Immunity to Growth Factor Receptor–Bound Protein 10, a Signal Transduction Molecule, Inhibits the Growth of Breast Cancer in Mice

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Abstract

This study describes the application of a unique strategy to identify breast cancer antigens [tumor-associated antigen (TAA)]. In a mouse model, the strategy led to the identification of growth factor receptor–bound protein 10 (Grb10) as a newly identified TAA. Grb10 is a signal transduction molecule associated with multiple transmembrane tyrosine kinase receptors. It was discovered by comparing microarrays of cellular breast cancer vaccines highly enriched for cells that induced breast cancer immunity in tumor-bearing mice with nonenriched vaccines. The vaccines were prepared by transferring a cDNA expression library derived from SB5b cells, a breast cancer cell line C3H/He origin (H-2^k), into LM mouse fibroblasts (H-2^k). As the transferred cDNA integrates spontaneously into the genome of the recipient cells, replicates as the cells divide, and is expressed, the vaccine could be prepared from microgram amounts of tumor tissue. Relatively few cells in the transduced cell population, however, incorporated cDNA fragments that included genes specifying TAA. (The vast majority specified normal cellular constituents.) A unique strategy was used, therefore, to enrich the vaccine for immunotherapeutic cells. Twenty genes were overrepresented in the enriched vaccines. One, the gene for Grb10, was ~100-fold overrepresented. To determine if Grb10 in the enriched vaccine was partly responsible for its therapeutic benefits, the gene was transferred into the fibroblast cell line, which was then used as a vaccine. Mice with established breast cancer treated solely by immunization with the modified fibroblasts developed robust immunity to the breast cancer cells, which, in some instances, was sufficient to result in tumor rejection. [Cancer Res 2008;68(7):2463–70]

Introduction

Each year, ~500,000 women worldwide develop breast cancer. This disease is the second leading cause of cancer-related death in women. The frequency of death of patients with metastatic breast cancer has remained essentially constant for the last 30 years in spite of more sensitive methods of detection, novel chemotherapeutic agents, and improved hormonal, surgical, and radiotherapeutic techniques (1). The 5-year survival is poor. New treatment options are urgently needed.

The potential benefits of immunotherapy as an adjunct to conventional forms of treatment of various types of cancer, including metastatic breast cancer, are well established (2–6). Activated CTLs, which recognize and can destroy cancer cells, are generated in patients receiving various forms of immune-based therapies. In most instances, the immunity is directed toward unique MHC class I–restricted tumor-associated antigens (TAA) expressed by the malignant cells.

Multiple TAAs were identified in various human malignancies by comparing microarrays of primary cancers or cancer cell lines with cells from the homologous nonmalignant tissue (7–10). Although numerous antigens have been identified by this method, there is no assurance that they are "therapeutic," in the sense that immunity to the identified antigen will result in rejection of the tumor. Another concern is that many cellular cancer vaccines, including vaccines prepared by transfer into dendritic cells of tumor extracts [mRNA (11, 12), tumor cell lysates (13, 14), apoptotic cell bodies (15, 16), among others (17)], may not contain sufficient numbers of cells that express TAA. It is likely that the vast majority of determinants expressed by cancer cells and by vaccines derived from the malignant cells are normal cellular constituents, which are unrelated to the induction of immunity to the neoplasm. In contrast, immunization with a vaccine highly enriched for cells that express TAAs that characterize the patient's cancer can result in enhanced anti-immune responses, improving its therapeutic benefits.

Relatively few tumor antigens, including antigens associated with breast cancer, have been identified. T-cell clones from cancer patients have been used to identify tumor antigens (18). Genetic mutations resulting in the aberrant expression or overexpression of determinants commonly expressed by nonmalignant cells seem to underlie their immunogenic properties. MUC1 (19, 20), HER2/neu (21), p53 (22, 23), and NY-BR-1 (24–26) are aberrantly expressed by the malignant cells of breast cancer patients. It is likely that these are only a few representations of an array of tumor antigens that characterize the malignant cell population. The cancer cell genome is notoriously unstable (27–29).

In prior reports (30), we described studies designed to enrich a cellular breast cancer vaccine for immunotherapeutic cells. The vaccine was prepared by transfer of a cDNA library from SB5b cells, a highly aggressive breast cancer cell line derived from an adenocarcinoma that arose spontaneously in the mammary gland of a female C3H/He mouse in our animal colony (H-2^k), into LM cells (H-2^k), a mouse fibroblast cell line. As the transferred cDNA segments integrate spontaneously into the genome of the recipient cells, replicate as the cells divide, and are expressed, sufficient cDNA to prepare the vaccine could be obtained from unusually small neoplasms. DNA isolated from as few as 10 million cells (4-mm tumor) was sufficient (31).

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In this report, we investigated a novel strategy for the discovery of breast cancer–associated antigens, which resulted in the identification of growth factor receptor–bound protein 10 (Grb10), a signal transduction molecule, as a breast cancer antigen. Grb10 is an adaptor protein that associates with multiple transmembrane tyrosine kinase receptors. The strategy consisted of enriching the cDNA-based cellular breast cancer vaccine for cells that induced therapeutic immune responses in tumor-bearing mice. Microarrays of vaccines highly enriched for the immunotherapeutic cells were compared with the arrays of nonenriched vaccines. The gene for Grb10 was ~100-fold overrepresented in cells from the enriched vaccine. C3H/He mice with established breast cancer treated solely by immunization with fibroblasts modified to express Grb10 survived significantly longer than mice in various control groups. In some instances, the treated tumor-bearing animals seemed to have rejected the breast cancer cells. They survived indefinitely. Toxic effects in the treated mice were not observed. These data establish Grb10 as a therapeutic target expressed by mammary carcinoma cells.

Materials and Methods

Experimental animals, tumor cell lines, and monoclonal antibodies. Pathogen-free C3H/He female mice (H-2^k) between 10 and 14 wk old were from The Jackson Laboratory. They were maintained according to NIH Guidelines for the Care and Use of Laboratory Animals. SB5b cells were a breast cancer cell line established from an adenocarcinoma that arose spontaneously in the mammary gland of a C3H/He mouse in our animal colony. LM fibroblasts, of C3H/He mouse origin, were from the American Type Culture Collection (ATCC). B16F1 cells, a melanoma cell line of C57BL/6 mouse origin (H-2^b), were from the ATCC. Each of the cell types was maintained at 37°C in a humidified 7% CO_2/air atmosphere in DMEM (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (growth medium; Life Technologies). Monoclonal antibodies (mAb) for $CD4^+$, $CD8^+$, and NK1.1 cells and fluorescein-conjugated mAbs for H-$2K^b$ class I determinants were from BD PharMingen.

