Myeloma Cells Express Fas Antigen/APO-1 (CD95) but Only Some Are Sensitive to Anti-Fas Antibody Resulting in Apoptosis

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To find out which cytokines are involved in the pathogenesis of multiple myeloma, we investigated cytokine receptor expression on myeloma cells using a panel of monoclonal antibodies (MoAbs). Flow cytometric analysis of five myeloma cell lines (RPMI8226, ARH77, KMM-1, U266, and Hs) and myeloma cells freshly isolated from eight patients showed that interleukin-1 receptor (IL-1R) type I and type II, IL-2Rα and β chains, IL-4R, IL-6R, IL-7R, IL-8R, granulocyte macrophage colony-stimulating factor receptor (GM-CSFR), c-kit (stem cell factor receptor [SCFRI]), membrane bound stem cell factor (MBSCF), and tumor necrosis factor (TNF) receptors type I and type II were not always detected on the myeloma cells. However, interferon-γ receptor, gp130, and Fas antigen were constitutively expressed, except one sample. To determine the role of Fas antigen on myeloma cells, these cells were cultured with anti-Fas MoAb. Apoptotic changes characterized by loss of cell volume, membrane blebbing, fragmentation of nuclei, and condensed chromatin were observed in three of five myeloma cell lines. When bcl-2 expression was examined, it was seen in all the cell lines regardless of the sensitivity to anti-Fas MoAb. Furthermore, anti-Fas MoAb not only induced apoptosis of freshly isolated myeloma cells but also inhibited the DNA synthesis, although such effects varied from patient to patient. The data indicate that only some myeloma cells undergo apoptosis in response to the signal mediated by the Fas antigen.

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Multiple Myeloma is a B-cell neoplasia characterized by slow growth and accumulation of myeloma cells. Some cytokines have been reported to be involved in the pathogenesis of this disease. Interleukin-(IL)-6 is known as a potent growth factor of human myeloma cells. IL-1α, IL-1β, and tumor necrosis factor α (TNFa), and granulocyte macrophage colony-stimulating factor (GM-CSF) can accelerate growth of myeloma cells through IL-6. In addition, IL-1, TNF, and IL-6 are responsible for the progressive bone resorption characteristic of this disease. IL-3 has also been identified as a proliferation and differentiation factor of myeloma cells synergistically with IL-6. On the other hand, Anderson et al reported that myeloma cells neither proliferate nor secrete Ig in response to GM-CSF, granulocyte colony-stimulating factor, macrophage colony-stimulating factor, IL-1α, IL-1β, IL-2, or IL-4. For further study of cytokines involved in the pathogenesis of multiple myeloma, we used a panel of MoAbs against cytokine receptors to investigate the expression of cytokine receptors on both human myeloma cell lines and myeloma cells freshly isolated from patients. Immunofluorescence analysis showed that all the tested cell lines and freshly isolated myeloma cells expressed interferon-γ receptor (IFN-γR), gp130, and Fas antigen, except one fresh sample. IFN-γ was previously reported to inhibit growth of myeloma cells in vitro, while gp130 is an essential signal transducing molecule for IL-6 to stimulate myeloma cell growth. However, the role of Fas antigen on myeloma cells is not clear.

Fas antigen (identical to APO-1) is a cell surface protein, which belongs to the protein family of TNF receptors (TNFRs), nerve growth factor receptor, and human B cell antigen CD40. Fas antigen/APO-1 was recently assigned CD95. It can mediate apoptosis of various human cells including myeloid cells, T and B lymphoblastoid cells, and diploid fibroblasts. Therefore, to determine whether or not Fas antigen on myeloma cells is able to transduce a signal for apoptosis, we performed morphologic analysis and cell proliferation analysis on myeloma cells stimulated by anti-Fas MoAb. Furthermore, we examined the correlation between the bcl-2 expression, which has been shown to prolong the survival of various hematopoietic cells, and the anti-Fas MoAb-induced apoptosis in myeloma cells.

