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CASE REPORTS

Objective Clinical Regression of Metastatic Breast Cancer in Disparate Sites after Use of Whole-Cell Vaccine Genetically Modified to Release Sargramostim

Charles L. Wiseman, MD and Alex Kharazi, MD

Immunotherapy Laboratory, St. Vincent Medical Center, Los Angeles, California

■ **Abstract:** A patient with recurrent breast cancer metastases following initial response to chemotherapy and hormonal maintenance was treated with a whole-cell tumor vaccine, resulting in a prompt objective complete remission of a lung lesion on computed tomography (CT) scans and near-complete regression of multiple breast lesions on magnetic resonance imaging (MRI). Three months after completion of the protocol, metastases were again found in the breast and lung, with new lesions in the brain and liver. Reinstitution of vaccine inoculation resulted in major regression of the brain and breast lesions, improvement in all other areas, and no indication of new lesions. Therapy consisted of inoculation of 20×10^6 SV-BR-1-GM cells, a unique breast cancer cell line transfected to release sargramostim (granulocyte macrophage colony-stimulating factor [GM-CSF]). Following lethal irradiation to 200 cGy, vaccine was injected intradermally in four divided doses to the back and thighs, every 2 weeks × 3, then every month × 3. Each treatment was preceded 48 hours earlier with low-dose cyclophosphamide 300 mg/m^2 to abrogate regulatory T-cell activity. Interferon (IFN)-α, 20,000 IU, was injected into each inoculation site at 48 and 96 hours postinoculation to provide an additional "danger signal." The patient developed positive delayed-type hypersensitivity responses and also antibody reactivity to the vaccine cells. ■

Key Words: breast cancer, GM-CSF, whole-cell vaccine

This investigation involves a new whole-cell vaccine genetically engineered to release sargramostim (granulocyte macrophage colony-stimulating factor [GM-CSF]) for up-regulation of professional antigen-presenting cells together with use of low-dose cyclophosphamide 2–3 days prior to inoculation to down-regulate the activity of regulatory T-cells (1,2). Further, unique to our program is the use of interferon (IFN)-α injected into the inoculation site 2 and 4 days after vaccine injection. A combination of IFN-α and GM-CSF has been shown to induce differentiation of dendritic cells (3). Furthermore, IFN-α has the potential to be a powerful vaccine adjuvant, in agreement with the "danger signal hypothesis" (4). This hypothesis claims that, in addition to antigen presentation, the initiation of an immune response requires some chemical signal indicating tissue injury.

We report a patient in whom lesions identified in soft tissue and lung showed disappearance or subtotal regression within 8 weeks of treatment. Recurrent and new metastases developed 3 months after the last vaccine treatment, and again regressed with re-treatment. This case provides evidence that argues against several generalizations held currently by many investigators, that immunotherapy requires many weeks or months for effect, that the immune system will be ineffective or inadequate to elicit regressions of macroscopic tumor, especially in the brain, and that the best objective for immunotherapeutic trials is targeting subclinical disease in patients at high risk of relapse.

It is important to review closely any positive evidence against the above generalizations, given that vaccine therapy in general has met with disappointing results. For example, a recent review of 1306 patients, insisting on clear definitions of response (World Health Organization [WHO] or Response Evaluation Criteria in Solid Tumors [RECIST]), reported objective responses (complete and partial) in 13 of 428 patients treated at the Surgery Branch of the National Cancer Institute and 29 responses in 765 patients culled from the literature, for an overall response rate of 3.5% (5). Melanoma was the diagnosis that accounted for the overwhelming majority of these programs, with heavy emphasis on peptide antigens. There is a possibility that immune responses may develop from lipid or carbohydrate antigens or other protein epitopes

Address correspondence and reprint requests to: Charles L. Wiseman, MD, Immunotherapy Laboratory, St. Vincent Medical Center, 201 S. Alvarado St., Suite 321, Los Angeles, CA 90057, USA, or e-mail: clwmd@aol.com.

that are not major histocompatibility complex (MHC) restricted. The recent publication of tumor control in the SR/CR mouse is also consistent with non-MHC-restricted antitumor recognition and supports a major role for cellular mechanisms of innate immunity as opposed to the CD8+ responses expected from peptide vaccines (6).

