 µg/ml Con A, and $[\text{Ca}^{2+}]_i$ was determined fluorometrically. Data are from a representative experiment repeated four times.
Glycine prevents proliferation of T lymphocytes in the MLC

Glycine caused a dose-dependent inhibition in proliferation of T lymphocytes in the MLC (Fig. 1). The IC$_{50}$ for glycine was only about 1.5 times normal blood concentration and was similar to the inhibitory effect of glycine on agonist-induced increases in [Ca$^{2+}$]$_i$ in Kupffer cells, neutrophils, and alveolar macrophages (1, 21, 22). Furthermore, the concentrations of glycine that reduced proliferation of T lymphocytes in the MLC are easily achievable by feeding a diet enriched with glycine (2). The effect of glycine was not as robust (maximal effect was around 50%) as the near complete inhibition of T lymphocyte proliferation that can be achieved with cyclosporine. Moreover, glycine inhibited the growth of T lymphocytes stimulated with anti-CD3 Ab (Fig. 2). These data suggest that glycine and cyclosporin A could be used in combination to prevent immune activation in vivo, especially rejection of transplanted organs (see Clinical significance).

Possible mechanism of glycine-induced prevention of cell proliferation

It is well known that increases in [Ca$^{2+}$]$_i$ are important in the activation and proliferation of T lymphocytes (5, 7). As shown schematically in Fig. 8, stimulation of the TCR by either histocompatibility Ags or Con A activates both the mitogen-activated protein kinase cascade and phospholipase C to activate transcription factors leading to IL-2 production (Fig. 8, left). Calcium is required to activate calcineurin for IL-2 production. Data from this laboratory showed that glycine prevented increases in [Ca$^{2+}$]$_i$ in other leukocytes, including Kupffer cells, neutrophils, and alveolar macrophages, by activating a glycine-gated chloride channel (1, 21, 22). This led to the hypothesis that glycine prevents increases in [Ca$^{2+}$]$_i$ in T lymphocytes by a similar mechanism. Indeed, glycine blunted increases in [Ca$^{2+}$]$_i$ due to Con A and anti-CD3 Ab (Figs. 2B and Fig. 5B) to a similar degree as observed in the MLC (Fig. 1A). Moreover, inhibition of increases in [Ca$^{2+}$]$_i$ was reversed by substituting chloride in the buffer with the impermeant ion gluconate (Figs. 2B and 4). These data show that the effect of glycine is dependent on extracellular chloride, as in other cell types (1, 21, 22). Furthermore, strychnine, an inhibitor of the glycine-gated chloride channel in the neuron and other leukocytes (1, 21, 22, 29), also reversed the effect of glycine in Jurkat cells and rat T lymphocytes, restoring the Con A-mediated increase in [Ca$^{2+}$]$_i$ to near control values (Figs. 2B and 4). These data are consistent with the hypothesis that glycine inhibits agonist-induced increases in [Ca$^{2+}$]$_i$ by activating a glycine-gated chloride channel in T lymphocytes.

Table I. Effect of glycine and cyclosporin A on IL-2 production in MLC

<table>
<thead>
<tr>
<th>Glycine (mM)</th>
<th>Cyclosporin A (ng/ml)</th>
<th>IL-2 (pg/ml)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>434 ± 76</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>496 ± 93</td>
<td>114</td>
<td></td>
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<tr>
<td>*</td>
<td>11 ± 4*</td>
<td>2</td>
<td></td>
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</tbody>
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* MLC were established as described in Materials and Methods. Rat IL-2 was measured in media after 72 h of culture using standard ELISA techniques (n = 5). * p < 0.05 by one-way ANOVA with Bonferroni’s post hoc test.
**FIGURE 8.** Proposed mechanism of the effect of glycine on proliferation of T lymphocytes. The cell on the left is representative of a T lymphocyte that responds to a stimulus, either Con A or via the T cell receptor, to produce IL-2. Based on the data presented in this study, glycine blunts agonist-induced increases in intracellular calcium, an effect reversed by low concentrations of strychnine. These data are consistent with the hypothesis that glycine activates a glycine-gated chloride channel, leading to the influx of chloride which hyperpolarizes the cell membrane and decreases the opening time of plasma membrane voltage-dependent calcium channels. Although the reduction in $[Ca^{2+}]_{i}$ is not sufficient to prevent synthesis of IL-2, the proliferative effect of IL-2 is reduced significantly. The data are consistent with the hypothesis that glycine blocks calcium-dependent steps between the IL-2 receptor and the kinases required for cell proliferation. It is possible that glycine blocks the activation of p70 S6 kinase and prevents initiation of translation, thereby slowing rates of proliferation.