Materials and Methods

Isolated myeloma cells and myeloma cell lines. Human myeloma cells were isolated from eight patients. The profiles of the patients are shown in Table 1. Seven samples (P-1, P-2, P-3, P-4, P-5, P-6, and P-7) were freshly isolated from patients, and one sample (P-8) was once transplanted into a severe combined immunodeficiency (SCID) mouse and harvested from a fully developed tumor of myeloma 6 weeks later. P-1 was a patient with plasma cell leukemia, and P-2 and P-8 were patients with extramedullary plasmacytomas. Bone marrow samples were collected by aspiration from the posterior iliac crest or sternum into heparinized tubes after obtaining informed consent, and myeloma cells were enriched by means of Iwato’s method with some modification. In brief, bone marrow mononuclear cells were isolated by ficoll-paque (Pharmacia, Uppsala, Sweden) centrifugation and rosetted with sheep red blood cells (RBCs) (E-rosetting). Non-E rosetting cells were fractionated on a discontinuous density gradient, which consisted of 2 mL of 90%, 60%, 50%, 45%, and 30% Percoll solutions (Pharmacia), and the cells were labeled with mixture of anti-CM, anti-CD8, anti-CD33, anti-CD20, and anti-CD14, and anti-CD15 MoAb, followed by the depletion using goat antimouse immunoglobulin (lg)-coated flasks (AIS, Santa Clara, CA).

Five human myeloma cell lines, RPMI8226, ARH77, KMM-1,
U266, and Hs, provided by Dr. J. Minowada (Hayashibara Biochemical Laboratories, Inc, Okayama, Japan) were also used for analysis in this study.

**Immunofluorescence analysis.** To analyze the expression of cytokine receptors on myeloma cells, the following MoAbs provided by the Fifth International Workshop and Conference on Human Leukocyte Differentiation Antigens were used: anti-IL-1 receptor (IL-1R) type I MoAb (hIL-1R-M1), anti-IL-1R type II MoAb (hIL-1R-M22), anti-IL-2R chain MoAb (B-10), anti-IL-2Rb chain MoAb (TU27), anti-IL-4R MoAb (hIL-4R-M57), anti-IL-6R MoAb (PM1), anti-gp 130 MoAb (AM64), anti-IL-7R MoAb (hIL-7R-M20), anti-IL-8R MoAb (B-F25), anti-IL-1R MoAb (hIL-1R-M1), anti-CD38 MoAb (YB5B8), anti-membrane bound stem cell factor (MBSCF) MoAb (4B10), anti-TNF type I MoAb (hr-9), anti-TNF type II MoAb (utr-1), anti-Fas antigen MoAb (Anti-Fas), and anti-IFN-γR MoAb (GIR-208). Mouse IgG and IgM (Cappel, Durham, NC) with irrelevant specificity were used as negative controls. Affinity purified goat antimouse Ig antibody conjugated with fluorescein isothiocyanate (FITC), which had been absorbed with solid phase human serum protein, was used as a developing reagent (Zymed Laboratories, Inc, San Fransisco, CA).

The cells were incubated with each of the MoAbs for 20 minutes, followed by culture with anti-Fas MoAb (100 ng/mL) at 39°C. and 18 hours after addition of anti-Fas MoAb. Freshly isolated myeloma cells were analyzed at 0, 2, and 18 hours. The anti-Fas MoAb was purchased from MBL Hitoecline (Nagoya, Japan). One hundred nanograms per milliliter of this MoAb induced apoptosis of the A673 cells pretreated with IFN-γ at 38.5°C. For the possible enhancement of the cytoidiac action of anti-Fas MoAb, cells were treated with 200 IU/mL recombinant human IFN-γ (Hayashibara Biochem Lab, Okayama, Japan) for 24 hours at 37°C followed by culture with anti-Fas MoAb (100 ng/mL) at 39°C, and then analyzed as described above. More than 1,000 cells were counted independently by two investigators under a light microscope after staining with the May-Grunwald-Giemsa method. In the same experiment, the percentages of viable cells were determined by trypan blue dye exclusion.

**Cell proliferation assay.** DNA synthesis of freshly isolated myeloma cells stimulated by anti-Fas MoAb was measured with the [3H]-thymidin (TdR) incorporation method. Briefly, 1 x 10^6 cells were cultured for 72 hours in 200 μL of RPMI-1640 with 10% FCS in 96-well plates in the presence or absence of 100 ng/mL anti-Fas MoAb. The cells were labeled with 18.5 kBq/well of [3H]-thymidin (370 GBq/mmol, Dupont, DE) in the last 16 hours of the culture and harvested for counting. The effect of IL-5, stem cell factor (SCF) and GM-CSF on the DNA synthesis was also analyzed at the same time.

