Treatment of Experimental Viral Myocarditis
With Interleukin-10

Ryosuke Nishio, MD, PhD; Akira Matsumori, MD, PhD; Tetsuo Shioi, MD, PhD; Hiroshi Ishida, MD, PhD; Shigetake Sasayama, MD, PhD

Background—The T helper cell type 2–associated cytokine interleukin (IL)-10 has a variety of immunomodulatory properties. However, the effects of the cytokine on viral myocarditis remain unclear.

Methods and Results—We studied the effects of recombinant human IL-10 (rhIL-10) fully active on mouse cells in a murine experimental model of acute viral myocarditis caused by the encephalomyocarditis virus (EMCV). Four-week-old DBA/2 mice were inoculated with EMCV (day 0). rhIL-10 (10 μg/mouse) was administered once daily, starting on day 0, and control mice received vehicle only. Survival rates were determined on day 14. Myocardial histopathology, cytokine levels in the heart by ELISA assay, and myocardial virus concentration were examined on day 6, and the expression levels of myocardial inducible nitric oxide synthase (iNOS) mRNA were measured by competitive polymerase chain reaction. The 14-day survival in mice treated with rhIL-10 was significantly higher (80%) than in the control group (30%, n=10 in each, P<0.05). rhIL-10 treatment significantly attenuated myocardial lesions and suppressed tumor necrosis factor-α and IL-2 in the heart. rhIL-10 treatment had little effect on myocardial virus concentration. The expression levels of myocardial iNOS mRNA were significantly decreased in the group treated with rhIL-10 (8.6±4.7 amol/mg total RNA in treated versus 26.5±7.1 amol/mg total RNA in control mice, P<0.05).

Conclusions—These findings provide new insights into the in vivo effects of IL-10 on viral infection and suggest a therapeutic effect of IL-10 on viral myocarditis. (Circulation. 1999;100:1102-1108.)

Key Words: interleukins ■ myocarditis ■ tumor necrosis factor ■ nitric oxide synthase

Current concepts of the immune responses focus on the cross-regulation between the 2 types of helper T cell.1 T helper type 1 (Th1) cells produce proinflammatory cytokines and contribute to cell-mediated immunity. The Th2-associated cytokines augment humoral immunity. The cytokines produced by one type of helper T cell regulate the others.

The Th2-associated cytokine interleukin-10 (IL-10) has a variety of immunomodulatory properties involving the inhibition of Th1 cells, macrophage function, and the production of proinflammatory cytokines.2,3 IL-10 inhibits the inflammatory response by inhibiting the activation of nuclear factor-κB through preservation of IkB-α.4 Recent reports have suggested that the profound immunosuppressive effects associated with IL-10 may be effective against transplanted organ rejection, immune complex diseases, and sepsis,5 and clinical trials of IL-10 have been carried out in patients with these disorders. However, its use in infectious diseases has produced mixed results, impairing immune activities and promoting infection while suppressing inflammation without reducing the host defense.6

Viral myocarditis is one of the clinically important causes of congestive heart failure and may lead to dilated cardiomyopathy.7 Recent reports have emphasized the important role of cytokines in the pathophysiology of viral myocarditis.8 We and others have reported the expression and the role of cytokines in a murine model of viral myocarditis resulting from encephalomyocarditis virus (EMCV) infection.9–12 These reports suggested that modulation of cytokines could be a successful approach in the treatment of the disease.

This study was designed to examine the effects of IL-10 in a murine experimental model of acute viral myocarditis caused by EMCV.

Interleukin-10

A rat IgG1 designated JES 2A5 monoclonal antibody specific for murine IL-10 (αIL-10 Ab), an isotype-matched control antibody (control Ab) designated GL113 (IgG1), and recombinant murine IL-10 (rmIL-10) were prepared as described previously.13,14 Recombinant human IL-10 (rhIL-10), which was fully active on mouse cells,15 was kindly provided by Schering-Plow Research Institute, Kenilworth, NJ. rmIL-10, rhIL-10, αIL-10 Ab, and control Ab were mixed in 0.1 mL of PBS for the purpose of the experiments.

