Disseminated Growth of a Human Multiple Myeloma Cell Line in Mice with Severe Combined Immune Deficiency

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ABSTRACT

We have successfully engrafted a human multiple myeloma cell line, ARH-77, into C.B. 17 SCID mice. When ARH-77 cells were injected s.c., tumors grew only at the site of inoculation (five of five). When ARH-77 cells were injected i.v., tumors did not grow in any of the mice (zero of five). However, when mice were given γ-irradiation with 150 rads and then inoculated i.v. with 10^7 ARH-77 cells, 100% (13 of 13) of the mice developed tumors. Hind leg paralysis was observed in 13 of 16 mice as a result of compression of the spinal cord by tumor. Histological analysis demonstrated that myeloma cells proliferated and formed osteolytic lesions (15 of 16) in the vertebrae and bones of the skull (14 of 16). Tumor cells also invaded the brain and meninges (14 of 16), lung (13 of 15), liver (seven of 15), and kidney (two of 15). Flow cytometric analysis demonstrated that the phenotype of 31% of the bone marrow cells in the vertebrae and 79% of s.c. tumor cells was similar to ARH-77 cells (CD38^+, PCA-1^+, HLA-Classes I and II^+). Furthermore, DNA hybridization with a human α-I probe confirmed their human origin. ARH-77-derived human immunoglobulin was detected in the serum of SCID/ARH-77 mice by ELISA. These observations demonstrate systemic involvement of human multiple myeloma following i.v. injection of ARH-77 cells into irradiated mice. This in vivo model should be useful for evaluating new therapeutic modalities for myeloma.

INTRODUCTION

Multiple myeloma is a malignant proliferation of plasma cells in bone marrow, manifested by skeletal destruction, bone fractures, the presence of circulating monoclonal immunoglobulins, Bence Jones proteinuria, impaired hematopoiesis, and renal disease. The disease accounts for about 10% of all hematological neoplasms (1), and the incidence appears to be increasing (2, 3). The activation of a series of oncogenes may govern the initiation and stepwise progression of this malignancy (4). Chemotherapy induces tumor regression in about 50% of patients and prolongs survival, but it fails to alter the course of the disease in a significant manner (4). Thus, the median survival of patients is 3 yr, and this has not improved markedly despite the addition of new drugs, such as doxorubicin, to the chemotherapeutic regimen (4, 5). Recent studies suggest that remissions and overall survival can be prolonged by maintenance therapy with interferon (4, 6). However, interferon is rarely of benefit to patients with resistant or relapsing disease (6). The use of supralethal doses of chemotherapy and total-body irradiation, followed by bone marrow rescue using syngeneic (7), allogeneic (8), or autologous (8), marrow or granulocyte-macrophage colony-stimulating factor (9), have failed to cure patients, although short-term remissions have been reported for patients refractory to other forms of treatment (8). However, even during complete remission, the M-protein can still be detected, and complete healing of lytic bone lesions is rare (9).

The evaluation of novel experimental therapeutic approaches for multiple myeloma has been hampered by the lack of an appropriate animal model. The pathophysiology of mouse plasmacytoma models is well characterized (10, 11) but is not directly applicable, because of the pattern of cell growth and the antigenic diversity between human and murine plasma cell dyscrasias.

The SCID mouse provides an attractive model for the study of a variety of human tumors, because SCIDs are severely deficient in functional B- and T-lymphocytes due to a defect in rearrangement of their antigen receptor genes (12, 13). Many human tumors that do not grow in nude mice grow in SCID mice (14). The s.c. or i.p. growth of human myeloma cell lines in SCID mice has been described (15, 16), but the cells do not grow in a disseminated manner analogous to primary multiple myeloma in humans, namely, in bone marrow.

In this paper, we describe the disseminated growth of a human myeloma cell line in the bone marrow, kidney, brain, and liver of irradiated SCID mice, resulting in a disease very similar to multiple myeloma in humans. The mice have a monoclonal gammapathy and succumb to paralysis following infiltration of tumor cells into the thoracolumbar vertebrae.