### RESULTS

**Expression of cytokine receptors.** To identify cytokine receptors expressed on myeloma cells, we performed flow cytometric analysis of both human myeloma cell lines and freshly isolated myeloma cells using a panel of MoAbs against cytokine receptors. Most of the fresh samples were purified highly enough for the detection of cytokine receptors on myeloma cells by single-color immunofluorescence analysis. Furthermore, to exclude contaminated nonmyeloma cells, two-color immunofluorescence analysis using a combination of FITC-conjugated anti-CD38 MoAb and biotinylated anticytokine receptor MoAb in conjunction with PE-labeled streptavidin was performed because all the freshly isolated myeloma cells were CD38-strong positive and distinguishable from nonmyeloma cells, as reported earlier.

**Table 1.** 3H-TdR Incorporation of Isolated Myeloma Samples Cultured With Anti-Fas MoAb

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex/Age</th>
<th>Stage</th>
<th>Isotype</th>
<th>% Purity*</th>
<th>Anti-Fas MoAb (cpm)</th>
<th>(%) (cpm)</th>
<th>% Inhibition†</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-1</td>
<td>F/74</td>
<td>IIa</td>
<td>IgG-κ</td>
<td>70</td>
<td>836 ± 53</td>
<td>3,621 ± 331</td>
<td>76.9</td>
</tr>
<tr>
<td>P-2</td>
<td>M/61</td>
<td>IIa</td>
<td>NS</td>
<td>95</td>
<td>17,487 ± 3,216</td>
<td>20,169 ± 896</td>
<td>37.8</td>
</tr>
<tr>
<td>P-3</td>
<td>F/37</td>
<td>IIa</td>
<td>IgG-λ</td>
<td>83</td>
<td>12,065 ± 85</td>
<td>11,665 ± 703</td>
<td>0.0</td>
</tr>
<tr>
<td>P-4</td>
<td>M/45</td>
<td>IIb</td>
<td>IgG-κ</td>
<td>82</td>
<td>1,425 ± 145</td>
<td>3,245 ± 155</td>
<td>57.4</td>
</tr>
<tr>
<td>P-5</td>
<td>M/46</td>
<td>IIb</td>
<td>IgA-κ</td>
<td>88</td>
<td>15,571 ± 848</td>
<td>21,681 ± 1,038</td>
<td>9.7</td>
</tr>
<tr>
<td>P-6</td>
<td>M/57</td>
<td>IIb</td>
<td>IgA-λ</td>
<td>99</td>
<td>88,666 ± 607</td>
<td>91,691 ± 2,201</td>
<td>3.3</td>
</tr>
<tr>
<td>P-7</td>
<td>F/54</td>
<td>IIa</td>
<td>IgG-κ</td>
<td>99</td>
<td>137,052 ± 1,474</td>
<td>149,047 ± 1,969</td>
<td>8.0</td>
</tr>
<tr>
<td>P-8</td>
<td>F/42</td>
<td>IIb</td>
<td>NS</td>
<td>99</td>
<td>67 ± 4</td>
<td>74,790 ± 68</td>
<td>99.9</td>
</tr>
</tbody>
</table>

Abbreviation: NS, nonsecretory multiple myeloma.

*Percentage of myeloma cells after purification.
†[3H-TdR incorporation with anti-Fas MoAb(−)] x 100.
The result of immunofluorescence analysis for 16 kinds of cytokine receptors is summarized in Table 2. IFN-γR, gp130, and Fas antigen were expressed on all the myeloma cells tested, except for P-5. Specifically, Fas antigen was strongly expressed as shown in Fig 1. IL-6R was detected on RPMI8226, ARH77, U266, Hs cell lines, and freshly isolated myeloma cells from seven of eight patients. Although IL-6 is a potent growth factor of some myeloma cells, growth of the cells expressing IL-6R did not necessarily depend on IL-6 (data not shown). IL-1R type I, IL-2Rα chain, IL-7R, GM-CSFR, c-kit, MBSCF, and TNFR type I could be detected only on a minority of the myeloma cell lines and freshly isolated myeloma cells. IL-1R type II, IL-2Rα chain, IL-4R, IL-8R, and TNFR type I were not detectable on any myeloma cells tested. Fas antigen belongs to the protein family of TNFRs and nerve growth factor receptors and both Fas antigen and TNFR type I transduce an apoptosis signal in various human cells. However, myeloma cells express only Fas antigen on their cell surface.