Modification of LM fibroblasts to secrete interleukin-2. The fibroblasts were modified to secrete interleukin-2 (IL-2) before transfection (LM-IL-2 cells) as a means of augmenting their nonspecific immunogenic properties, as described previously (30).

Modification of the cytokine-secreting fibroblasts to express H-$2K^b$ class I determinants, allogeneic in C3H/He mice. Allogeneic class I determinants are strong immune adjuvants (32, 33). To stimulate uptake of the vaccine by dendritic cells of the tumor-bearing host, and to ensure rejection, the fibroblasts (H-2^k) were modified to express H-$2K^b$ determinants, allogeneic in C3H/He mice (LM-IL-$2K^b$ cells), as described previously (34, 35).

Preparation of cDNA libraries from SB5b breast cancer cells. Guanidine isothiocyanate was used to recover total RNA from SB5b cells or from B16F1 melanoma cells. The mRNA, derived from ~1×10^7 cells, was isolated using a mRNA isolation system (Promega). The cDNA expression libraries were constructed with a Lambda Zap vector using a cDNA library kit (Stratagene). cDNAs >0.5 kb were selected by size fractionation via gel filtration and directionally cloned into a pBK-CMV vector with an *Eco*RI restriction site at the 5′-end and an *Xho*I site at the 3′-end. The expression libraries yielded ~4×10^5 plaque-forming units/μg cDNA with an individual cDNA insert. The size distribution of the cDNA transduced into the modified fibroblasts was 0.5 to 7.0 kb.

Preparation of the cDNA-based cellular vaccines. LM-IL-$2K^b$ cells were transduced with a cDNA library from SB5b cells or, for use as a specificity control, with a cDNA library from B16F1 cells using Lipofectamine 2000 (Invitrogen) to aid cDNA uptake. In brief, 30 μg cDNA from either of the cell types was mixed with 3 μg pcDNA6/Bla (Invitrogen), a plasmid specifying a gene conferring resistance to blasticidin, used for selection. Afterward, the cDNA/pcDNA6/Bla mixture was added to Lipofectamine 2000 and then to 2.0×10^7 LM-IL-$2K^b$ cells divided into four 100-mm plastic cell culture dishes 24 h previously. After incubation for 18 h, the cells were divided into 16 100-mm dishes and incubated for 14 d in fresh growth medium containing 5 μg/mL blasticidin and 500 μg/mL G418. The surviving blasticin/G418-resistant cells (at least 2×10^6 colonies) were pooled and maintained as cell lines for use in the experiments (LM-IL-$2K^b$/cSB5b and LM-IL-$2K^b$/B16F1 cells, respectively). For use as a control, the same procedure was followed except that the fibroblasts were transduced with pcDNA6/Bla alone (LM-IL-$2K^b$ cells).

Mouse enzyme-linked immunospot IFN-γ assays. Mouse enzyme-linked immunospot (ELISPOT) IFN-γ assays were used to determine the number of responding T cells in mice immunized with the transduced fibroblasts. The spots were counted by computer-assisted image analysis (ImmunoSpot Series 2 analyzer, Cellular Technology Ltd.).

^{51}Cr release cytotoxicity assays. Mononuclear cells from the spleens of C3H/He mice immunized with the cDNA-transduced cells, or with cells transduced with a plasmid encoding the gene specifying Grb10, were isolated by Ficoll-Hypaque density gradient centrifugation. After washing, the cells were cocultured at 37°C with mitomycin C–treated (45 min, 50 μg/mL) SB5b cells for 5 d (ratio of spleen cells to SB5b cells = 30:1). Afterward, the population that failed to adhere to the plastic cell culture flasks was collected and used as effector cells for the cytotoxicity determinations. Spleen cell–mediated cytotoxicity was determined in a standard ^{51}Cr release assay using ^{51}Cr-labeled SB5b cells as targets in the reaction.

The percent specific cytolysis was calculated as follows:

$$\frac{\text{Experimental } {}^{51}\text{Cr release} - \text{Spontaneous } {}^{51}\text{Cr release}}{\text{Maximum } {}^{51}\text{Cr release} - \text{Spontaneous } {}^{51}\text{Cr release}} \times 100$$

The spontaneous release of ^{51}Cr was <15% of the total release in each instance.

Enrichment of the vaccine for cells that induce immunity to (SB5b) mammary carcinoma cells. The vaccine was prepared by transfer of a cDNA library derived from SB5b cells into the modified mouse fibroblasts. Because only a small proportion of the transduced cell population was expected to have incorporated cDNAs that included genes specifying tumor antigens (TAA), a unique strategy was used to enrich the vaccine for TAA-positive cells, as described previously (30). In brief, aliquots of the suspension of transduced cells were added to each of 10 wells of a 96-well plate. Each pool contained a starting inoculum of 1×10^3 cells. Wells containing higher numbers of TAA-positive cells were detected by comparing the response of C3H/He mice to immunization with cells from the individual pools, as determined by both ELISPOT IFN-γ and ^{51}Cr release cytotoxicity assays. To obtain a sufficient number of cells for immunization, cells from the individual pools were allowed to increase to ~5×10^7 through periodic transfers to larger culture plates and eventually cell culture flasks. An aliquot of each of the expanded cell populations was maintained frozen/viable (for later recovery). The remaining portion was used for immunization. For immunization, naive C3H/He mice were injected s.c. twice at weekly intervals with cells from the individual pools. One week after the second injection, spleen cells from the immunized mice were analyzed by both ELISPOT IFN-γ and ^{51}Cr release assays for reactivity toward SB5b cells, as described previously (30). Frozen cells derived from the pool that stimulated immunity to the breast cancer cells to the greatest extent (immuno^{high}), and, for use as a control, from the pool that induced immunity to SB5b cells to the least extent (immuno^{low}), were recovered, reestablished in culture, and subjected to additional rounds of positive or negative immune selection according to the same protocol (30). [As an additional control, one pool was not subjected to either positive or negative selection (master pool).] After five rounds of selection, microarrays were used to compare the gene expression profiles of cells in the immuno^{high} and immuno^{low} pools.