MATERIALS AND METHODS

Cell Line. The human myeloma cell line ARH-77 was originally established by Burk et al. (17) and was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 100 μg/ml of penicillin, and 100 μg/ml of streptomycin. Prior to inoculation into mice, ARH-77 cells were washed and suspended at an appropriate concentration in sterile PBS.

Mice. SCID (C.B. -17 SCID/SCID) mice, 6 to 8 wk of age, were obtained from our specific-pathogen-free colony. All procedures were performed in a laminar flow hood.

Irradiation and Inoculation. Recipient mice were exposed to 150 cGy (rads) of radiation from a γ source, at a dose rate of 75 cGy/min. Cells were inoculated into mice 24 h after total-body irradiation. For s.c. administration of tumors, 10^7 ARH-77 cells were injected into the flank of nonirradiated mice. Irradiated or nonirradiated mice were given a single i.v. injection of 0.1 to 10 × 10^6 cells in the tail vein.

Histopathology. Animals were sacrificed, and excised tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin prior to routine histopathological examination.

For blood smears, the air-dried peripheral blood smears from SCID mice were stained with a Baxter staining kit (Baxter, Miami, FL). Briefly, smears were fixed with Diff-Quik fixative (1.8 mg/liter of triazine dye mixture: 100% PDC:buffer:0.01% NaN3) for 5 s and then stained with Diff-Quik Solution I (1 g/liter of xanthene dye: 100% PDC:buffer) for 5 s. Slides were then rinsed in distilled H2O.

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3 The abbreviations used are: SCID, severe combined immunodeficiency; PBS, phosphate-buffered saline; PDC, pyridinium dichromate; ELISA, enzyme-linked immunosorbent assay; GAHgf, goat anti-human immunoglobulin; BSA, bovine serum albumin; IgG, immunoglobulin G; GAMgf, goat anti-mouse immunoglobulin; FITC, fluorescein isothiocyanate; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; NK, natural killer; ALL, acute lymphoblastic leukemia.
Human Immunoglobulin Assays. An ELISA was used to quantify levels of human immunoglobulin in the culture supernatant of ARH-77 cells and in the sera of SCID mice with ARH-77 tumors. Mice were bled on Days 18 and 33 after s.c. inoculation and or after i.v. inoculation when the hind legs became paralyzed. For testing tissue culture supernatants, the cells were cultured at 2 × 10^6/ml for 5 days in medium, and the supernatant was obtained following centrifugation and stored at -20°C. Ninety-six-well ELISA plates (Fisher, Plano, TX) were incubated with 50 μl/well of 20 μg/ml of GAHlg in PBS overnight at 4°C. The plates were washed 3 times with PBS, blocked with 2% BSA in PBS overnight at 4°C, rinsed 3 times with PBS, and incubated for 1 h at 37°C with either 50 μl/well of mouse serum diluted 1:1 in PBS or serially diluted samples of human immunoglobulin. The plates were again washed 3 times with PBS and incubated for 1 h at 37°C with biotin-labeled GAHlg (Tago, 1:800 in 0.5% BSA:PBS; Burlingame, CA). After three washes with PBS, the plates were incubated for 1 h at 37°C with avidin-biotin-alkaline phosphatase (Pierce, Rockford, IL). The plates were washed 6 times with PBS and then developed for 30 min at room temperature with phosphate substrate paranitrophenylphosphate (Sigma, St. Louis, MO). Fifty μl/ml of 3 M NaOH were then added to stop the reaction. The absorbance of each well was read at 405 nm in an ELISA reader (Model NJ-2000; Nippon Intermed K.K., Tokyo, Japan). The serum from normal SCID mice served as a negative control. Concentrations of human IgG were calculated from the standard curve.

For isotyping human immunoglobulin light chains in the sera of SCID mice, ELISA plates were coated with GAHlg (20 μg/ml), washed, and blocked, and diluted SCID sera or human immunoglobulin was added. After incubation and washing, the plate was incubated with mouse anti-human κ or anti-λ antibodies (Becton-Dickinson Immunocytometry Systems, Mountain View, CA), washed, and incubated with an alkaline phosphatase:GAHlg conjugate (Tago). The relative amount of bound enzyme conjugates in the wells was ascertained by the addition of phosphate substrate (Sigma). The reaction was stopped, and the absorbance was determined as described above.