Anti-Fas MoAb induced apoptosis of myeloma cells. Anti-Fas MoAb is reported to induce apoptosis of the cells expressing Fas antigen, and immunofluorescence analysis showed that Fas antigen was expressed on all the myeloma samples, except one, that we analyzed. Thus, to determine whether or not Fas antigen on myeloma cells is able to induce apoptosis, RPMI8226, ARH77, KMM-1, U266, and Hs cell lines, as well as freshly isolated myeloma samples, were cultured with anti-Fas MoAb. In the RPMI8226 cell line, the start of the morphologic changes of apoptosis characterized by loss of cell volume, membrane blebbing, fragmentation of nuclei, and condensed chromatin was observed at 60 minutes after the stimulation with 100 ng/mL anti-Fas MoAb. The proportion of the cells with apoptosis gradually increased during the culture, and most of the cells were dead at 18 hours (Fig 2A-B and Fig 3). Similar morphologic changes were also observed in the ARH77 and KMM-1 cell lines. On the other hand, no more than 5% of the U266 or Hs cells showed apoptotic change through an 18-hour incubation (Fig 3), although these cell lines express Fas antigen on their cell surface similarly to the other cell lines. These cells proved to be resistant against even a 100 times higher dose of anti-Fas MoAb (10 μg/mL). In the freshly isolated samples, the morphologic changes of apoptosis were observed in various degrees. P-3 did not undergo apoptosis at all (Fig 2I and J), while P-8 died of apoptosis within 2 hours (Fig 2E) and no viable cell was observed at 18 hours (Fig 2F). Because the cytotoxic action of anti-Fas MoAb on target cells was enhanced when the
Fas-MEDIATED APOPTOSIS OF MYELOMA CELLS

Fig 3. Time course of cell death induced by anti-Fas MoAb. RPMI8226 and Hs cell lines were incubated with 100 ng/mL anti-Fas MoAb or control IgM MoAb at 37°C for 0 to 18 hours and viable cells were determined by trypan blue dye exclusion. Note that viability of RPMI8226 cells stimulated by anti-Fas MoAb (O—O) gradually decreased during the 18-hour culture in comparison with control IgM (O—O). However, Hs cells survived in the presence of anti-Fas MoAb (■—■) or control IgM MoAb (□—□).

Myeloma cells were pretreated with IFN-γ or incubated with the factors at a higher temperature. U266 and Hs cells were pre-treated with IFN-γ for 24 hours, followed by incubation with anti-Fas MoAb at 39°C. U266 and Hs cells, however, did not undergo apoptosis even after treatment with IFN-γ and anti-Fas MoAb at 39°C, suggesting some myeloma cells are not susceptible to the cytocidal action of anti-Fas MoAb.

bcl-2 protein expression in myeloma cell lines. To examine whether bcl-2 expression in responsive to anti-Fas MoAb-induced apoptosis, we analyzed intracytoplasmic bcl-2 protein. As shown in Fig 4, U266 and Hs cells expressed bcl-2. However, RPMI8226, ARH177, and KMM-1 cells, which underwent apoptosis in response to anti-Fas MoAb, also expressed bcl-2 at similar levels found in U266 and Hs cells, so that no correlation was found between the expression of bcl-2 protein and the sensitivity to anti-Fas MoAb.