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Experimental Infection

Four-week-old inbred male DBA/2 mice were inoculated with 0.1 mL IP of the M variant of EMCV diluted in Eagle’s modified essential medium (Nissui Pharmaceutical Co) to a concentration of 100 pfu/mL.7

The day of virus inoculation was defined as day 0.

Time Course of IL-10 Levels in the Heart

The time course of IL-10 mRNA and protein levels in the heart of surviving infected animals were examined. IL-10 mRNA and protein levels in the heart of uninfected mice are indicated as “day 0” values.

Treatment Protocols

Protocol 1: Dose-Dependent Effects of rhIL-10

rhIL-10 was administered in a dose of 1 or 10 µg·mouse−1·day−1 for 6 or 14 consecutive days, starting on day 0, 1, or 3.

Protocol 2: Effects of Timing of rhIL-10 Administration

rhIL-10 (10 µg·mouse−1·day−1) was administered daily consecutively to day 6 or 14, starting on day 0, 1, or 3.

Protocol 3: Effects of rmIL-10

rmIL-10 was administered in a dose of 3 µg·mouse−1·day−1 for 6 consecutive days, starting on day 0.

All recombinant IL-10 was administered once daily, while control mice received vehicle only.

Protocol 4: Effects of αIL-10 Ab

αIL-10 Ab was administered in a dose of 500 µg·mouse−1·day−1 on alternate days starting on day 0, 1, or 3, while control mice received control Ab on equivalent days. All antibodies were administered once daily.

Histological Examination

The hearts were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The extent of cellular infiltration and myocardial necrosis was graded blindly by 2 observers and scored as follows: 0, no lesions; 1+, lesions involving <25% of the myocardium; 2+, lesions involving 25% to 50%; 3+, lesions involving 50% to 75%; and 4+, lesions involving 75% to 100%.

Assay of Cytokine Levels in the Heart

Cytokine levels in the heart were measured as previously described.10,11 Briefly, the hearts of surviving animals were homogenized with PBS at 4°C and centrifuged, and the resultant supernatants were collected to measure cytokine levels with an ELISA kit (Genzyme for IL-1α, IL-6, IL-12, and tumor necrosis factor-α; BioSource International for IL-4 and IL-6; 15 pg/mL for IL-10, TNF-α, and IFN-γ; and Endogen Inc for IL-2 and interferon-γ (IFN-γ)). The sensitivity of the kit is 5 pg/mL. The expression of iNOS mRNA in the heart was measured by a competitive PCR experiment shown in A. Log of ratio of absorbance of target and MIMIC (350-bp iNOS competitor and 497-bp iNOS) was plotted against log of (molecules MIMIC added to PCR reaction. Line was drawn from a linear regression analysis of data points. Amount of target molecule was determined as point of an equimolar ratio between internal control and target.

Figure 1. Quantification of murine iNOS mRNA in heart by competitive PCR. A, Lane 1 contains 50×1017 amol iNOS-MIMIC; lane 2, 25×1017; lane 3, 12.5×1017; lane 4, 6.1×1017; lane 5, 3.1×1017; lane 6, 6.1×1016; lane 7, 3.1×1016; and lane 8, 1.5×1016. B, Quantitative analysis of competitive PCR experiment shown in A. Log of ratio of absorbance of target and MIMIC (350-bp iNOS competitor and 497-bp iNOS) was plotted against log of (molecules MIMIC added to PCR reaction. Line was drawn from a linear regression analysis of data points. Amount of target molecule was determined as point of an equimolar ratio between internal control and target.