Studies addressing advanced breast cancer have been receiving increasing attention, with some positive results (7–9). However, our observation of breast cancer vaccine associated with a rapid response in virtually all identified measurable and evaluable metastases is a notable and uncommon event in itself. Furthermore, it is most uncommon to observe an additional induction of response in sites of recurrence, together with responses in new areas, most especially including brain metastases.

CASE REPORT

The patient is a 58-year-old postmenopausal woman, G4P3 Ab1, who presented in September 2003 with impending superior vena cava syndrome, pulmonary densities, and a 3 cm × 2.5 cm mass at 6 o'clock in the right breast (T2N3M1) with right axillary adenopathy. Core biopsy identified infiltrating ductal carcinoma of the right breast, modified Bloom-Richardson grade II/III. Histochemistry studies reported estrogen positive/progesterone weak; HER-2/*neu* histochemistry weak, fluorescent in situ hybridization (FISH) negative. Bony metastases were identified in the manubrium. The patient had a past history of carcinoma in situ in 1998 for which she used herbal remedies. Additional clinical problems at presentation included a history of mitral valve prolapse, lumbar degenerative osteoarthritis, allergic rhinitis controlled with fexofenadine, and possible old granulomatous disease.

The patient had a prompt and complete remission after treatment with doxorubicin and cyclophosphamide. Subsequently the patient was placed on letrozole; regional irradiation (5800 cGy) was directed to the manubrium and node-bearing areas (but not to the breast).

On April 24, 2005, there were multiple new and recurrent lesions in the right breast on mammography and increased activity in the sternal metastasis by isotope bone scan. Letrozole was discontinued. No treatment was initiated, however, pending repeat evaluation after discontinuation of hormonal therapy. By June 24, 2005, a pulmonary lesion measuring 2.3 cm had clearly progressed. Positron emission tomography (PET), magnetic resonance imaging (MRI), and computed tomography (CT) scans were confirmatory, identifying characteristic lesions in breast, axillae, and lung, the sites previously involved at presentation.

Written informed consent was obtained and the patient began treatment on an Institutional Review Board (IRB) and U.S. Food and Drug Administration (FDA)-approved phase I–II clinical trial. The patient received intradermal injections of SV-BR-GM-1 cells, transfected to secrete GM-CSF. A total of 20,000 viable irradiated vaccine cells in four aliquots were applied to the back and thighs, and repeated every 2 weeks × 3, then monthly × 3. Forty-eight to 72 hours before inoculation, the patient received low-dose cyclophosphamide 300 mg/m^2 intravenously. IFN-α2b (Schering-Plough, Kenilworth, NJ) was injected into the inoculation sites to provide a "danger signal" 2 and 4 days after vaccine injection. Within 48 hours the patient developed generous erythema and induration at the injection sites on the thighs and shoulders on the order of 5 cm and 1–2 cm, respectively. The IFN injections did not seem to exacerbate the cutaneous response and there was virtual disappearance of inflammation after 10–14 days. Treatment was well tolerated, although the patient had a self-limited, untreated recurrence of benign positional vertigo and also developed hemorrhoids with biopsy-defined mild "nonspecific colitis."

Repeat scanning after three inoculations showed substantial regression of virtually all identified tumor sites. CT scans showed only some residual, but nonmeasurable density in the lung, corresponding to a change in PET standardized uptake values (SUVs) from 10.2 to 5.3. The lesion was improved on MRI, which also showed regression of all the lesions in the breast. After an additional three inoculations, the pulmonary lesion was undetectable on CT and PET. The MRI studies of the breast lesions showed a sequential decrease in aggregate volume from, initially, 15.1 ml to 2.46 ml after three inoculations and to 1.6 ml after six inoculations. In parallel, the reduction in enhancement changed from more than 250% to 150% to 131% (Fig. 1). The right axillary lesions were undetectable on PET and CT. Left axillary lesions, indeterminate as to inflammatory or neoplastic origin, regressed only slightly in volume, although showing reduced enhancement on MRI and PET. Isotope bone scans before and after treatment showed activity in the sternum, marginally less, without evidence of new lesions. No new lesions were identified, nor were any previously suspicious sites found to enlarge. Active disease was not confirmable on any imaging. The first sequence of regressions of all lesions, as evaluated by different imaging methods, is summarized in Table 1.