Anti-Fas MoAb inhibited the proliferation of isolated myeloma cells. Because the proportions of the cells with anti-Fas MoAb-induced apoptosis defined by May-Grünwald-Giemsa stain were different in each freshly isolated sample, the cytoidical effect of anti-Fas MoAb on freshly isolated myeloma cells was confirmed by ³H-TdR incorporation. Anti-Fas MoAb inhibited ³H-TdR incorporation of these cells, but the inhibitory effect varied from patient to patient as shown in Table 1. The maximum growth inhibition was 99.9%, observed in the cells from P-8, but this sample was not directly isolated from a patient as described in Materials and Methods. The minimum growth inhibition was 0% in the cells from patient P-3. The average inhibitory effect was 27.6% ± 28.0 (n = 7), except for P-8. No correlation was observed in any sample between the ability of spontaneous proliferation and the growth inhibitory effect, nor was any correlation observed between the purity of myeloma cells and the growth inhibitory effect. In this study, GM-CSF did not stimulate the growth of these purified cells, confirming that few proliferating myeloid cells were contaminated and that the ³H-TdR incorporation probably reflected the myeloma cell proliferation.

DISCUSSION

Immunofluorescence analysis using a panel of MoAbs against cytokine receptors has suggested that some cytokines may participate in the pathogenesis of multiple myeloma, although fluorescence-activated cell sorter (FACS) analysis may not be sensitive enough for the detection of minimal numbers of functional cytokine receptors. It is natural that IL-6R and its signal transducer, gp130, are expressed on the majority of the cells, because IL-6 is a potent growth factor for myeloma cells. IFN-γR was also detected on all the cells tested, and Portier et al. reported that IFN-γ inhibited the myeloma cell growth through the downregulation of IL-6R. However, IL-1R type II, IL-2Ra chain, IL-4R, IL-8R, and TNFR type I were not detected at all, while IL-1R type I, IL-2R β chain, IL-7R, GM-CSFR, c-kit (stem cell factor receptor [SCFR]), MBSCF, and TNFR type II were detected on the minority of myeloma cells. These various patterns of cytokine receptor expression suggest the heterogeneity of multiple myeloma. Among these receptors, we observed that the myeloma cells expressing c-kit could proliferate in response to SCF in vitro (data not shown). Taken together with the evidence that SCF is abundantly produced by marrow stromal cells, this finding may be one of the reasons why multiple myeloma develops primarily in bone marrow.

Fas antigen, identical to APO-1, was expressed by all the myeloma cells, including plasmacytoma and plasma cell leukemia cells, except for one sample, while Möller et al. reported that APO-1 was not detected on plasma cells in lymph nodes. This may represent a difference between malignant and normal plasma cells. While Fas antigen could not be detected on P-5 cells, no significant difference was observed in his personal profile including clinical course, stage, sex, or age.

Next, we addressed the question whether myeloma cells were susceptible to Fas antigen-mediated apoptosis in vitro.

Fig 2. Morphologic analysis of myeloma cells stimulated by anti-Fas MoAb. RPMI8226 cell line and freshly isolated samples (P-3 and P-8) cultured with anti-Fas MoAb were examined by light microscopy after staining with May-Grünwald-Giemsa. RPMI8226: (A) anti-Fas MoAb (2 hours), (B) anti-Fas MoAb (18 hours), (C) control IgM (2 hours), (D) control IgM (18 hours), P-3: (E) anti-Fas MoAb (2 hours), (F) anti-Fas MoAb (18 hours), (G) control IgM (2 hours), (H) control IgM (18 hours), P-8: (I) anti-Fas MoAb (2 hours), (J) anti-Fas MoAb (18 hours) (original magnification × 1,000). P-3 cells cultured with control IgM were not shown because no difference was observed. Note that (A) and (E) contain apoptotic cells with membrane blebbing, condensed and fragmented nuclei. (B) and (F) contain the cells with further morphologic changes after apoptosis.
Log Fluorescence Intensity

Fig 4. *bcl-2* expression in the myeloma cells. Five myeloma cell lines were stained with FITC-conjugated anti-*bcl-2* or control IgG1. RPMI8226, ARH77, and KMM-1 cell lines, sensitive to anti-Fas MoAb, expressed *bcl-2* similarly to the U266 and Hs cell lines, which were resistant to anti-Fas MoAb. The unshaded histogram shows control staining.