Semiquantitative PCR Analysis for Time Course of IL-10 mRNA

The semiquantitative analysis of IL-10 and β-actin mRNA was performed as described.10,11 Briefly, cDNA was amplified for 24 cycles (for IL-10) or 21 cycles (for β-actin). The PCR products were resolved by electrophoresis and analyzed by the Southern blotting method with a Fujix (Japan) bioimaging analyzer BAS 2000. The amounts of IL-10 PCR products were normalized to β-actin. The results are expressed as arbitrary units.

Quantification of Murine iNOS mRNA in the Heart by Competitive PCR

The expression of iNOS mRNA in the heart was measured by a modification of the methods described by Li et al.15 The 350-bp iNOS competitor DNA construct was created for the quantification of iNOS mRNA by PCR with a PCR MIMIC construction kit (Clontech Laboratories, Inc.). A constant amount of the cDNA (2 µL) was then coamplified with known concentrations of the competitor DNA construct (2 µL) for 35 cycles of 94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 90 seconds with 0.2 µmol/L of inos gene-specific primer pairs in a reaction mixture (46 µL) containing 0.25 U AmpliTaq Gold polymerase (Roche Molecular Systems, Inc.). The PCR products were resolved by 2% agarose gel electrophoresis, visualized by ethidium bromide staining, and photographed. The negatives of the photographs were analyzed by an image analyzing system (NIH Image) (Figure 1). The concentration of mRNA was expressed as amol iNOS mRNA/mg total RNA.

Assay of Myocardial Virus Concentration

The hearts of surviving animals were homogenized in PBS and centrifuged. The supernatant was assayed by FL plaque assay.16 The myocardial virus concentration was expressed as log pfu/mg of heart.

Statistical Analysis

Survival was analyzed by the Kaplan-Meier method. Statistical comparisons were performed by ANOVA with Bonferroni’s
The 14-day survival rate was significantly higher in the 10-μg/mouse group (80%) than in the control group (30%, n=10 each, P<0.05, Figure 3). The 14-day survival rate in the 1-μg/mouse group was intermediate (6 of 10, 60%).

**HW/BW Ratio and Myocardial Histology on Day 6**

The ratio of heart weight to body weight (HW/BW) and the pathological scores were lower in the low-dose rhIL-10 group and significantly lower in the high-dose rhIL-10 group than in the control group (Table 1 and Figure 4).

**Cytokine Levels in the Heart on Day 6**

The differences in TNF-α and IL-2 levels measured between the high-dose rhIL-10 group and the control group were statistically significant (P<0.05, Figure 5). On day 6, compared with a mean control value of 125.6±13.2 pg/mg heart, rhIL-10 had suppressed TNF-α levels by 81.8±4.8% in the low-dose rhIL-10 group and by 76.1±5.1% in the high-dose rhIL-10 group (n=5 each). Likewise, compared with a mean control measurement of 15.4±1.0 pg/mg heart, rhIL-10 had suppressed IL-2 levels by 82.4±4.2% and 72.9±10.4% in the low- and high-dose groups, respectively (n=5 each). No such effects were measured with respect to IL-1β, IL-4, IL-6, IL-12, and IFN-γ production (n=5 each, Table 2).

**iNOS mRNA Levels in the Heart on Day 6**

The difference in iNOS mRNA levels measured between the high-dose rhIL-10 group (8.6±1.0 log pfu/mg total RNA) and the control group (26.5±7.1 amol/mg total RNA) was statistically significant (P<0.05, Figure 6). On day 6, iNOS mRNA in the low-dose rhIL-10 group was 16.9±4.1 amol/mg total RNA (n=5 each).

**Myocardial Virus Concentration on Day 6**

On day 6, the myocardial virus concentration was unchanged by rhIL-10. Myocardial virus concentration was 2.8±0.4 log pfu/mg heart in the high-dose rhIL-10 group, 3.0±0.4 log

![Figure 2.](image-url)

**Figure 2.** Time course of IL-10 levels in heart. A, Representative analysis of IL-10 and β-actin mRNA by semiquantitative PCR. B, Semiquantitative PCR analysis of IL-10 mRNA. n=5 each. C, IL-10 protein levels. n=5 each. Results are expressed as mean maximal response±SEM. *P<0.05.