Approximately 3 months (106 days) after the last inoculation, PET, CT, and MRI studies identified multiple areas of recurrence in the right breast. Also noted were several brain metastases, lesions in the lung and mediastinum,

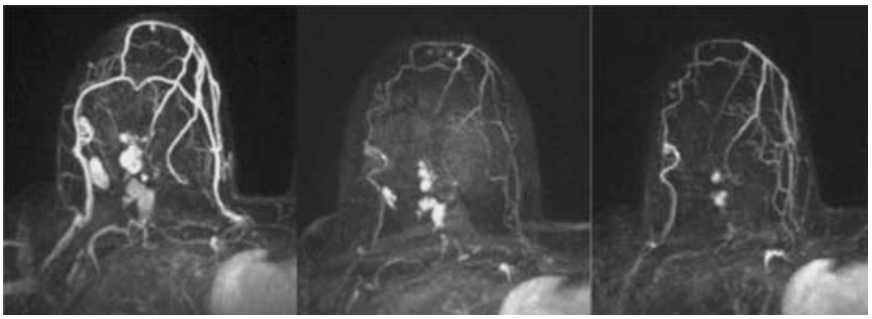

Figure 1. Reinduction treatment series. Breast MRI: maximum intensity projections (MIPs) of contrast-enhanced T1 gradient echo images of the right breast. Left image: baseline prevaccine. Multiple enhancing masses with increased vascularity to the breast; peak enhancement greater than 250% with washout perfusion pattern consistent with neoplasm. Middle image: after inoculation no. 3 (2 months); note the regression of multiple lesions and reduced vascularity; peak enhancement 150%. Right image: After inoculation no. 6 (5 months); note the progressive decrease in size of masses and enhancement; peak enhancement 131%.

and probable involvement in the liver. Permission was obtained from the FDA to reinitiate vaccine treatments, as the protocol initially permitted only six inoculations. Evaluation following three more inoculations demonstrated marked measurable regression of multiple brain and breast lesions, and improvement in the liver and chest. PET scan activity confirmed interval improvement of the size and intensity of previously seen multiple foci of increased uptake. No new lesions were identified and no areas showed enlargement or growth by any imaging method. Figures 2 and 3 show MRI scans before reinoculation and after three reinoculations, for the breast and brain, respectively.

Addendum: Since this manuscript was accepted, the patient completed a total of 10 vaccine inoculations over 4 months. Repeat imaging studies report only normal findings on MRI and PET, consistent with a complete remission of the previous multiple central nervous system metastases.

Immune monitoring to date includes serology and cutaneous hypersensitivity testing to recall antigens and to nontransfected SV-BR-1. The maximum delayed-type hypersensitivity (DTH) response to nontransfected SV-BR-1 cells was 5 mm × 3 mm at 72 hours after the second inoculation; the patient was nonresponsive to purified protein derivative (PPD) and reactive to only one of two recall antigens. There was a sustained 1:100 increase in enzyme-linked immunosorbent assay (ELISA) serology against SV-BR-1 cells. In vitro cell-mediated immune response studies are pending.

Table 1. Site and Size of Metastases

	Prevaccine	Three inoculations (8 weeks)	Six inoculations (20 weeks)
Right breast			
MRI volume	15.1 ml[a]	2.46 ml	1.6 ml
MRI enhancement	>250	150	131
PET	Positive	Improved (SUV 4.4)	Improved (SUV 4.2)
Mammogram	Recurrence, calcifications	Not done	No change
Right axilla			
MRI	Not identified	Not identified	Not identified
PET	Positive	Marked improvement	Absent
CT	Not identified	Not identified	Not identified
Sternal lesion			
Isotope bone scan	Positive		Less prominent
Right lower lung lesion			
MRI	Positive	Decreased	Absent
PET (SUV)	10.2	5.3	Absent
CT	2.3 cm	Minimal	Absent
Left axilla			
MRI	1.3 cm	Less prominent	Less prominent
PET	Positive	Positive, new (SUV 3.0)	Improved (SUV 2.1)
CT	0.8 cm	Not identified	0.7 cm

[a]Computer-generated aggregate volume.