Microarrays of cellular vaccines enriched for transduced fibroblasts that induced immunity to SB5b cells. cRNA microarrays were used to compare the gene expression profiles of transduced fibroblasts from the

immuno^{high} and immuno^{low} pools, as described previously (36). (Series record accession number is GSE10095.).

Reverse transcription-PCR of *Grb10*, a candidate gene specifying a breast cancer antigen, identified by comparing microarrays of the enriched and nonenriched vaccines. Grb10 was highly overrepresented in cells from immuno^{high} pools. Reverse transcription-PCR (RT-PCR) was used to determine if the gene was expressed. Approximately 6×10^6 cells from the immuno^{high} pool in monolayer culture were disrupted and homogenized. One volume of 70% ethanol was added before the extracts were loaded onto RNeasy mini columns. RT-PCR was performed on RNA eluted from the column with a One-Step RT-PCR kit (Qiagen) according to the manufacturer's instructions. RNA (1 μg) was mixed with buffer containing 1.25 mmol/L $MgCl_2$, 40 μmol/L deoxynucleotide triphosphates, 0.6 μmol/L of each forward and backward primers, and 2 μL of a mixture containing reverse transcriptase and Taq polymerase. The reverse transcriptase reaction was at 50°C for 45 min. The PCR was at 94°C. The denaturation step was for 2 min at 58°C. The annealing step was for 1 min at 72°C and extension was for 2 min for 35 cycles. A DNA Thermal Cycler 480 (Perkin-Elmer) was used for the reactions. The primers used were the following: Grb10, 5′-CGTGGTCCAGTGGAGAGTA (forward) and 5′-TCCGGTCTTCGGCGTAACTGA (backward).

Preparation of an expression vector that specified the gene for Grb10 (pcDNA6/V5-HisA/Grb10). An expression vector that specified the gene for Grb10 was prepared by ligation of the gene into a pCR2.1 vector using a TA 2.1 cloning kit (Invitrogen). In brief, 50 ng pCR2.1 and 2 μL of the PCR product containing 10 ng Grb10 were mixed with buffer and 1 μL T4 DNA ligase in 10 μL total volume and incubated at 14°C for 4 h. Ligation mixture (5 μL) containing pCR2.1/Grb10 was transferred into 50 μL DH5α-competent cells followed by 30-min incubation on ice. Afterward, the cells were subjected to a 20-s heat shock at 37°C and 2-min additional incubation on ice. As a control, pUC 19 DNA (5 μL) was also transferred into DH5α-competent cells. The transformation complex was mixed with 950 μL SOC medium and incubated at 37°C for 1 h. The cell pellets were plated on LB agar plates containing 100 μg/mL ampicillin and 1 mg 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and incubated at 37°C overnight. White colonies indicating insertion of the *Grb10* gene in the lacZ site of pCR2.1 were selected and amplified. DNA from each amplified clone was extracted and digested with *Eco*RI enzyme to verify the presence of the 430-bp portion of the *Grb10* gene. The resulting 430-bp band was recovered from the gel and purified from a gel purification kit (Qiagen). The 430-bp portion of the gene was ligated into the expression vector pcDNA6/V5-HisA (Invitrogen). In brief, 170 ng of pcDNA6/V5-HisA digested with *Eco*RI were mixed with 30 ng Grb10 and 3 μL T4 DNA ligase with buffer and incubated at 14°C for 4 h. Ligation mixtures containing pcDNA6/V5-HisA/Grb10 were transformed into 50 μL of chemically competent *Ecoli*DH5α cells with 30-min incubation on ice followed by 20-s heat shock at 37°C and 2-min additional incubation on ice. As a control, pUC 19 DNA (5 μL) was also transferred into DH5α-competent cells. The transformation complex was mixed with 950 μL SOC medium and incubated at 37°C for 1 h. The cell pellets were plated on LB agar plates containing100 μg/mL ampicillin and incubated at 37°C overnight. Colonies were selected and amplified in 2 mL cultures for DNA isolation and Grb10 verification through *Eco*RI digestion. The identified pcDNA6/V5-HisA/Grb10 clone was amplified in 2 L cultures and 1.26 mg of pcDNA6/V5-HisA/Grb10 DNA were obtained using a plasmid maxi prep kit (Qiagen).

Preparation of a vaccine for the treatment of mice with breast cancer by transfection of modified fibroblasts with the vector specifying Grb10 (LM-IL-2K^b/Grb10). The vaccine was prepared by transfection of modified fibroblasts with the Grb10 vector according to an analogous procedure reported previously (36). In brief, 2×10^6 LM-IL-2K^b cells were added to four 100-mm plates in minimal growth medium without antibiotics. Afterward, 30 μg of pcDNA6/V5-HisA/Grb10 DNA in 2 mL Opti medium were mixed with 100 μL Lipofectamine 2000 followed by incubation for 20 min at room temperature. One milliliter of the 4 mL transfection complex (pcDNA6/V5-HisA/Grb10 DNA and Lipofectamine 2000) was added to each of the four plates and incubated overnight at 37°C in a 7% CO_2/air incubator. The number of plates was expanded to 16. The transduced cells were selected in growth medium containing 5 μg/mL blasticidin. The blasticidin-resistant cells were allowed to proliferate for an additional 7 d and pooled. One half of the cell suspension was maintained frozen/viable; the remaining portion was maintained at 37°C in a 7% CO_2/air incubator in selection medium. For use as a control, the same procedure was followed except that the LM-IL-2K^b cells were transduced with the "empty" vector pcDNA6/V5-HisA.

Statistical analyses. Kaplan-Meier log-rank analyses were used to determine the statistical differences between the survival of mice in the various experimental and control groups. A P value of <0.05 was considered significant. Student's t test one-way ANOVA was used to determine the statistical differences between experimental and control groups in the experiments performed *in vitro*.