**Glycine acts independent of IL-2**

Proliferation of lymphocytes in culture is dependent on the production of IL-2 (27). Moreover, as shown in Fig. 8, IL-2 production is dependent on $[Ca^{2+}]_{i}$ (5, 30). Indeed, the calcineurin inhibitors cyclosporin A and FK-506 inhibit the calcium-dependent production of IL-2, leading to immunosuppression (Fig. 8, left; Ref. 28). Therefore, it was hypothesized that glycine would blunt increases in $[Ca^{2+}]_{i}$, and thereby reduce IL-2 production. However, glycine had no effect on the production of IL-2 and inhibited proliferation in the presence of IL-2 (Table I and Fig. 7). The exact reason for the lack of effect of glycine on IL-2 production is not fully understood. Calcium-chelating agents and calcium channel blockers, which prevent sustained increases in $[Ca^{2+}]_{i}$, totally block IL-2 production (31). In contrast, glycine does not inhibit small sustained increases in $[Ca^{2+}]_{i}$, completely (Fig. 3). Dolmetch et al. (32) have shown that a sustained increase in $[Ca^{2+}]_{i}$ is required to activate calcineurin, leading to activation of NF-AT in B lymphocytes. Activation of the transcription factor NF-AT is a critical event in the activation of the IL-2 gene in T lymphocytes. IL-2 production occurs even in the presence of glycine, most likely because glycine does not prevent small sustained increases in $[Ca^{2+}]_{i}$.

Another compound that is being developed as an immunosuppressive drug (i.e., rapamycin) does not affect IL-2 production by T lymphocytes; rather, it prevents proliferation by inhibiting IL-2-dependent cell growth (20). Rapamycin inhibits the activity of Frap kinase and blocks activation of p70 S6 kinase and the cascade required for cell growth (Fig. 8, right). Large sustained increases in $[Ca^{2+}]_{i}$ are required for IL-2-mediated proliferation of T lymphocytes (11, 33). Furthermore, the activation of p70 S6 kinase may be $Ca^{2+}$ dependent in some cells (34). Therefore, it was hypothesized that glycine inhibited cell growth of activated T cells by blocking IL-2-mediated proliferation by affecting $Ca^{2+}$. Indeed, glycine blunted IL-2-dependent growth of a murine cytotoxic T lymphocyte cell line by around 40% (Fig. 6). Furthermore, the IC50 values were nearly identical in the MLC and in the cytotoxic T lymphocyte cell line, suggesting that glycine may function by the same mechanism in both systems. These data are also consistent with the hypothesis that glycine inhibits cell proliferation by opening a glycine-gated chloride channel in the activated T lymphocyte that blunts the increase in calcium required for cell proliferation by hyperpolarizing the cell membrane and decreasing the open probability of plasma membrane calcium channels.

**Clinical significance**

Data presented here demonstrate that glycine has immunosuppressive effects and suggest that it could be used in combination with reduced doses of cyclosporin A to maintain effective immunosuppression and prevent rejection of transplanted organs. There are several advantages to the use of glycine in this manner. It can be fed in the diet and has no reported side effects in a clinical trial (35). More important, glycine has been shown to prevent the nephrotoxic side effects of cyclosporin A in the rat (36). These observations suggest that glycine could be used in combination with cyclosporin A to prevent organ rejection and limit the nephrotoxic side effects of this widely used immunosuppressive drug.

**Acknowledgments**

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**References**

GLYCINE AND T LYMPHOCYTES


