In Vivo Modulation With Anti-Interleukin-1 (IL-1) Receptor (p80) Antibody 35F5 of the Response to IL-1. The Relationship of Radioprotection, Colony-Stimulating Factor, and IL-6

By R. Neta, S.N. Vogel, J.M. Plocinski, N.S. Tare, W. Benjamin, R. Chizzonite, and M. Pilcher

Interleukin-1 (IL-1) is radioprotective and induces both circulating colony-stimulating factor (CSF) and IL-6 in mice. We evaluated the relationship among these three responses to IL-1 using anti-IL-1 receptor antibody 35F5. This antibody in vitro blocks responses of T cells and fibroblasts, but not of B cells or myeloid cell lines, to IL-1. Administration of 35F5 alone before irradiation reduced the number of surviving mice compared with those not treated with 35F5, demonstrating that endogenous IL-1 participates in the natural resistance to radiation. Thirty micrograms of 35F5 per mouse also reduced by 92% the survival of irradiated mice pretreated with 0.3 μg of IL-1. Similarly, 30 μg of 35F5 reduced by 96% to 98% the induction of IL-6 by IL-1. In contrast, 30 μg of 35F5 resulted in only moderate reduction of circulating CSF. Consequently, the level of circulating CSF after 35F5 treatment was still equivalent to levels of CSF that were induced by doses of IL-1 in the radioprotective range. Because treatment with 35F5 antibody resulted in the blocking of IL-1-reduced radioprotection, the above results suggest that circulating CSF, by itself, may not be sufficient for radioprotection. This conclusion supports our previous results which showed that granulocyte-macrophage CSF (GM-CSF) and G-CSF were radioprotective only when administered with suboptimal doses of IL-1.

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THE MECHANISM(S) that underlie the action of biologic radioprotectors (inflammatory or immunomodulatory agents) remain speculative, despite decades of research. The use of interleukin-1 (IL-1), a radioprotective cytokine that has a recognized range of activities and activates cells by identified specific receptors, allows the study of these mechanisms in more detail. Several of the recognized activities of IL-1 have been suggested to provide a basis for its radioprotective effect. IL-1 induces the appearance of colony-stimulating factors (CSFs) and IL-6 in the circulation, and stimulates the production of CSF and IL-6 by cultured fibroblasts, endothelial cells, and macrophages. Although not radioprotective when administered alone, both CSFs (granulocyte-CSF [G-CSF] and granulocyte-macrophage-CSF [GM-CSF]) and IL-6 synergized with suboptimal doses of IL-1 to induce radioprotection. Furthermore, IL-1 administration initiates cycling of myeloid progenitor cells in the marrow. This cycling is thought to depend on the presence of hematopoietic growth factors, such as CSFs and IL-6, and on direct interaction of IL-1 and IL-6 with early progenitor cells. However, direct evidence that the above mechanisms are essential for radioprotection remains to be established.

Recently, a monoclonal antibody to the IL-1 receptor was developed. This agent binds only to T lymphocytes and fibroblasts but not to B lymphocytes, neutrophils, and myeloid cell lines. This antibody may provide a means to dissociate those events induced by IL-1 that occur independent of radioprotection. We used this antibody in mice and compared its effect on the activity of IL-1 as a radioprotector with its effect as an inducer of circulating CSF and IL-6.

MATERIALS AND METHODS

Mice. CD2Fl male mice were purchased from the Animal Genetics and Production Branch, National Cancer Institute, National Institutes of Health (Frederick, MD). Mice were quarantined on arrival and screened for evidence of disease before being released from quarantine. They were maintained in an AAALAC-accredited facility in plastic Micro-isolator cages on hardwood chip contact bedding, and given commercial rodent chow and acidified (HC1 to a pH of 2.5) tap water ad libitum. Animal holding rooms were maintained at 70°F ± 2°F with 50% ± 10% relative humidity, using at least 10 air changes per hour of 100% conditioned fresh air. The mice were on a 12-hour light-dark full-spectrum lighting cycle with no twilight. Mice were 8 to 12 weeks old when used. All cage cleaning, handling, and injections were performed in a laminar flow clean air unit.