Flow Cytometry. Mouse anti-human CD38 was purchased from Coulter Immunology (Hialeah, FL), anti-PCA-1 monoclonal antibody was a generous gift from Dr. K. Anderson (Dana Farber Cancer Center, Boston, MA), anti-HLA class I (W6/32) was kindly provided by Dr. P. Stastny (University of Texas Southwestern Medical Center, Dallas, TX), and anti-HLA-DR was a gift from Dr. P. Lipsky (University of Texas Southwestern Medical Center, Dallas, TX). The MOPC-21 myeloma protein was obtained from Cappel (West Chester, PA), and FITC-GAMlg (fluorescent isothiocyanate-labeled GAMlg) was obtained from Sigma (St. Louis, MO).

Spleens, subcutaneous tumors, and vertebral columns were removed and placed in RPMI-1640. Single-cell suspensions were prepared by teasing the tissues with a pair of scissors and filtering cells through a nylon mesh. For eliminating erythrocytes, the cell pellets were incubated for 4 min at 37°C in 0.5 ml of 0.8% ammonium chloride:0.01% KH₂PO₄ and then washed 3 times with PBS. Fresh cell suspensions were stained with appropriate amounts of test antibodies and FITC-GAMlg. Samples were analyzed on a fluorescence-activated cell sorter (Becton-Dickinson).

DNA Hybridization. Genomic DNA was extracted from fresh cell suspensions of vertebrae, spleens, and subcutaneous tumors as well as paraffin tissue sections of tissues from SCID mice. The fresh bone marrow, spleen, and subcutaneous tumor cell suspensions were lysed with 0.34 M lithium acetate.1mm EDTA:10 mm Tris-HCl (pH 8.0):74 mm lithium deoxyctydlate and extracted with phenol:chloroform. The DNA was concentrated by ethanol precipitation, washed twice with 70% ethanol, dried, and reconstituted in sterile water. For paraffin-embedded tissues, five sequential 10-μm-thick sections were dewaxed in xylene and rehydrated by passage through graded ethanol solutions. The remaining ethanol was evaporated under vacuum. The sections were resuspended in 100 μl of 0.1 mm Tris-HCl (pH 7.8):0.005 mm EDTA containing 0.05% SDS and digested at 50°C for 72 h with proteinase K (500 μg/ml). The DNA was extracted by phenol:chloroform, concentrated, washed, dried, and reconstituted in the same manner as fresh DNA.

DNA samples were diluted in 250 μl of EDTA buffer (0.4 mm NaOH: 10 mm EDTA) and denatured in boiling water for 10 min. Added to each sample were 250 μl of cold 2 x ammonium acetate, and samples were placed on ice. The samples were serially diluted, loaded onto a prewetted nitrocellulose membrane, and pulled through the wells of a dot blot apparatus (Biorad, Richmond, CA). The membrane was rinsed with a 2 x concentrated solution of SSC (3 x NaCl:0.3 x trisodium citrate), air dried for 15 min, and baked in a vacuum oven for 2 h at 80°C. DNA hybridizations were carried out using the denatured biotinylated human-specific DNA genome probe Afal (Blur 8) (Oncor, Gaithersburg, MD) in the presence of 6 x SSC buffer, 0.5% SDS, and 5 x Denhert's solution. The hybridized samples were washed, air and vacuum oven dried, and incubated for 5 to 10 min with a 1-μg/ml streptavidin-alkaline phosphatase conjugate solution (1 mg/ml of stock; BluGENE nonradioactive nucleic acid detection system; BRL, Gaithersburg, MD) after appropriate washings. The filter was developed with nitroblue tetrazolium:5-bromo-4-chloro-3-indolyl phosphate substrate solution for 5 to 30 min. It was then placed in a stop-reaction solution containing 20 mm Tris-HCl:0.5 mm EDTA and dried at 80°C in a vacuum oven for 1 to 2 min.

RESULTS

Growth of Subcutaneous ARH-77 Tumors. All five nonirradiated mice developed palpable tumors 9 to 11 days (mean, 9.4 days) after s.c. injection of 1 x 10⁶ human myeloma cells from the ARH-77 cell line. The tumors grew to a diameter of 12 cm² 21 days after the tumor appeared. At necropsy the tumors invaded surrounding tissues including muscle (Fig. 1A), but there were no metastases. The growing s.c. tumor could be successfully passaged by serial s.c. injections in SCID mice.