Results

IL-2 secretion and expression of H-2K^b determinants by modified LM fibroblasts, used as recipients of the cDNA library from the mammary carcinoma cells. Among other advantages, the use of a nonmalignant cell line as the recipient of the cDNA library enabled the recipient cells to be conveniently modified beforehand to augment their nonspecific immunogenic properties. In this instance, the fibroblasts, of C3H/He mouse origin, were modified to secrete IL-2, a T-cell growth factor, and to express H-2K^b determinants, allogeneic in C3H/He mice (LM-IL-2K^b cells). The transduced cells formed 3.3 ± 0.3 ng IL-2/10^6 cells/48 h. Nontransduced fibroblasts failed to form detectable quantities of IL-2. The mean fluorescence index of LM cells transduced with pBR327H-2K^b, the plasmid that specified H-2K^b, stained with fluorescein-conjugated H-2K^b mAbs, was significantly higher than that of cells stained with the isotope control sera (1.0 ± 0.2 and 0.3 ± 0.1, respectively). The secretion of IL-2 and the expression of H-2K^b determinants by the modified cells were essentially unchanged after 3 months of continuous culture (these data are not presented).

Grb10 was relatively overrepresented in cells from the immuno^{high} pool. cRNA microarrays were used to compare the gene profiles of cells from the immuno^{high} and immuno^{low} pools. After five rounds of positive or negative immune selection, 20 genes were relatively overrepresented in cells from the immuno^{high} pool (Table 1). The gene for Grb10 was ~100-fold overrepresented. Other highly overrepresented genes included tripartite motif protein 13 (71.5-fold overrepresented), serum amyloid A3 (44-fold), and Xlr-related, meiosis-regulated gene (39-fold), suggesting that multiple genes in the immuno^{high} pool of transduced cells specified an array of immunogenic TAA.

Expression of Grb10 by LM fibroblasts transduced with pcDNA6/V5-HisA/Grb10, an expression vector that specified Grb10 (LM-IL-2K^b/Grb10 cells). A vaccine for breast cancer was prepared by transduction of LM-IL-2K^b cells with pcDNA6/V5-HisA/Grb10, an expression vector that specified Grb10. As a first step, RT-PCR was used to determine if the transduced cells expressed Grb10. As indicated (Fig. 1), Grb10 was strongly expressed by LM-IL-2K^b/Grb10 cells, by cells from the immuno^{high} pool, and by cells from the (nonselected) master pool. Grb10 was also expressed, but to a lesser extent, by cells from the immuno^{low} pool, by the breast cancer cells, by nontransduced fibroblasts (LM-IL-2K^b), and by fibroblasts transduced with a cDNA library from B16F1 melanoma cells (LM-IL-2K^b/cB16F1). Quantitative RT-PCR was used to compare the relative expression of Grb10 by cells from the immuno^{high} and the immuno^{low} pools. The results indicated that the expression of Grb10 by cells from the immuno^{high} pool was 75.6-fold higher than that of cells from the immuno^{low} pool (these data are not presented).

Table 1. Genes relatively overrepresented in the cellular vaccine selected for its enhanced immunotherapeutic properties in mice with breast cancer

H/L	Description
99.92	Growth factor receptor–bound protein 10
71.57	Tripartite motif protein 13
44.25	Serum amyloid A3
39.19	Xlr related, meiosis regulated
35.22	Pentaxin-related gene
29.81	CD36 antigen
24.65	RIKEN cDNA 9030625A04 gene
22.54	Prostaglandin-endoperoxide synthase 2
15.32	RIKEN cDNA E030003E18 gene
13.47	Tumor-associated calcium signal transducer 1
12.70	RIKEN cDNA 2310005E10 gene
12.17	DEAD (Asp-Glu-Ala-Asp) box polypeptide 25
11.51	Neuropeptide Y receptor Y1
9.93	GRP1 (general receptor for phosphoinositides 1)-associated scaffold protein
9.53	Nuclear receptor subfamily 4, group A, member 1
9.39	SRY box-containing gene 5
8.27	Carbonic anhydrase 9
7.24	Aldehyde dehydrogenase family 1, subfamily A7
7.16	Thymoma viral proto-oncogene 3
7.08	RIKEN cDNA D130020G16 gene

NOTE: Comparative microarrays of immuno^{high} (after five rounds of positive immune selection) and immuno^{low} (after five rounds of negative immune selection) LM-IL-2K^b/cSB5b cells.

Immunity to breast cancer in mice immunized with LM-IL-2K^b/Grb10 cells. ELISPOT IFN-γ assays were used to compare the number of responding T cells in C3H/He mice immunized with LM-IL-2K^b/Grb10 cells with that of mice immunized with cells from various control groups. Naive mice were injected s.c. twice at weekly intervals with 5×10^6 LM-IL-2K^b/Grb10 cells. One week later, spleen cells from the immunized mice were coincubated for 18 h in the presence or absence of (mitomycin C treated) SB5b cells. At the end of the incubation, the number of responding T cells was determined in a standard ELISPOT IFN-γ assay. As controls, the same protocol was followed except the mice were injected with an equivalent number of cells from the (nonenriched) master pool, with nontransduced fibroblasts (LM-IL-2K^b/−) or, as a specificity control, with fibroblasts transduced with a cDNA library derived from B16F1 cells (LM-IL-2K^b/cB16F1). [Our prior experience (30) indicated that the immunity in tumor-bearing mice immunized with DNA-based vaccines was specific for the type of tumor from which the DNA was obtained].

One group of mice was not injected (naive). As indicated (Fig. 2*A*), the highest number of responding cells (number of spots/10^6 spleen cells) was in the group immunized with LM-IL-2K^b/Grb10 cells. Lesser numbers of responding cells were detected if the spleen cells were from mice immunized with cells from the (nonenriched) master pool or from mice immunized with nontransduced fibroblasts. The number of responding T cells in mice immunized with LM-IL-2K^b/cB16F1 cells was not significantly different than that of mice immunized with cells from the (nonenriched) master pool, suggesting that the two cellular vaccines share determinants in common. Significantly lesser responses were obtained if the cells were from mice immunized with (nontransduced) LM-IL-2K^b/− cells or if the spleen cells were from naive mice ($P < 0.001$).

The types of cells activated for immunity to the breast cancer cells in mice immunized with LM-IL-2K^b/Grb10 cells was investigated by determining the effect of CD4$^+$, CD8$^+$, and NK 1.1 mAbs on the number of responding cells. Each of the mAbs inhibited the response. CD4$^+$ mAbs inhibited the response to the greatest extent (Fig. 2*B*). Analogous effects were obtained if the mice were immunized with cells from the nonenriched master pool (LM-IL-2K^b/cSB5b) or with LM-IL-2K^b/cB16F1 cells (Fig. 2*B*). Whether or not combined depletion with CD4/CD8 mAbs would result in a greater suppression of the responding cells than either mAb alone was not determined.