Reagents. Human recombinant IL-1α and anti-IL-1 receptor antibody (35F5), a rat anti-mouse monoclonal immunoglobulin G, [IgG], were prepared at Hoffmann-La Roche (Nutley, NJ). Control rat IgG was purchased from Sigma Chemical Co (St Louis, MO). The antibody and the recombinant IL-1α were diluted in pyrogen-free saline on the day of injection. The antibody was given intraperitoneally (ip) according to the protocol previously developed at Hoffmann-La Roche (unpublished results, 1989), 6 hours before ip injection of IL-1. Mice were anesthetized with methapane and were exsanguinated 2 to 3 hours after IL-1 administration.

Irradiation. Mice were placed in Plexiglass containers and were given whole-body irradiation at 40 cGy/min by bilaterally positioned 60Co elements. The number of surviving mice was recorded daily for 30 days.

Stromal cell culture. Murine bone marrow stromal cell cultures

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were established using a method described by Dexter et al. for long-term marrow culture. Briefly, bone marrow cells from one femur (approximately $2 \times 10^7$ cells) of a 6-week-old C57BL/6J female mouse (Jackson Laboratory, Bar Harbor, ME) were cultured in a 25-cm$^2$ tissue culture flask (Costar, Cambridge, MA) containing 5 mL minimum essential $\alpha$ medium (GIBCO, Grand Island, NY) supplemented with 25% horse serum, $1 \times 10^{-6}$ mol/L hydrocortisone 21-hemisuccinate (Sigma, St Louis, MO), and antibiotics. After 1 week of incubation at 33°C in a humidified atmosphere containing 5% CO$_2$, 95% air, half of the culture medium was replaced. After an additional 2 weeks of culture, a sub-confluent monolayer of adherent stromal cells, as well as hematopoietic colonies, could be observed. At this time, the medium was changed to contain 10% fetal bovine serum (HyClone, Logan, UT), plus 10% horse serum and $5 \times 10^{-4}$ mol/L hydrocortisone, and the cultures were transferred to a 37°C incubator. Once confluent, cultures were trypsinized and passed at 1- to 2-week intervals. During a 6-month selection period, several phenotypically distinct homogenous adherent stromal cell cultures were isolated. To determine the effect of 35F5 antibody on production of IL-1-stimulated cytokines, stromal cell cultures were incubated for 72 hours with 130 pg/mL of IL-1 in the presence of 100 µg/mL 35F5 antibody or rat IgG. Supernatants were assayed as described.

**Measurement of IL-6 activity.** IL-6 activity in the serum and stromal cell supernatants was determined by using the hybridoma growth factor assay described by Aarden et al. This method uses the IL-6-dependent hybridoma, B9, in a conventional microproliferation assay. Briefly, individual samples were initially treated to assay for CSF activity has been described in detail. Briefly, a growth factor assay described by Aarden et al. This method uses culture cells were incubated for 72 hours with 130 pg/mL of IL-I in a humidified atmosphere containing 5% CO$_2$, 95% air, half of the culture medium was replaced. After an additional 2 weeks of culture, a sub-confluent monolayer of adherent stromal cells, as well as hematopoietic colonies, could be observed. At this time, the medium was changed to contain 10% fetal bovine serum (HyClone, Logan, UT), plus 10% horse serum and $5 \times 10^{-4}$ mol/L hydrocortisone, and the cultures were transferred to a 37°C incubator. Once confluent, cultures were trypsinized and passaged at 1- to 2-week intervals. During a 6-month selection period, several phenotypically distinct homogenous adherent stromal cell cultures were isolated. To determine the effect of 35F5 antibody on production of IL-1-stimulated cytokines, stromal cell cultures were incubated for 72 hours with 130 pg/mL of IL-1 in the presence of 100 µg/mL 35F5 antibody or rat IgG. Supernatants were assayed as described.