Disseminated Tumor Growth in SCID Mice. Preliminary experiments demonstrated that ARH-77 cells injected i.v. into nonirradiated SCID mice failed to grow (0 of 5) (Table 1). However, when 10⁷ tumor cells were injected i.v. into irradiated mice, 100% of mice (13 of 13) developed disseminated tumors with a mean latent period of 33.4 days. In addition, two of the three mice given injections i.v. of 10⁸ cells and one of the three mice given an injection of 10⁹ cells developed tumors. In mice with disseminated tumors, 81% (13 of 16) of the animals developed hind leg paralysis following infiltration of tumor cells into the thoracolumbar vertebrae and compression of the spinal cord. These mice died 2 to 7 days after the onset of paralysis. Histopathological observation confirmed that the mice had tumor cells in the vertebrae. The lumbar region was most severely affected; in contrast, the cervicothoracic vertebrae were often free of lesions. The mice also had tumor cells in the skull (14 of 16), brain and meninges (8 of 16), lung (13 of 15), liver (7 of 15), and kidney (2 of 15) (Table 1; Fig. 1). The myeloma cells caused osteolytic lesions in the vertebrae and the skull. Often, the bones of the calvarium were free of infiltrate, whereas the mandible was destroyed. The mandibular infiltrates were commonly unilateral, suggesting that only widely scattered inoculated cells survived and produced tumors. Tumor cells were not found in the peripheral blood.

Human Immunoglobulin in the Sera of SCID/ARH-77 Mice. The presence of human immunoglobulin in the supernatant of cultured ARH-77 cells and in the sera of mice given injections of tumor cells was confirmed by an ELISA assay (Table 2). Human immunoglobulin (3.50 μg/ml) was detected in the serum 18 days after s.c. inoculation (Table 2). In mice given i.v. injections of tumor cells, serum levels of human immunoglobulin were 4.37 μg/ml at the time when these mice had hind leg paralysis. The ARH-77 origin of the human immunoglobulin was confirmed by analysis of the light chain. Hence, both the culture supernatant and sera contained human immunoglobulin with κ L-chain.

Flow Cytometric Analysis of the Phenotype of ARH-77 Cells in SCID Mice. Data comparing the phenotype of cultured myeloma cells and tumor cells from xenografts are shown in Table 3 and Fig. 2. The original ARH-77 cells expressed human Class I and Class II antigens and the plasma cell markers CD38 and PCA-1. After transplantation into SCID mice, the tumor cells were phenotypically similar to the parent cells, comprising an average of 30.5% of the bone marrow cells in the vertebrae from mice given i.v. injections and 78.7% of s.c. tumor cells.
HUMAN MULTIPLE MYELOMA XENOGRAFTS IN SCID MICE

Fig. 1. ARH-77 myeloma xenografts in SCID mice. A, subcutaneous tumor (× 400); B, liver; myeloma cells have replaced hepatic parenchyma (× 200); C, vertebra; myeloma cells have replaced hematopoietic cells and surround a bony spicule (× 400); D, mandible; myeloma cells have replaced periodontal bone. The pulp of the tooth is necrotic (× 400); E, lung; myeloma cells form a subpleural plaque (× 400); F, cerebral cortex; myeloma cells expand the Virchow-Robin space (× 200).

DNA Hybridization. Dot blot DNA hybridization with the human specific genome probe AluI (Blur 8) confirmed the presence of myeloma cells in heterotransplanted SCID mice (Fig. 3). DNA from the ARH-77 cells hybridized with the probe as did s.c. ARH-77 tumor and bone marrow from mice given injections i.v. of ARH-77. DNA from the bone marrow and liver of control SCID mice was negative.

Taken together, these experiments show the presence of human myeloma cells in the tissues and bone marrow of irradiated mice given injections i.v. of ARH-77 cells.