^{51}Cr release cytotoxicity assays were used to measure CTL responses toward SB5b cells in mice immunized with LM-IL-2K^b/Grb10 cells. Tumors were first established following a s.c. injection of 0.4×10^6 SB5b cells. One week later, when the tumors were ~3 mm in diameter, the mice received the first of two s.c. injections at weekly intervals of 5×10^6 LM-IL-2K^b/Grb10 cells. One week after the second injection, spleen cells from the immunized mice were tested for the presence of CTL. As controls, the same procedure was followed except that the tumor-bearing mice were immunized with cells from the master pool (LM-IL-2K^b/cSB5b), with (nontransduced) LM-IL-2K^b/− cells, or with LM-IL-2K^b/cB16F1 cells. One group of mice injected with SB5b cells alone was not treated. The results (Fig. 3) indicated that the highest responses (percent specific lysis of SB5b cells) were in mice immunized with LM-IL-2K^b/Grb10 cells. Equivalent responses were detected in mice immunized with cells from the nonenriched master pool (LM-IL-2K^b/cSB5b). Lesser responses were present in mice immunized with LM-IL-2K^b/− cells. The response in tumor-bearing mice immunized with LM-IL-2/cB16F1 cells was not significantly different than that of untreated tumor-bearing mice.

Thus, augmented immunity to the breast cancer cells was generated in mice immunized with the fibroblasts modified to express the gene specifying Grb10, as determined by two independent assays.

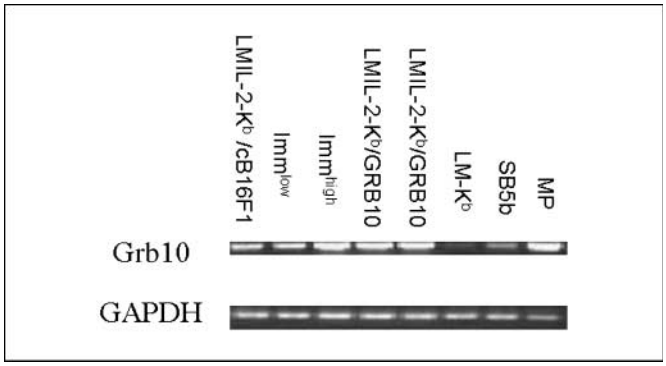

Figure 1. RT-PCR of LM-IL-2K^b/Grb10 cells and cells from various control groups for expression of the gene specifying Grb10. *Lane 1,* LM-IL-2K^b/cB16F1 (LM-IL-2K^b cells transduced with pcDNA6/cB16F1); *lane 2,* immuno^{low} (cells from the immuno^{low} pool after five rounds of negative immune selection); *lane 3,* immuno^{high} (cells from the immuno^{high} pool after five rounds of positive immune selection); *lane 4,* LM-IL-2K^b/Grb10 (LM-IL-2K^b cells transduced with pcDNA6/Grb10); *lane 5,* LM-IL-2K^b/Grb10 (LM-IL-2K^b cells transduced with pcDNA6/Grb10); *lane 6,* LM-K^b (LM cells transduced with a plasmid specifying K^b determinants); *lane 7,* SB5b (SB5b breast cancer cells); *lane 8,* MP (cells from the nonenriched master pool of LM-IL-2K^b/cSB5b cells). *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase.