**Measurement of CSF activity in the serum and supernatant.** Mice were bled 2 to 3 hrs after IL-1 injection, and serum was collected by centrifugation after clot formation. CSF activity was measured in pooled serum samples collected from 4 to 5 mice per treatment group per experiment. Supernatants were collected from the stromal cell cultures as described above. The bone marrow colony assay for CSF activity has been described in detail. Briefly, C3H/HeJ bone marrow cells were enriched for mononuclear cells by density gradient centrifugation on lymphocyte separation medium (Litton Bionetics, Charleston, SC). The cells collected from the interface of the gradient were washed and resuspended in RPMI 1640 supplemented with antibiotics, glutamine, sodium bicarbonate, HEPES buffer, and 15% FCS. Three serial twofold dilutions of each serum sample (30%, 15%, and 7.5% vol/vol) or two fourfold dilutions of stromal cells supernatant sample (20% and 5% vol/vol) were prepared in this medium, and $0.2 \text{ mL}$ of each dilution was added to duplicate well in a 6-well tissue culture plate. A final cell suspension was prepared of $1 \times 10^5$ cells/mL in complete medium supplemented with 0.35% Bacto-Agar (Difco, Detroit, MI) and maintained at 41°C. Immediately after resuspension of the cells in the agar-medium mixture, 1 mL of this medium was added to each well. Once solidified, the cultures were incubated at 37°C, 6% CO$_2$, for 6 to 7 days, at which time colonies ($\geq 25$ cells per colony) were counted under a dissecting microscope. CSF activity was expressed as colony-forming units per milliliter, based on colony count within the linear part of the dilution curve.

**Statistical analysis.** Statistical evaluation of the results was done using chi-square analysis.

### RESULTS

#### Effect of anti-IL-1 receptor antibody 35F5 on radiation sensitivity of mice.

We have shown that pretreatment with IL-1 confers radioprotection on mice. Conversely, others have shown that radiation results in a subsequent increase in IL-1. To determine if endogenous IL-1 is important in protection from damage by ionizing radiation, mice were administered the 35F5 antibody, control rat Ig, or saline before midlethal irradiation (Table 1). The results show clearly that pretreatment with the 35F5 antibody reduces the survival of mice significantly.

The effect of 35F5 antibody on radioprotection induced by IL-1. The 35F5 antibody has been shown to block the effect of IL-1 only on selected cell populations: fibroblasts and T-lymphoid cells, but not myeloid cells and B lymphocytes. Therefore, we determined whether treatment with 35F5 could reduce the radioprotective effect of IL-1. Experimental mice were administered the 35F5 antibody, while control mice were treated with either rat Ig at equivalent concentrations or saline 6 hours before injection of 0.3 µg of IL-1, and exposed to lethal irradiation 20 hours later. The results demonstrate that even the 30-µg dose of the antibody almost completely blocked the radioprotective action of IL-1 (Table 2).

#### Dose-dependence of the radioprotective effect and CSF-inducing effects of IL-1.

We have previously shown that IL-1 induces the release of CSF into circulation. The increase in circulating CSF and the corresponding increase in radioprotection is dependent on the dose of IL-1 (Table 3). The results show that at IL-1 doses above 0.1 µg, the titers of CSF approach plateau values (Tables 3 and 4). However,

- **Table 1. Radiosensitization of Mice With Anti-IL-1 Receptor Antibody 35F5**

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Dead/Total</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>7/16</td>
<td>56</td>
</tr>
<tr>
<td>Rat Ig</td>
<td>4/8</td>
<td>50</td>
</tr>
<tr>
<td>35F5</td>
<td>21/26</td>
<td>20*</td>
</tr>
</tbody>
</table>

*CD2F1 mice received 100 µg of 35F5 antibody, vehicle, or equivalent amount of rat Ig 6 hours before irradiation with 825 cGy. Combined results of three experiments.

+P < .05 compared with saline control.

- **Table 2. Effect of Anti-IL-1 Receptor Antibody 35F5 on Radioprotection With IL-1**

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Dead/Total</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Ig</td>
<td>53/54</td>
<td>2</td>
</tr>
<tr>
<td>IL-1</td>
<td>15/78</td>
<td>81</td>
</tr>
<tr>
<td>IL-1 + 35F5 (30 µg)</td>
<td>44/48</td>
<td>8</td>
</tr>
<tr>
<td>IL-1 + 35F5 (150 µg)</td>
<td>48/52</td>
<td>8</td>
</tr>
</tbody>
</table>