Three (RPMI-8226, ARH77, and KMM-1) of five myeloma cell lines and some of the freshly isolated myeloma cells underwent apoptosis in response to anti-Fas MoAb. On the other hand, U266 and Hs cell lines were resistant against anti-Fas MoAb, although they expressed Fas antigen similarly to the other cell lines. Even a high dose of MoAb (10 μg/mL) and a higher culture temperature (39°C) could not induce apoptosis. Consequently, we examined the *bcl-2* protein expression in these cell lines, because the protein product of proto-oncogene *bcl-2* is reported to inhibit the apoptosis mediated by Fas antigen. As shown in Fig 4, the U266 and Hs cell lines, resistant to anti-Fas MoAb, expressed *bcl-2* protein. But the RPMI8226, ARH77 and KMM-1 cell lines sensitive to anti-Fas MoAb also expressed it, so that no correlation was observed between the susceptibility to anti-Fas MoAb and the *bcl-2* protein expression. The data indicate that *bcl-2* is not able to interfere with Fas antigen-mediated apoptosis in some myeloma cells. Further study is required to elucidate the mechanism for the acquisition of resistance against anti-Fas MoAb by the U266 and Hs cell lines. They may feature defects, especially in the signal-transducing domain in the cytoplasmic region of Fas antigen, or a blockade of apoptosis by molecules other than *bcl-2*. The existence of *bcl-x* or *Bax*, a *bcl-2*-independent regulator of apoptosis, was not examined in this study. This protein product might regulate Fas antigen-mediated apoptosis in some myeloma cells.

It is also interesting that freshly isolated myeloma cells strongly expressed CD38 antigen. Lagresle et al. reported that *bcl-2* was expressed in CD38- B cells but not detected in CD38+ B cells in the tonsil. The former cells proliferate and produce Igs with the proper stimulus, while the latter cells were only stimulated for DNA synthesis. Myeloma cells seem to have the characteristics of both subsets, and it may, therefore, be useful to identify the origin of multiple myeloma.

When we performed a proliferation assay of freshly isolated myeloma samples, anti-Fas MoAb inhibited their DNA synthesis in vitro, though the inhibitory effect varied from patient to patient (Table 1), reflecting the various proportions of apoptotic cells observed in the morphologic analysis of freshly isolated myeloma samples. Taken together with the data for cell lines, this finding suggests that freshly isolated myeloma samples might consist of two subpopulations in terms of apoptosis, even though they showed the same rearrangement pattern of Ig genes. This idea was supported by the observation that anti-Fas MoAb completely inhibited the growth of P-8. P-8 was harvested from a fully developed tumor in a SCID mouse into which fresh myeloma cells were transplanted. The complete inhibition may have been due to the selection of myeloma cells in the SCID mouse where sensitive cells may not be exposed to Fas ligand resulting in the accumulation of them. As shown in Table 1, freshly explanted myeloma cells are relatively resistant. For this study, only advanced stage myeloma cells were used to obtain highly purified cells. Therefore, it remains to be proven whether early stage myeloma cells undergo apoptosis.

Another question we wish to address here is how the myeloma cells sensitive to anti-Fas MoAb can survive in vivo. Several explanations can be given for it. (1) Apoptosis may, in fact, occur in vivo, but the cells may proliferate more rapidly than they undergo apoptosis. (2) Fibroblast conditioned medium decreased the apoptosis of human immunodeficiency virus (HIV) infected mononuclear cells, suggesting the possible existence of a factor that inhibited the apoptosis. The possible existence of a soluble form of the Fas antigen in vivo may interfere with the interaction...
between Fas antigen and its ligand.25 (3) Because most myeloma cells express CD38 antigen, a signal through this molecule may protect the cells from apoptosis.26 (4) Recently, cDNA of Fas ligand was cloned and expressed in activated splenocytes and thymocytes,27 which involve T-cell–mediated cytotoxicity.28 Another attractive explanation is that impaired T-cell–mediated cytotoxicity, including Fas ligand, may be involved in the pathogenesis of this disease.

In this report, we examined the expression of 16 cytokine receptors on human myeloma cell lines and freshly isolated myeloma cells. We first demonstrated that human myeloma cells expressed Fas antigen, which in turn could mediate apoptosis in only part of the myeloma cells. This evidence could be important, not only for understanding the mechanism of growth control, but also for determining a therapeutic approach for this disease. Additionally, it would be useful to understand normal plasma cell development, because myeloma cells with different characteristics reflect various developmental stages of normal plasma cells.

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is encoded by the SI locus and is the ligand of the c-kit receptor, the gene produce of the W locus. Cell 63:225, 1990


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