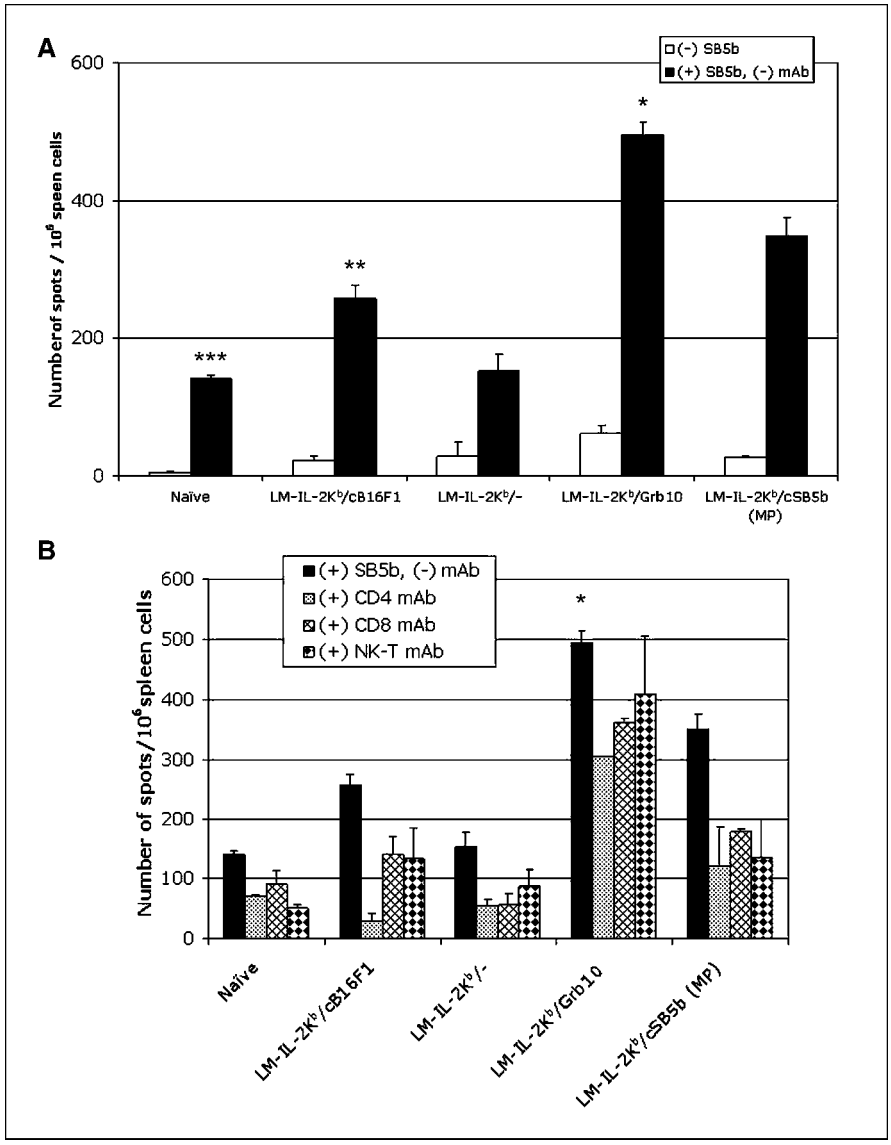

Figure 2. Cellular immune responses toward SB5b cells in mice immunized with LM-IL-2K^b/Grb10 cells. *A,* ELISPOT IFN-γ assays: naive C3H/He mice received two s.c. injections at weekly intervals of LM-IL-2K^b/Grb10 cells. Each injection contained 4×10^6 cells. As controls, the same procedure was followed except that cells from the nonenriched master pool were substituted for LM-IL-2K^b/Grb10 cells. As additional controls, the mice were injected with equivalent numbers of LM-IL-2K^b/− cells, or with LM-IL-2K^b/cB16F1 cells, or the mice were not injected. One week after the last injection, spleen cells from the immunized mice were coincubated for 18 h with (mitomycin C treated) SB5b cells (E:T ratio = 10:1) before the ELISPOT IFN-γ assays were performed. *, $P < 0.0002$, for the number of spots/10^6 spleen cells in cultures of spleen cells from mice immunized with LM-IL-2K^b/Grb10 cells coincubated with SB5b cells versus the number of spots/10^6 spleen cells incubated alone; **, $P < 0.001$, for differences in the number of spots/10^6 spleen cells in cultures of cells from mice immunized with LM-IL-2K^b/Grb10 cells cocultured with SB5b cells relative to the number of spots in spleen cell cultures from mice immunized with LM-IL-2K^b/cB16F1 cells cocultured with SB5b cells; ***, $P < 0.001$, for differences in the number of spots/10^6 spleen cells in cultures of cells from mice immunized with LM-IL-2K^b/Grb10 cells cocultured with SB5b cells relative to the number of spots in cultures from mice immunized with LM-IL-2K^b/− cells cocultured with SB5b cells. Pooled spleen cells were from three mice in each group. *B,* ELISPOT IFN-γ assays in the presence of mAbs for CD8$^+$, CD4$^+$, or natural killer T (*NK-T*) cells and complement. The same protocol described in the legend to *A* was followed except that the mAbs were added 1 h and complement was added 30 min before the ELISPOT IFN-γ assays were performed. *, $P < 0.05$, for the difference in the number of spots in the presence or absence of CD4 or CD8 mAb in cocultures of spleen cells from mice injected with LM-IL-2K^b/Grb10 cells and (mitomycin C treated) SB5b cells.

Survival of C3H/He mice with breast cancer treated by immunization with LM-IL-2K^b/Grb10 cells. Enhanced T-cell immunity toward SB5b cells was generated in mice immunized with modified fibroblasts transduced with the gene for Grb10. To determine if the immunogenic properties of the vaccine observed *in vitro* were reflected by the immunotherapeutic properties of the cells in mice with breast cancer, tumors were first established in C3H/He mice injected with 4×10^5 SB5b cells followed by the first of three weekly injections of LM-IL-2K^b/Grb10 cells 7 days later. The size of the tumor at the injection site was 5 ± 2 mm at the time of the first injection. As controls, the same procedure was followed except that LM-IL-2K^b/cSB5b cells, (nontransduced) LM-IL-2K^b/− cells, or cells transduced with cDNA from B16F1 melanoma cells were substituted for LM-IL-2K^b/Grb10 cells. One group of tumor-bearing mice was not treated.

As indicated (Fig. 4*A*), tumor-bearing mice treated by immunization with LM-IL-2K^b/Grb10 cells survived significantly ($P < 0.01$) longer than mice in any of the control groups, including mice immunized with LM-IL-2K^b/cB16F1 cells, except mice treated by immunization with LM-IL-2K^b/cSB5b cells. Although differences in the survival of treated and untreated mice with breast cancer were highly significant, the immunotherapeutic properties of LM-IL-2K^b/cSB5b cells and LM-IL-2K^b/Grb10 cells were not statistically different. Analogous results were obtained if tumor-free mice were immunized with LM-IL-2K^b/Grb10 cells before injection of the breast cancer cells (Fig. 4*B*).

Discussion

Under appropriate circumstances, immunizations with cell-based vaccines activate antitumor immune responses that can result in the killing of various different histologic types of cancer cells, including breast cancer. Successful immunotherapy can become an important part of the overall management of patients with neoplastic disease. Among other advantages, the immunity is tumor specific, and there is little or no toxicity. Various clinical trials are in progress, which are designed to test immune-based therapies for patients with various types of malignancies.

However, not all vaccines are equally successful in inducing therapeutic immune responses. Often, tumor progression continues

following immunization with tumor vaccines. Secondary considerations, such as immune tolerance to the antigen chosen for therapy or the presence of regulatory T cells, which inhibit tumor immunity, are important variables. Malignant cells, including breast cancer cells, can be highly immunosuppressive.

In this report, we applied a new strategy to identify antigens associated with breast cancer cells that induced therapeutic immune responses. The effects of the vaccine were tested in mice bearing tumors derived from SB5b cells, a highly malignant breast cancer cell line. The vaccine was prepared by transfer of a cDNA library derived from the breast cancer cells into a modified mouse fibroblast cell line. This approach was an extension of classic studies indicating that the phenotype of the cells that take up the exogenous DNA is altered as the transferred DNA integrates spontaneously into the genome of the recipient cells and is expressed (37, 38). Oncogenes were first discovered by this approach (39, 40). Fibroblasts, rather than dendritic cells, were chosen as recipients of the tumor-derived cDNA for two complementary reasons. As a cell line, they are readily transfected and the number of transfected cells can be conveniently expanded as required for multiple immunizations. Leukophoresis, required for the recovery of syngeneic dendritic cells, is not required. The growth of dendritic cells *in vitro* can be technically challenging. The fibroblasts used as cDNA recipients can be modified to augment their immunogenic properties. In this instance, the cells were modified to express allogeneic MHC determinants to stimulate uptake of the vaccine by dendritic cells of the host where TAAs are expressed (cross-priming).