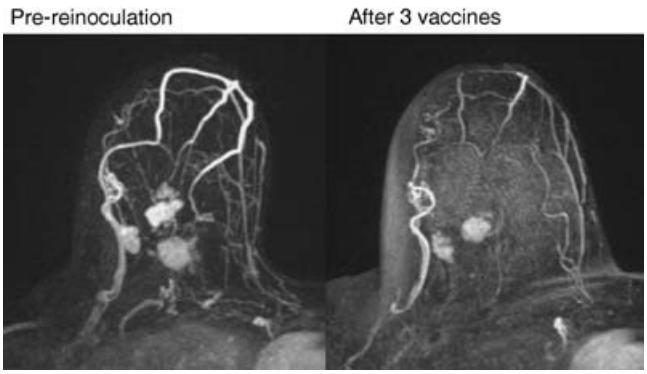

Figure 2. Reinduction treatment series. Left image: Breast preinoculation baseline study showing relapse. Note the multiple areas of contrast enhancement. Reported total volume of the tumor is 6.2 ml. Right image: Postinoculation. Note the diminution in size and number of lesions. Total volume is 3.6 ml.

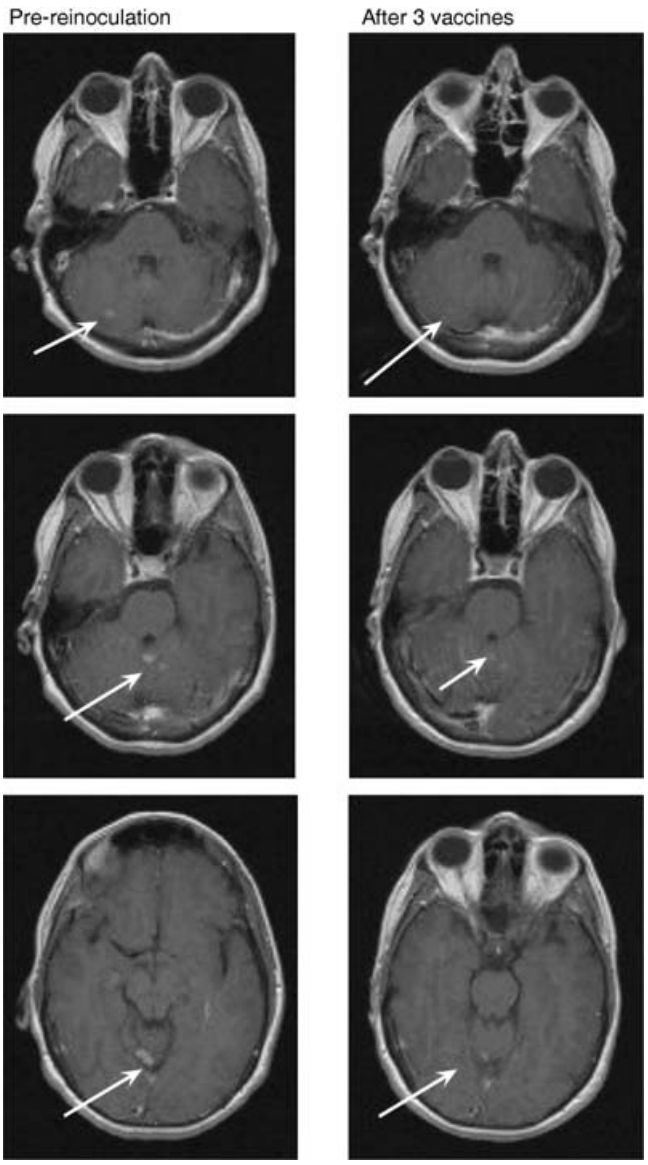

Figure 3. Reinduction treatment series. Left image: Brain preinoculation baseline study shows metastases. Right image: Postinoculation. Note the regression of three different lesions. Overall summed maximal diameters regressed from 28 mm at baseline to 11 mm after three inoculations. Note slight variation in imaging owing to patient orientation and selection of images most representative of the actual enhancing lesion.