**Figure 3.** Dose-related effects of rhIL-10 on survival. Significant increase in 14-day survival rate in 10-μg/mouse rhIL-10 group vs control group (n=10 each). ○ indicates control group; □, 1-μg/mouse rhIL-10 group; and △, 10-μg/mouse rhIL-10 group. *P<0.05 vs control.

**Table 1.** Dose-Dependent Effects of rhIL-10 on HW/BW and Myocardial Histology on Day 6

<table>
<thead>
<tr>
<th>Group</th>
<th>HW/BW (×10^-7)</th>
<th>Necrosis</th>
<th>Infiltration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.3±0.7</td>
<td>1.5±0.4</td>
<td>1.3±0.3</td>
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<tr>
<td>Low dose</td>
<td>6.3±0.4</td>
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<td>1.0±0.1</td>
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<tr>
<td>High dose</td>
<td>5.7±0.3*</td>
<td>0.6±0.2</td>
<td>0.6±0.2*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *P<0.05 vs control. n=5 each.

**Results**

**Time Course of IL-10 Levels in the Heart**

Both IL-10 mRNA and protein levels were detectable in the hearts of control mice (Figure 2, day 0). These cytokine levels increased 1 day after virus inoculation to reach peak levels 7 days after inoculation and had decreased significantly by day 14 after inoculation, although they remained significantly higher than in uninfected controls. IL-10 mRNA levels on days 3, 7, and 14 were significantly higher than those on day 0 (P<0.05), and IL-10 protein levels on day 7 were significantly higher than those on day 0 (P<0.05). Compared with the value of 48.2±17.8 U measured in infected mice on day 7, mean IL-10 mRNA level was 2.1±5.4% in the uninfected control, 3.9±8.3% on day 1 after infection, 12.2±8.1% on day 3, and 14.7±7.9% on day 14 (n=5 each, Figure 2B). Compared with the value of 2.8±0.5 pg/mg heart measured in infected mice on day 7, IL-10 protein levels were 52.5±10.6% in the uninfected control, 73.9±22.4% on day 1 after infection, 81.5±18.1% on day 3, and 56.5±12.6% on day 14 (n=5 each, Figure 2C).

**Dose-Dependent Effects of rhIL-10**

**Survival Rate**

The 14-day survival rate was significantly higher in the 10-μg/mouse group (80%) than in the control group (30%, multiple-comparison correction. Measurements of myocardial virus concentration were tested by the Kruskal-Wallis test. Values are expressed as mean±SEM. A value of 48.2±17.8 U measured in infected mice on day 7, mean IL-10 mRNA level was 2.1±5.4% in the uninfected control, 3.9±8.3% on day 1 after infection, 12.2±8.1% on day 3, and 14.7±7.9% on day 14 (n=5 each, Figure 2B).

**Histological Score**

<table>
<thead>
<tr>
<th>Group</th>
<th>HW/BW (×10^-7)</th>
<th>Necrosis</th>
<th>Infiltration</th>
</tr>
</thead>
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<tr>
<td>Control</td>
<td>7.3±0.7</td>
<td>1.5±0.4</td>
<td>1.3±0.3</td>
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<tr>
<td>Low dose</td>
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<td>1.1±0.2</td>
<td>1.0±0.1</td>
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<tr>
<td>High dose</td>
<td>5.7±0.3*</td>
<td>0.6±0.2</td>
<td>0.6±0.2*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *P<0.05 vs control. n=5 each.
pfu/mg heart in the low-dose rhIL-10 group, and 2.9±0.5 log pfu/mg heart in the control group (n=5 in each).

Effects of Timing of rhIL-10 Administration

Survival Rate

The 14-day survival rate increased significantly in the rhIL-10 groups treated from day 0 (80%) or from day 1 (80%) compared with the control group (30%, n=10 each, P<0.05, Figure 7). The 14-day survival rate was not changed by rhIL-10 in the group treated from day 3 (4 of 10, 40%).