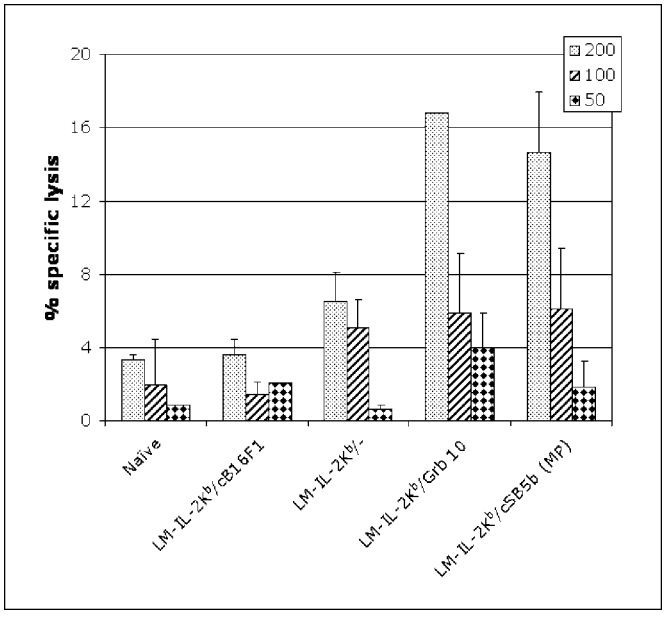

Figure 3. CTLs reactive with SB5b cells were generated in C3H/He mice immunized with LM-IL-2K^b/Grb10 cells. Naive C3H mice received a s.c. injection of 0.4×10^6 viable SB5b cells. One week later, the mice received the first of two s.c. injections at weekly intervals of LM-IL-2K^b/Grb10 cells. Each injection consisted of 4×10^6 cells. As controls, the mice were injected with LM-IL-2K^b/– cells, with cells from the nonenriched master pool, or the mice were not injected (naive). One week after the second injection, spleen cells from the immunized mice were cocultured for 5 d with (mitomycin C treated) SB5b cells (ratio of spleen cells to SB5b cells = 30:1). After incubation, the nonadherent cells were collected and coincubated for 5 h with ^{51}Cr-labeled SB5b cells before the specific cytotoxicity was determined. Approximately 15% of maximum ^{51}Cr release released spontaneously (background). $P < 0.03$, for the specific release of isotope above background from SB5b cells coincubated with spleen cells from mice immunized with LM-IL-2K^b/Grb10 cells versus that of spleen cells from nontreated tumor-bearing mice. Pooled spleen cells were from three mice in each group.

The protocol used to prepare the vaccine was designed to identify antigens expressed by the mammary carcinoma cells that induced therapeutic immune responses in mice with established breast cancer. The vaccines were enriched for cells that induced breast cancer immunity by identifying subpools of the cell population that induced breast cancer immunity. After five rounds of positive enrichment, microarrays of cell pools that stimulated immunity to the breast cancer cells to the greatest and, as a control, to the least extent (immuno^{high} and immuno^{low}, respectively) were compared. At least 20 genes were overrepresented in cells from the immuno^{high} pool. The gene for Grb10, a signal transduction molecule, was overrepresented to the greatest extent. The vaccine was prepared by introducing the gene for Grb10 into the modified fibroblasts (LM-IL-2K^b/Grb10). After confirming that the molecule was expressed, the immunotherapeutic properties of the vaccine were tested in mice with established tumors derived from the breast cancer cells. The therapeutic properties of the vaccine were clearly evident. The treated mice survived significantly longer than mice in various control groups. At times, the tumor-bearing animals seemed to have rejected the breast cancer cells. They survived indefinitely. The finding that each of the cell pools tested expressed Grb10 to a greater or lesser extent was consistent with its wide distribution among various cells in the transduced cell population. The observation that the immunotherapeutic properties of LM-IL-2K^b/Grb10 cells were equivalent or at times exceeded those of a vaccine prepared with unfractionated cDNA derived from SB5b cells may be an indication that 100% of LM-IL-2K^b/Grb10 cells expressed the molecule.

Grb10 is a member of the family of adaptor proteins (other family members include Grb7 and Grb14) that interact with various receptor tyrosine kinases, such as epidermal growth factor, ErB2/Her2, platelet-derived growth factor, insulin-like growth factor-1, vascular endothelial growth factor receptors, and the kinase insert domain containing receptor (41–45). Attachment of the adaptor protein to the receptor activates Ras, resulting in gene activation through the mitogen-activated protein kinase (MAPK) cascade. In addition to intracellular receptor tyrosine kinases, Grb10 also links cell surface receptor tyrosine kinases with non–receptor-associated tyrosine kinases, such as Raf-1 and the MAPK (MAPK/extracellular signal-regulated kinase kinase).

Grb7, another member of the Grb family of adaptor proteins, has been implicated in cancer progression. Pero et al. (46) identified a novel peptide that binds to the SH2 domain of Grb7 and inhibits its association with receptor tyrosine kinases. The proliferation of various breast cancer cell lines was impaired by treatment with Grb inhibitors, including an inhibitor of Grb10. Grb10 was overexpressed in squamous carcinoma cells derived from primary tumors of patients with cervical cancer and that inhibition of the molecule with a Grb10-specific small interfering RNA markedly reduced tumor growth (47). Thus, this central molecule affects multiple cellular processes primarily associated with the control of cell division. Immunity to this class of cell regulatory molecules may have profound effects on the growth of various types of rapidly proliferating neoplastic cells.