MATERIALS AND METHODS

Vaccine

The cell line SV-BR-1 is characterized as estrogen receptor (ER) negative, progesterone receptor (PR) negative, and HER-2/*neu* very strong. The human leukocyte antigen (HLA) phenotype was A11a, A24c, B34(3514), B55a, DR11i, DR13f, DR52b, DQ3a. The cell line grew well in nude mouse and showed routine microscopy consistent with breast cancer. Likewise, electron microscopy was consistent with malignant cells of breast cancer origin. Histochemical stains showed the cell line was positive for human β-actin. The cell line has a remarkable number of chromosomal abnormalities: 57–60,XX, +1add(1)(36.3), del(1)add(1)(p36.3) add(1)(q32), i(3)(q10), add(4)(p16), +6,–10,–10,+11,+12,–14,+15,+16, add(19)(q13.4), +20,–21,–21,+11–13mar[cp20]. The cytogenetic diagnostic impression confirms human female aneuploid karyotype with multiple abnormalities involving chromosomes 1, 3, 4, 6, 10, 11, 12, 14, 15, 16, 19, 20, and 21. Quality control tests included sterility (bacterial, mycoplasma, and adventitial viruses negative), endotoxin negative, hepatitis B and C, Epstein-Barr virus, cytomegalovirus (CMV), parvovirus, human immunodeficiency virus (HIV), and *Treponema pallidum* negative by polymerase chain reaction (PCR).

Cell Transfection and Lot Release Preparation

SV-BR-1 cells were transfected with the pcDNA 3.1/GS plasmid containing open reading frame of human GM-CSF using LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA). The stable transfected cell line (SV-BR-1-GM) was propagated and the Master Cell Bank (MCB) was prepared and frozen in liquid nitrogen. The MCB was validated according to the FDA-approved biosafety panel (see above). Each lot release was generated from an MCB vial and tested for microbial contamination and GM-CSF production. The value of GM-CSF secretion for the reported lot by ELISA was 115 ng/1 $\times 10^6$ cells/24 hours.

Vaccine Formulation

Following thaw and wash procedures, 20×10^6 viable irradiated (20,000 cGy) SV-BR-1-GM cells were resuspended in 2.0 ml lactated Ringers solution (LRS) and distributed into four 1 cc syringes, 0.5 ml in each syringe.

Skin Test Methodology

An aliquot of 1.0 (± 0.1) $\times 10^6$ viable nontransfected irradiated SV-BR-1 tumor cells, each in 0.1 ml of LRS was injected intradermally into the patient's arm on the day of vaccine injection. Delayed-type hypersensitivity response (the two largest diameters of induration and erythema) was assessed at 48 and 72 hours after injection.

ELISA Antibody

Serum antibody titers to SV-BR-I antigens were determined by whole-cell ELISA. Patient's serum samples before and after vaccine treatment were preincubated with fetal bovine serum, serially diluted, and reacted with

SV-BR-1 cells. The cells were washed and incubated with peroxidase-labeled antihuman Ig antibody followed by treatment with the developing solution. The end titer of antibodies was defined as the last dilution with optical density values two times above the background level (peroxidase-labeled antibody alone).

DISCUSSION

This patient showed multiple sites having progressive disease identified by CT, MRI, and PET scans. There was clear evidence of disease progression 2 months after cessation of letrozole and the rare possibility of withdrawal response therefore seems unlikely. Also, the PET and MRI scan characteristics before immunotherapy make it unlikely these lesions at presentation were explainable by anything other than progressive metastatic breast cancer. Following the administration of whole-cell vaccine and adjuvant IFN-α, there was substantial regression with consistent findings in all three imaging methods. Multiple areas in the breast regressed in size and number, and the ipsilateral axillary node became undetectable. Figure 1 provides visual evidence of these findings.

The MRI findings show tumor regression and also changes in peak enhancement. The vascularity seems much reduced in the postvaccine image also. MRI is receiving much attention for assessing early tumor response, as both morphologic and dynamic information may be obtained (10).

This case is more remarkable because recurrence with metastases occurred following cessation of vaccine therapy, but reinitiation of vaccine inoculations led again to tumor regression, including regression of brain metastases.