HW/BW Ratio and Myocardial Histology on Day 6

The HW/BW ratio and the pathological scores were significantly lower in the mice treated with rhIL-10 from day 0 or from day 1 than in the control group. The scores were not changed in the group actively treated from day 3 (Table 3).

Effects of rmIL-10

The HW/BW ratio and the pathological scores were significantly lower in the rmIL-10 group than in the control group (Table 4).

Table 2. Absence of Effects of rhIL-10 on IL-1β, IL-4, IL-6, IL-12, and IFN-γ Production

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Low Dose</th>
<th>High Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>45.7±3.5</td>
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<td>46.2±2.4</td>
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<td>IL-4</td>
<td>19.0±2.6</td>
<td>21.0±1.3</td>
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<td>IL-6</td>
<td>8.5±0.3</td>
<td>8.4±0.6</td>
<td>8.4±0.3</td>
</tr>
<tr>
<td>IL-12</td>
<td>2.5±1.2</td>
<td>2.2±0.6</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>7.1±3.0</td>
<td>6.8±3.7</td>
<td>7.0±2.7</td>
</tr>
</tbody>
</table>

Values are pg/mg heart, mean±SEM, n=5 each.
Effects of αIL-10 Ab

Survival Rate
The 14-day survival rate was significantly lower in the αIL-10 Ab groups treated from day 0 (0%) and from day 1 (0%) than in the group treated from day 3 (30%) or the control group (30%, n=10 each, P<0.05, Figure 8).

HW/BW Ratio and Myocardial Histology on Day 6
The HW/BW ratio and the pathological scores were higher in the αIL-10 Ab groups than in the control group, but no significant differences were found between the 4 groups in the HW/BW ratio and the pathological scores (Table 5).

Discussion
IL-10 improved the survival and attenuated myocardial lesions in mice suffering from acute myocarditis induced by EMCV. On the basis of previous reports, several mechanisms may explain this favorable effect of the cytokine. First, IL-10 can suppress TNF-α release by macrophages and lymphocytes. In our murine model of viral myocarditis, immunohistochemical studies have shown that TNF-α immunostaining in the heart is localized to macrophages, lymphocytes, and endothelial cells. The effect of rhIL-10 on TNF-α production in heart tissue homogenates is probably due to inhibition of TNF-α production by macrophages and lymphocytes. We have reported that pretreatment with anti–TNF-α antibody reduces myocardial damage and prolongs survival in the same murine model. In addition, TNF-α appears to be an important element in the pathophysiology of congestive heart failure. These findings suggest that, in this model of viral myocarditis, IL-10 may prolong survival and attenuate myocardial lesions by inhibiting TNF-α.

Second, IL-10 can inhibit IL-2 production by lymphocytes. In a murine model of coxsackievirus B3–induced myocarditis, survival was prolonged and myocardial injury was lessened by the exogenous administration of IL-2 during the first week after viral inoculation. In contrast, administration of IL-2 during the second week exacerbated the course of the disease. In another report, exogenous treatment by IL-2 accentuated the myocardial damage caused by murine coxsackievirus B3–induced myocarditis. These findings suggest that in this model of viral myocarditis, IL-10 may prolong survival and attenuate myocardial injury in part by inhibiting IL-2.

Third, IL-10 can reduce NO production by inhibiting macrophage function. In our animal model of viral myocarditis, NO production was enhanced in response to EMCV infection, and N\textsuperscript{G}-monomethyl-L-arginine, an inhibitor of NO synthesis, attenuated myocardial lesions. We reported that the third-generation calcium channel blocker amlodipine had beneficial effects and decreased iNOS-positive macrophages by immunohistochemistry in the same model. In this study, rhIL-10 may inhibit the excessive production of NO by suppression of iNOS mRNA. In addition, TNF-α and IL-2 are strong inducers of iNOS. Thus, rhIL-10 may indirectly suppress iNOS gene expression by inhibiting production of TNF-α and IL-2. NO is a mediator of the negative inotropic effects of cytokines, including TNF-α and IL-2. IL-10 may also suppress the negative inotropic effects of NO by directly and indirectly inhibiting iNOS gene expression.