We hypothesize that immunization of mice with breast cancer with the fibroblasts modified to express Grb10 resulted in the induction of immunity to rapidly proliferating breast cancer cells that overexpressed Grb10, identifying the molecule as a potential therapeutic target. It is likely that Grb10 is only one of an array of breast cancer antigens expressed by the

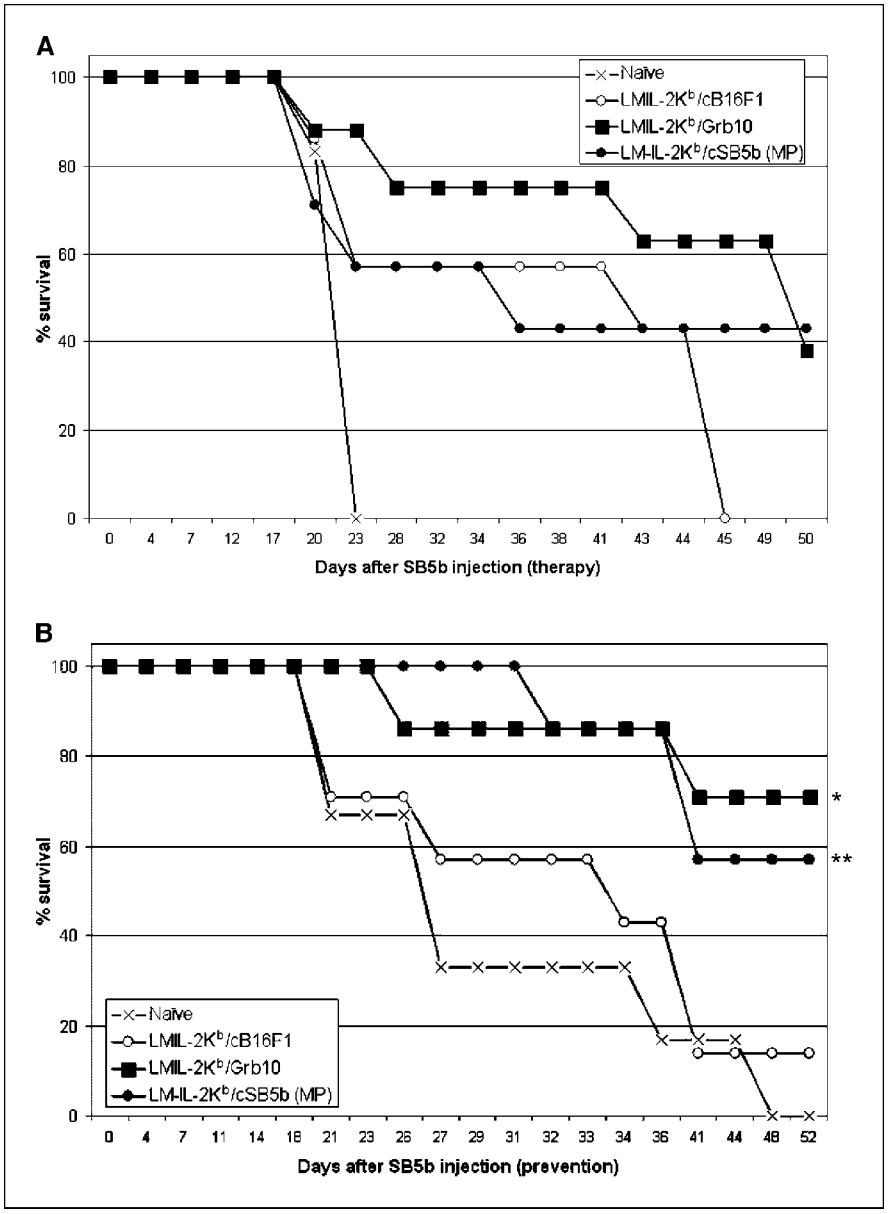

Figure 4. *A,* immunization with LM-IL-2K^b/Grb10 cells prolonged the survival of C3H/He mice with breast cancer. Tumors were established in naive C3H/He mice following a s.c. injection of 0.4×10^6 SB5b cells. Seven days later, the mice received the first of two s.c. injections at weekly intervals of 4×10^6 LM-IL-2K^b/Grb10 cells. As controls, the same procedure was followed except that LM-IL-2K^b/− cells, LM-IL-2K^b/cB16F1 cells, or cells from the master pool were substituted for LM-IL-2K^b/Grb10 cells. One group of mice was injected with SB5b cells alone. The surviving mice were euthanized 50 d after injection of the breast cancer cells. Mean survival time (MST) for mice injected with SB5b cells followed by the injections of LM-IL-2K^b/Grb10 cells, 41.0 ± 11.0 d; MST for mice injected with SB5b cells followed by the injections of nonenriched LM-IL-2K^b/cSB5b (master pool) cells, 40.0 ± 14.0 d; MST for mice injected with SB5b cells followed by the injections of LM-IL-2K^b/cB16F1 cells, 34.0 ± 12.0 d; MST for naive mice injected with SB5b cells alone, 22.0 ± 1.3 d. $P < 0.01$, for survival of mice with breast cancer treated by immunization with LM-IL-2K^b/Grb10 cells versus naive mice. $P < 0.02$, for survival of mice with breast cancer treated by immunization with LM-IL-2K^b/Grb10 cells and mice with breast cancer treated by immunization with LM-IL-2K^b/cB16F1 cells. There were eight mice in each group. *B,* immunization with LM-IL-2K^b/Grb10 cells prevented the growth and prolonged the survival of C3H/He mice injected with SB5b cells. C3H/He mice received two s.c. injections at weekly intervals of 4×10^6 LM-IL-2K^b/Grb10 cells. One week after the last injection, the mice were injected s.c. with 0.4×10^6 SB5b cells. As controls, the same procedure was followed except that equivalent numbers of LM-IL-2K^b/− cells or nonselected cells from the master pool or LM-IL-2K^b/cB16F1 cells were substituted for LM-IL-2K^b/Grb10 cells. One group of mice was injected with SB5b cells alone. The experiment was terminated 52 d after injection of the breast cancer cells. MST for mice injected with LM-IL-2K^b/Grb10 cells followed by the injection of SB5b cells, 47.0 ± 10.0 d; MST for mice injected with cells from the master pool followed by the injection of SB5b cells, 45.0 ± 9.1 d; MST for mice injected with LM-IL-2K^b/cB16F1 cells, 34.0 ± 11.0 d; MST for naive mice injected with SB5b cells alone, 29.0 ± 9.0 d. *, $P < 0.001$, for survival of mice immunized with LM-IL-2K^b/Grb10 cells or cells from the master pool versus naive mice; **, $P < 0.01$, for survival of mice immunized with LM-IL-2K^b/Grb10 cells or cells from the master pool and mice immunized with LM-IL-2K^b/cB16F1 cells. There were seven mice in each group.

vaccine. At least 19 other genes were relatively overrepresented in the highly enriched vaccines. This strategy, which combined the identification of immunogenic tumor antigens with the therapeutic response in tumor-bearing animals, raises the possibility that an analogous approach could be used to generate vaccines of enhanced effectiveness for use in the treatment of patients with breast cancer and possibly other histologic types of cancer.

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