*CD2F1 mice received ip injection of 35F5 antibody followed 6 hours later with ip injection of 0.3 µg of IL-1. Control mice received rat IgG (100 µg), followed by saline injection. Twenty hours after injection of IL-1, the mice were irradiated with 950 cGy.
MODULATION OF IL-1 RESPONSE WITH ANTI-IL-1 RECEPTOR

Table 3. Relationship of the Effect of Increasing Doses of IL-1 on Radioprotection and CSF in Serum of Mice

<table>
<thead>
<tr>
<th>Dose of IL-1 (μg)</th>
<th>CSF Titer* (± SEM)</th>
<th>Dose/Total*</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>&lt;20</td>
<td>15/16</td>
<td>6</td>
</tr>
<tr>
<td>0.05</td>
<td>1,430 ± 450</td>
<td>14/19</td>
<td>26</td>
</tr>
<tr>
<td>0.10</td>
<td>2,140 ± 520</td>
<td>12/20</td>
<td>40†</td>
</tr>
<tr>
<td>0.30</td>
<td>3,220 ± 570</td>
<td>5/20</td>
<td>75†</td>
</tr>
<tr>
<td>0.50</td>
<td>2,880 ± 280</td>
<td>2/12</td>
<td>83†</td>
</tr>
</tbody>
</table>

*The results are the means (± SEM) of three experiments using pooled serum from three mice per group. Radioprotection results are derived from two experiments (950 cGy).

†p < .05 compared with saline controls.

Radioprotection increases more gradually, with 0.1 pg of IL-1 conferring 40%; 0.3 μg, 75%; and 0.5 μg, 83% protection. A dose of 0.05 μg of IL-1 conferred only limited radioprotection, but a significant increase in circulating CSF.

The effect of 35F5 antibody on levels of CSF in circulation. To determine if induction of CSF with IL-1 was also blocked by 35F5, we used IL-1 in concentrations of 0.1 μg and 0.5 μg, doses just above and below those used in the radioprotection experiments (0.3 μg). Table 4 shows that both 0.1 μg and 0.5 μg of IL-1 induced high titers of CSF in circulation. The use of 35F5 antibody reduced CSF in a dose-dependent manner: 30 μg of 35F5 reduced by 16% the titers that were induced by a 0.5-μg dose, and reduced by 52% the titers induced by a 0.1-μg dose of IL-1. Thus, we assume that the 35F5 reduction of CSF induced with 0.3 μg of IL-1 is within the above-mentioned percentage range. Thirty micrograms of the 35F5 antibody with 0.1 μg of IL-1 reduced titers of CSF to levels comparable with those induced with 0.05 μg of IL-1 given alone. The same dose of antibody (30 μg) used with 0.5 μg of IL-1 reduced CSF to a level similar to that induced with 0.1 μg of IL-1.

The effect of 35F5 antibody on levels of circulating IL-6. Our previous work established that IL-1 induces high titers of IL-6 in circulation within 2 to 4 hours after injection. We examined the effect of 35F5 antibody on the IL-1-induced increase in serum IL-6. Low doses of 35F5 (30 μg) resulted in 96% and 98% reductions in IL-6 titers at 0.5 μg and 0.1 μg of IL-1, respectively (Table 4). Thus, we observed a more complete abrogation in IL-6 than in CSF.

Table 4. Effect of Anti-IL-1 Receptor Antibody 35F5 on IL-1-Induced CSF Production

<table>
<thead>
<tr>
<th>Treatment (μg)</th>
<th>CSF Titer (± SEM)</th>
<th>Reduction (%)</th>
<th>IL-6 Titer (± SEM)</th>
<th>Reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1 (0.1)</td>
<td>2,300 ± 347</td>
<td>—</td>
<td>7,940 ± 4,860</td>
<td>—</td>
</tr>
<tr>
<td>IL-1 (0.5)</td>
<td>2,885 ± 278</td>
<td>—</td>
<td>44,800 ± 6,400</td>
<td>—</td>
</tr>
<tr>
<td>IL-1 (0.1) + 35F5 (30)</td>
<td>1,110 ± 330</td>
<td>52</td>
<td>132 ± 68</td>
<td>98</td>
</tr>
<tr>
<td>IL-1 (0.5) + 35F5 (30)</td>
<td>2,412 ± 97</td>
<td>16</td>
<td>2,110 ± 1,090</td>
<td>96</td>
</tr>
<tr>
<td>IL-1 (0.1) + 35F5 (150)</td>
<td>160 ± 70</td>
<td>95</td>
<td>&lt;100</td>
<td>100</td>
</tr>
<tr>
<td>IL-1 (0.5) + 35F5 (150)</td>
<td>1,160 ± 725</td>
<td>60</td>
<td>1,310 ± 290</td>
<td>97</td>
</tr>
<tr>
<td>Control</td>
<td>&lt;20</td>
<td>—</td>
<td>&lt;100</td>
<td>100</td>
</tr>
</tbody>
</table>