The above patient response occurred in the second individual registered in our program, a program with several unique features. GM-CSF has multiple effects on the tumor-immune response equilibrium. GM-CSF has a direct effect on tumor growth (11) and activates dendritic cell recruitment and maturation and most effectively improves vaccine effects in animal models (12). The protocol employs a novel use of IFN, that is, local injection into the inoculation sites. The rationale for this injection is based on the notion that antigen alone is not sufficient to evoke a useful immune response without an additional stimulus of pathogenicity (the "danger hypothesis"). IFN-α also has a number of other physiological effects on the immune response. Work done in our group indicates that high doses of IFN-α can augment interleukin (IL)-12 p70 release and down-regulate IL-10 secretion by CD40 ligand-stimulated monocyte-derived dendritic cells (Kharazi et al., manuscript in preparation, 2006). IFN-α induces dendritic cell differentiation (3), polarizes immune response toward TH1 in animal models, facilitates effector cell function (13,14), and extends the survival of such activated cytotoxic lymphocytes (15). Therefore IFN-α may have a promising role as an adjuvant to stimulate immune response associated with vaccine strategies.

In this study, patients are not selected for HLA type or HER-2/*neu* status, although the breast cancer cell line is notable for strong overexpression of HER-2/*neu*, as well as having multiple cytogenetic abnormalities. However, the patient was recently tested for HLA phenotype, reported as A2,11; B18,44; C5,7 Bw4.6; DR11,13; DQ1,3; DR51,52,53.

The safety of the nontransfected cell line SV-BR-1 was reported in a previous study (16). In that small clinical trial, there were no grade 4 toxicities and the most severe adverse events were grade 3 vomiting in one patient, likely due to cyclophosphamide and not the vaccine. No objective responses were found, although 4 of 14 had subjective improvement and median survival of 12.1 months (range 1.2–38.7 months).

The rationale for use of low-dose cyclophosphamide, employed by our group in several previous studies, derives from reports of possibly beneficial immunomodulatory properties (2). Furthermore, low-dose cyclophosphamide was associated with improved survival in a randomized breast cancer vaccine study (17).

It is unusual for whole-cell breast cancer vaccines to elicit objective clinical regression. Wittig et al. (18) immunized 10 metastatic breast cancer patients with autologous tumor cells transfected with an IL-7 and GM-CSF construct and reported one complete, one partial, and one mixed response. Another study, using mingled autologous/allogeneic whole-cell vaccine admixed with IL-2 and GM-CSF, described partial remissions in 2 of 42 patients treated (19). Dols et al. (20) described a study with a cell line genetically modified to express the costimulatory molecule CD80, with some patients showing disease stabilization (4 of 30), but none showing tumor regression. Of interest is a recent article describing vaccination with autologous whole-cell/dendritic cell fusion preparation with the near-complete regression of a "large chest wall mass," although evidently liver, bone, and soft tissue lesions stabilized but did not diminish (9). A study by Emens et al. (21) has been entering patients to be treated with whole-cell vaccine transfected to produce GM-CSF, together with various chemotherapy agents, but definitive reports of their results are still pending.

The report by Peoples et al. (8) and the accompanying editorial by Salazar and Disis (22) argue for the application

of vaccines only for subclinical, low tumor burden subjects. That study addressed patients who were HER-2/*neu* positive and HLA A2 positive who were treated with an HER-2/*neu* peptide admixed with GM-CSF. At 22 months follow-up, the recurrence rate was 8%, compared to 22% in the control group of HLA A2-negative patients.

Our patient's tumor did not display HER-2/*neu* overexpression at diagnosis, but apparently up-regulated this marker according to histochemical findings in a small subcutaneous breast nodule biopsied at relapse (FISH data negative). The vaccine cell line, SV-BR-1-GM, was notable for strong HER-2/*neu* overexpression, but it still may be that the antigen was irrelevant or that an immune response to HER-2/*neu* triggered a more generalized set of responses, described by Disis et al. (23) as a process of "epitope spreading." This case is only the second patient enrolled in this trial; the first was characterized as stable after three inoculations and progressing after five.

Immune responses were indeed measurable, but certainly not robust. The skin test reactivity to nontransfected tumor cells was at best weak, but so too was the response to recall antigens. Evaluations of in vitro cell-mediated immune responses are pending. Whatever the mechanism, there was objective response to metastases in the breast, in lymph nodes, and in the lung after initial vaccine treatment. This response to three disparate sites is encouraging for the study of whole-cell vaccine therapy in clinically manifested metastatic breast cancer. Even more encouraging is the finding that after recurrence, restarting vaccine inoculations once again produced objective regression, even in brain metastases.

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