DISCUSSION

The human myeloma cell line ARH-77 was derived from peripheral plasma cells of a patient with IgG plasma cell leukemia (17, 18). Heterotransplantation of these cells into nude mice has been unsuccessful using different routes of inoculation and mice of different ages (18). Intracerebral inoculation did, however, result in xenogeneic growth in nude mice (19). The profound immunodeficiency of SCID mice made it possible to establish a human multiple myeloma mouse model for further study of this disease. Thus, 1 × 10⁷ ARH-77 cells induced s.c. tumors in 100% of SCID mice. However, the s.c. myeloma grew only at the site of inoculation and did not metastasize. Similar results have been reported using s.c. inoculation of the human myeloma cell lines RPMI-8226 and U-266 (15), the Burkitt’s lymphoma Daudi (20), and BL-60 (21). Hill et al. (22) and Muller et al. (23) have reported that s.c. xenografts of fresh metastatic melanoma and a melanoma cell line spontaneously metastasized to organs such as lung, liver, spleen, and pancreas. Nomura et al. (24) observed that...
the growth of s.c. human tumors appears to depend on both the route of injection. SCID mice are more sensitive to irradiation due to the defective repair of DNA double-strand breaks (13). Although most investigators have irradiated mice with 400 rads to induce the growth of ALL cell lines and bone marrow samples from ALL patients (27, 29), we found that lower doses of irradiation (150 rads) prior to inoculation were sufficient to induce disseminated growth of the myeloma cell line.

Our experiments have also shown that cell dose is an important variable for tumor growth and survival of the heterotransplanted mice. The optimal dose of ARH-77 cells for i.v. inoculation is 1 x 10^7. This dose induced tumors in 100% of the mice. In contrast, when 10^6 or 10^5 cells were injected, tumors grew in only 33 and 67% of the mice, respectively. In addition, their latency period was longer, and survival was prolonged. Similar dose-dependent growth of lymphoma xenografts has been reported by Ghetie et al. (20).

Histopathological studies showed that, after i.v. injection, the myeloma cells proliferated in the bone marrow of vertebrae and the bones of skull, resulting in osteolytic bone lesions. Cells also grew in various organs including lung, liver, brain, and kidney. The tumor cells infiltrated thoracolumbar vertebrae and compressed the spinal cord resulting in hind leg paralysis. This growth pattern is similar to that of human multiple myeloma.

Flow cytometric analysis demonstrated that in vivo adaptation of ARH-77 did not result in any significant variation in cell surface markers of the s.c. growth of the human germinoma tumor YST-2 in SCID mice was rapid, and the frequency of spontaneous metastases was high. Hence, the growth of s.c. human tumors appears to depend on both the metastatic capacity of the tumor cells and the immune status of the host. Since the growth of s.c. myeloma in SCID mice does not mimic that of multiple myeloma in humans, we initiated studies to determine whether i.v. injection would result in myeloma growth in SCID mice in a manner similar to the human disease (25). Our studies indicate that the disseminated growth of ARH-77 cells occurs in irradiated SCID mice, but not in unirradiated mice. Other investigators (20, 26) have reported that some human hematological malignancies grow in a disseminated fashion in SCID mice following i.v. injection. However, in some instances prior irradiation of the mice was required (12, 27, 28). Fulop et al. (28) and Dick (29) have also demonstrated that prior irradiation of mice promoted stem cell engraftment and facilitated the reconstitution of immune function. Previous studies have suggested that SCID mice have normal macrophages, NK cells, and neutrophils capable of rejecting engrafted cells (13). Hence, the failure of engraftment in unirradiated mice may be due to the fact that tumor cells are susceptible to NK-mediated lysis and T-cell-independent macrophage activation. Sublethal irradiation would decrease the activity of these cells and enhance acceptance of xenografts (13, 29). SCID mice are more sensitive to irradiation due to the defective repair of DNA double-strand breaks (13). Although most investigators have irradiated mice with 400 rads to induce the growth of ALL cell lines and bone marrow samples from ALL patients (27, 29), we found that lower doses of irradiation (150 rads) prior to inoculation were sufficient to induce disseminated growth of the myeloma cell line.