*CD2F1 mice were bled 2 to 3 hours after IL-1 injection and serum was assayed for the presence of CSF or IL-6 (using the IL-6-dependent hybridoma B9). The results represent the means (± SEM) of three experiments using pooled serum from 3 to 5 mice in each experiment.

The type of IL-1 receptor on these cells has not been determined. Therefore, the effect of 35F5 antibody on IL-1-induced production of CSF and IL-6 by stromal cells was examined in vitro. IL-1—induced production of IL-6 and CSF was found to be reduced in the presence of 35F5 antibody (Table 5). Rat IgG, alone or in the presence of IL-1, did not affect CSF or IL-6 production (results not shown). These results show that 35F5 anti-IL-1 receptor antibody blocks stromal cell responses to IL-1, indicating that these cells express receptors similar to those on EL-4 cells.

DISCUSSION

Anti–IL-1 receptor antibody (35F5) administered to mice before irradiation with 825 cGy rendered them more susceptible to the lethal effects of radiation. This result provides the first direct evidence that endogenously produced IL-1 plays an important role in protection from radiation injury. It has been observed previously that radiation induces production of IL-1,23-26 In view of the present results, the presence of endogenously produced IL-1 after irradiation may represent a natural defense mechanism of the host against damage from ionizing radiation. Furthermore, our previous finding that treatment with pharmacologic doses of IL-1 (10 μg/mouse) after irradiation protects mice from death,27 suggests that insufficient amounts of IL-1 may be produced endogenously because an additional exogenous supply of IL-1 is required for a greater degree of protection.

Figure 1 summarizes the effects of 35F5 on radioprotection (Table 2) and on serum CSF and IL-6 production (Table 4). Anti-IL-1 receptor antibody 35F5 administered to mice before injection of recombinant IL-1 reduced the radioprotective effects of IL-1 from 81% survival to 8% survival. Increasing doses of antibody (from 30 μg to 150 μg) had no additional effect. However, the same dose of antibody (30 μg) that blocked the radioprotective effect of IL-1 resulted in a comparatively small (between 16% and 50%) reduction in the titers of circulating CSF. Thus, despite the remaining high titers of circulating CSF, 92% of IL-1-treated mice given antibody succumbed to radiation-induced lethality. Increasing the dose of antibody to 150 μg reduced the CSF production, which was induced by 0.1 μg of IL-1, by 95%. Therefore, it appears that higher doses of 35F5 antibody can block the appearance of CSF in the circulation.

The 30-μg dose of 35F5 antibody not only blocked IL-1–induced radioprotection, but also greatly reduced the level of IL-6 in circulation. These results suggest that cells with
Table 5. Inhibition of IL-1 Stimulated Release of IL-6 and CSF From Bone Marrow Stromal Cells by 35F5 Antibody

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>IL-1 (U/mL) (± SD)</th>
<th>CSF (U/mL) (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−−</td>
<td>60 ± 20</td>
<td>1,650 ± 850</td>
</tr>
<tr>
<td>−+</td>
<td>60 ± 20</td>
<td>1,425 ± 825</td>
</tr>
<tr>
<td>++</td>
<td>22,400 ± 4,200</td>
<td>5,125 ± 875</td>
</tr>
<tr>
<td>+−</td>
<td>180 ± 60</td>
<td>2,075 ± 475</td>
</tr>
</tbody>
</table>