In this study, treatment with IL-10 reduced mortality and myocardial injury when begun on the day of virus inoculation or 1 day later. However, when administered later, the cytokine did not influence the survival rate or prevent the development of myocardial lesions. In a murine model of viral myocarditis, IL-10 may prolong survival and attenuate myocardial lesions by inhibiting TNF-α.
fatal group B streptococcus sepsis, IL-10 improved survival when administered 20 or 4 hours before inoculation but had no effect on mortality if given at later times.\textsuperscript{27} Mortality and inflammation were increased in IL-10 knockout mice with virus-induced encephalomyelitis.\textsuperscript{28} Collectively, these data suggested that IL-10 plays a major role in the early commitment of the immune response in vivo. In our animal model of viral myocarditis, the amplitude of increase in cytokine mRNA suggests that the early responses, including growth and differentiation of T cells, may occur as early as 24 hours after inoculation.\textsuperscript{10} The immune responses enter the next stage by 3 days after infection. IL-10 may represent an important regulator of the early immune response to viral infection.

This study showed that IL-10 administration suppressed inflammation without altering virus replication. These results suggest that IL-10 does not impair the host defense against intracellular pathogens in vivo. In this model of viral myocarditis, administration of IL-12\textsuperscript{11} and IFN-\gamma\textsuperscript{12} also decreased mortality and myocardial injury. However, from the results and data presented here, we could not determine whether the beneficial effects on viral myocarditis were associated with a predominant response of Th1 or Th2. At this point, these results merely point to the important role of these cytokines in the immune responses and to their potential clinical applications. Exogenous cytokines or neutralizing antibodies influence many effectors of immune responses in vivo, including the induction or suppression of the other endogenous cytokines. In addition, it has been reported that IL-10 acts in a Th-subset-independent fashion.\textsuperscript{29} A simple Th1-Th2 dichotomy may not explain the mechanisms of viral myocarditis. It has been recognized that the immune system may be inherently toxic to the host and that the negative regulation of an immune response prevents the toxicity caused by excessive inflammatory responses. This study shows that IL-10 may be a key regulatory cytokine to protect the organism against dangerous inflammatory responses.

**Table 5. Effects of \( \alpha \)-IL-10 Ab on survival. Lower 14-day survival rate in group treated with \( \alpha \)-IL-10 Ab from day 0 or from day 1 than in control group. Survival was not changed in group treated with \( \alpha \)-IL-10 Ab from day 3 \((n=10 \text{ each})\). \( \bigcirc \) indicates control group; \( \triangle \), \( \alpha \)-IL-10 Ab from day 0 group; \( \square \), from day 1 group; and \( \Diamond \), from day 3 group. \( ^{*}P<0.05 \) vs control.**

<table>
<thead>
<tr>
<th>Histological Score</th>
<th>HW/BW ( (\times 10^{-3}) )</th>
<th>Necrosis</th>
<th>Infiltration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.1±0.5</td>
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<td>1.8±0.2</td>
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<td>( \alpha )-IL-10 Ab from</td>
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<td></td>
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<tr>
<td>Day 0</td>
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<tr>
<td>Day 1</td>
<td>7.5±0.3</td>
<td>2.4±0.2</td>
<td>2.2±0.3</td>
</tr>
<tr>
<td>Day 3</td>
<td>7.2±0.6</td>
<td>2.0±0.4</td>
<td>2.0±0.6</td>
</tr>
</tbody>
</table>

Values are mean±SEM. \( n=5 \) each. The differences between the 4 groups are not statistically significant.

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