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Flow cytometric analysis demonstrated that in vivo adaptation of ARH-77 did not result in any significant variation in cell surface markers.

| Table 1 Growth and distribution of human myeloma cells in SCID mice |
|-------------------|-------------------|-------------------|
| Route of injection | s.c. | l.v. |
|                   | Nonirradiated | Irradiated |
|                   | 10^2 | 10^3 | 10^3 | 10^6 | 10^6 |
| Latency period (days) | 9.4 | 33.4 | 28.5 | 50 |
| Hind leg paralysis | 0/5 | 0/5 | 1/13 | 1/13 | 0/3 |
| Vertebrae | 0/5 | 0/5 | 1/13 | 1/13 | 0/3 |
| Skull | 0/5 | 0/5 | 1/13 | 1/13 | 0/3 |
| Lung | 0/5 | 0/5 | 1/13 | 1/13 | 0/3 |
| Liver | 0/5 | 0/5 | 1/13 | 1/13 | 0/3 |
| Spleen | 0/5 | 0/5 | 1/13 | 1/13 | 0/3 |
| Kidney | 0/5 | 0/5 | 1/13 | 1/13 | 0/3 |
| Heart | 0/5 | 0/5 | 1/13 | 1/13 | 0/3 |
| Peripheral blood smear | 0/3 | 0/3 | 1/13 | 1/13 | 0/3 |

- a Number of cells injected.
- b Positive mice/total mice.
- c NT, not tested.

| Table 2 Human immunoglobulin in the sera of SCID/ARH-77 mice |
|-------------------|-------------------|-------------------|
| Sample | Human immunoglobulin (µg/ml) |
| ARH-77 supernatant | 1.17 ± 0.35 |
| Sera: mice given i.v. injections | 4.37 ± 1.40 |
| Sera: mice given s.c. injections | 3.50 ± 0.14 |
| Sera: normal SCID/control | <0.01 |
| Sera: normal BALB/c | <0.01 |

- a Cells (2 x 10^5)/ml for 5 days in medium.
- b Mean ± SD.
- c Serum obtained at the onset of hind leg paralysis.
- d Numbers in parentheses, number of mice tested.
- e Sera obtained 18 days after s.c. inoculation of 1 x 10^7 cells.

| Table 3 Flow cytometric analysis of cells in lymphoid tissues from SCID/ARH-77 mice. Two to five mice were tested. |
|-------------------|-------------------|-------------------|
| Staining antibody | Bone marrow | Spleen | s.c. tumor |
| ARH-77 | W6/32 | 91.47 | 28.34 | 0.74 | 0.46 | 1.34 | 76.99 | 1.74 | 0.75 | 0.52 |
| | PCA-1 | 84.20 | 20.91 | 1.56 | 1.90 | 3.08 | 21.75 | 1.85 | 1.36 | 0.10 |
| | CD38 | 51.79 | 8.67 | 1.75 | 0.49 | 1.90 | 6.87 | 3.89 | 1.96 | 0.52 |
| | HLA-DR | 92.30 | 30.52 | 0.55 | NT | NT | 78.73 | 0.91 | NT | NT |

- a Percentage of positive cells above that observed in control samples incubated with the MOPC-21 control.
- b MFI, mean fluorescence intensity of positive cells from tissue indicated.
- c NT, not tested.

Fig. 2. Flow cytometric analysis of fresh s.c. tumor cells and bone marrow cells from the vertebrae of SCID mice, stained with anti-human W6/32, PCA-1, CD38, HLA-DR antibodies, and irrelevant antibody MOPC-21: A, ARH-77 cells in vitro; B, s.c. ARH-77 tumor cells; C, bone marrow cells derived from a control SCID mouse; D, bone marrow cells derived from an SCID/ARH-77 mouse. ——, MOPC-21; --, CD38; ----, PCA-1; ——, W6/32; ■, HLA-DR.
The growth pattern of ARH-77 cells in SCID mice depends on the excellent recipient for the human multiple myeloma cell line ARH-77. The myeloma cells were of human origin.

In summary, these observations suggest that a SCID mouse is an excellent recipient for the human multiple myeloma cell line ARH-77. The growth pattern of ARH-77 cells in SCID mice depends on the route of inoculation, dose of injected cells, and prior irradiation. This in vivo model should be useful for studying human multiple myeloma and the evaluation of therapeutic agents.

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