*Bone marrow stromal cell cultures were incubated with 130 pg/mL IL-1 and/or 100 μg/mL 35F5 antibody. Culture supernatants were collected after 72 hours, and IL-6 and CSF were determined as described in Materials and Methods. Rat IgG in doses equivalent to 35F5 was used as control and had no effect. Results are the average from two experiments.

identical IL-1 receptor participate in radioprotection and IL-1–induced IL-6 release. Although combined therapy with IL-1 and IL-6 achieved synergistic radioprotection, a close correlation of radioprotection and the presence of IL-6 in circulation does not necessarily indicate a role of circulating IL-6 in radioprotection. The importance for radioprotection of serum versus tissue levels of cytokines, such as IL-6 or CSFs, requires further analysis.

It is interesting to note that, in culture, 35F5 interacted only with the type of IL-1 receptor present on T lymphocytes and fibroblast cells, but not with the second type of IL-1 receptor detected on B cells, neutrophils, and myeloid cells. In view of these findings, the nearly complete elimination of circulating IL-6 and, at high concentration of 35F5, of CSF, is surprising because macrophages are known to be a source of G-CSF, GM-CSF, and IL-6, and are not expected to be blocked by this antibody. One possible explanation for the observed blocking of IL-6 and CSF may be based on the negligible contribution of macrophages to the levels of IL-6 and CSF in circulation. Another explanation may be based on the need for a second factor, in addition to IL-1, to stimulate the macrophages.

The fact that IL-6 has been shown to synergize with a variety of CSFs to enhance colony growth of specific progenitor populations may underlie the apparent preferential inhibition of 35F5 on IL-6 versus CSF activity. The whole serum, which presumably contained a mixture of CSFs, may have shown reduced colony-forming activity, not due to decreased production of CSF, but rather due to elimination of synergizing IL-6. Alternatively, a specific species of CSF may have been affected by treatment. Clearly, the use of CSF-type specific antibodies will be necessary to determine whether a lower dose of 35F5 antibody blocks only the release of selected hematopoietic growth factors.

Our results show that an increase of CSF in circulation was initially a function of the dose of IL-1, but reached a plateau at doses exceeding 0.1 μg of IL-1 (Tables 3 and 4), with differences not observed at dose increases ranging from 0.1 to 0.5 μg. In contrast, radioprotection was dose-dependent in a more gradual fashion, with 0.05-μg doses conferring protection to 25% of mice, 0.1 μg protecting 40%, and 0.3 to 0.5 μg protecting 75% to 83% of mice. Comparison of the results leads to the conclusion that the mere presence of high titers of CSF in circulation is not sufficient for radioprotection (Tables 2 through 4). This conclusion is also supported by the observation that IL-1–induced radioprotection is strain-specific, whereas IL-1–induced CSF is observed in all mouse strains.

The finding that 35F5 antibody blocks the release of IL-6 and CSF by IL-1–stimulated stromal cells suggests that stromal cells express T-type IL-1 receptors. Hence, 35F5...
may directly block CSF and IL-6 production in the bone marrow. 35F5 may also have an indirect effect on bone marrow cells, as indicated by the observation that in IL-1-treated mice, 35F5 greatly reduces eflux of neutrophils (Benjamin et al, unpublished results, 1989). As indicated above, neutrophils are not blocked by 35F5 from binding IL-1. Thus, it appears that release of other IL-1-induced factors, such as IL-8, a chemoattractant of neutrophils, may be blocked or reduced by 35F5.

It is notable that, in vitro, stromal cells produce CSF in substantial quantities within 72 hours, even in the absence of IL-1 (Table 5). However, addition of IL-1 results in much higher CSF titers, which are decreased to background levels by 35F5. This leads to the conclusion that 35F5 blocks IL-1-induced CSF production. These results support the hypothesis that IL-1 stimulates bone marrow stromal cells to produce other cytokines, such as IL-6 and CSF, which synergize with IL-1 to stimulate bone marrow progenitor cells and thus counteract the damaging effects of lethal irradiation.

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In vivo modulation with anti-interleukin-1 (IL-1) receptor (p80) antibody 35F5 of the response to IL-1. The relationship of radioprotection, colony-stimulating factor, and IL-6

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