

Generation of Monoclonal Antibodies to Surface Proteins of Human Multiple Myeloma Employing Membrane Extracts for Murine *In Vivo* Immunizations

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Multiple myeloma is a malignancy of plasma cells that accounts for 1% of cancers worldwide and is treatable, yet incurable. U266 is an IgE-secreting, IL-6-producing human multiple myeloma cell line, against whose unique surface markers we have tried to raise monoclonal antibodies (MAbs). Female Balb/c mice (6 weeks old) were immunized through two routes of administration, with three different antigenic concentrations. Fusion efficiencies and specific efficiencies for the three antigenic concentrations were calculated. It was found that while an initial immunogen concentration of 10 µg gave the best fusion efficiency of 63.89%, an initial immunogen concentration of 15 µg resulted in the best specific efficiency of 54.54%. Out of the 147 supernatants selected for screening, 66 were found reactive to common surface proteins of non-malignant human plasma cells and seven were myeloma-specific MAbs. These MAbs have a range of potential applications in multiple myeloma imaging, phenotyping, and possible targeted therapy.

Introduction

MULTIPLE MYELOMA (MM) is a fatal hematological malignancy characterized by the accumulation of terminally differentiated plasma cells in the bone marrow of patients.⁽¹⁾ Multiple myeloma is the most common primary cancer of the bones in adults and accounts for about 1% of all cancers worldwide. The incidence of multiple myeloma is highly age and race dependent.^(1,2) Therapy for this disease has been largely palliative. It has so far included the use of alkylating agents in combination with corticosteroids, bisphosphonates for prevention of early bone disease, and most recently thalidomide, the hypnotic sedative, which is best known for its teratogenicity.⁽³⁾ Recent innovations also aim at interrupting the paracrine and autocrine secretions of the bone marrow stromal cells and myeloma cells, inhibiting angiogenesis⁽⁴⁾ and inducing innate immunity against malignant plasma cells. Despite development of new therapeutic modalities, the median survival time of patients with multiple myeloma is 2.5 to 3.5 years.⁽⁵⁾

U266 is an IgE-secreting,⁽⁶⁾ autocrine IL-6-producing,⁽⁷⁾ human multiple myeloma cell line. It is resistant to death by apoptosis due to a constitutive upregulation of NF-κB and STAT3 pathways.⁽⁷⁾ Studies on U266 have so far revealed the role of HMG-CoA reductase inhibitors,⁽⁸⁾ proteasome inhibitors such as withaferin A, IL-6 antagonists, Sant 7,⁽⁷⁾ and CDK inhibitors,⁽⁹⁾ to name a few, in inducing apoptosis in the cell line. The targeting of unique surface markers of multiple

myeloma cells, however, has not been extensively studied, probably due to the large number of surface markers that are shared among normal as well as malignant plasma cells.^(10,11) Not only is there variation among patient populations with respect to the degree of surface marker expression, but cell-surface phenotypes may vary in different treatment phases as the malignant clone becomes more or less differentiated.⁽¹¹⁾ Our present study therefore aims at the production of MAbs unique to surface markers of the U266 cell line using *in vivo* primed murine splenocytes.

Materials and Methods

U266 cells were cultured in RPMI 1640 with 20% FBS. Sp2/0 cells were cultured in DMEM with 10% FBS (Gibco, Invitrogen, Grand Island, NY). Extraction of U266 membrane-bound proteins was carried out by sequential Ready Prep protein extraction reagents (Bio-Rad Labs, Hercules, CA). Briefly, a pellet of healthy U266 cells was obtained by centrifugation at 1000 rpm for 10 min at room temperature. This was followed by a two-step extraction. First the cytosolic proteins were extracted using the Ready Prep protein extraction reagent 1, followed by the extraction of the membrane bound proteins using the Ready Prep protein extraction reagent 2. The extracts were then subjected to buffer exchange in PBS and sucrose concentrated.

Protein estimation was performed for the membrane extracts using a modified Bio-Rad protein assay. Briefly, two

TABLE 1. IMMUNIZATION SCHEDULE WITH MEMBRANE PROTEIN EXTRACTS OF HEALTHY U266 CELLS

Day of immunization	Dosage (μ g)	Route of administration	Remarks
Day 0	20	s.c.	Without adjuvant
	15	i.p.	With FCA
	10	i.p.	With FCA
Day 9	20	s.c.	Without adjuvant
	15	i.p.	With FIA
	10	i.p.	With FIA
Day 23	2	i.v.	Without adjuvant
	1.5	i.v.	Without adjuvant
	1	i.v.	Without adjuvant
Day 24	2	i.v.	Without adjuvant
	1.5	i.v.	Without adjuvant
	1	i.v.	Without adjuvant
Day 25	2	i.v.	Without adjuvant
	1.5	i.v.	Without adjuvant
	1	i.v.	Without adjuvant

concentrations (100% and 50%) of the protein extracts were prepared in the Bio-Rad Ready Prep protein extraction reagent 2. Bovine gamma globulin of 1.5 mg/mL was used as standard.

Three 6-week-old, female Balb/c mice were immunized with the membrane protein extracts of healthy U266 cells. An immunization schedule of 4 weeks with two routes of administration and three different antigenic doses was followed (Table 1). Five days prior to fusions, feeder cells were prepared from splenocytes of two non-immunized healthy female Balb/c mice (6 weeks old).

On the 28th day of immunization, splenocytes of the immunized mice were fused with Sp2/0 cells in a ratio of 5:1, with Hybri-Max (MO63103, Sigma, St Louis, MO) containing 50% [w/v] polyethylene glycol and 10% DMSO [v/v] in PBS without Ca^{++} as fusogen. Limiting dilutions were performed into four 96-well culture plates containing feeder cells per antigenic concentration. The cultures were replenished every 5 days with DMEM-HAT (HAT supplement, Gibco) medium containing 20% spent medium from syngenic mouse splenocyte cultures as conditioned medium. The fusion and specific efficiencies for each antigen concentration were calculated on

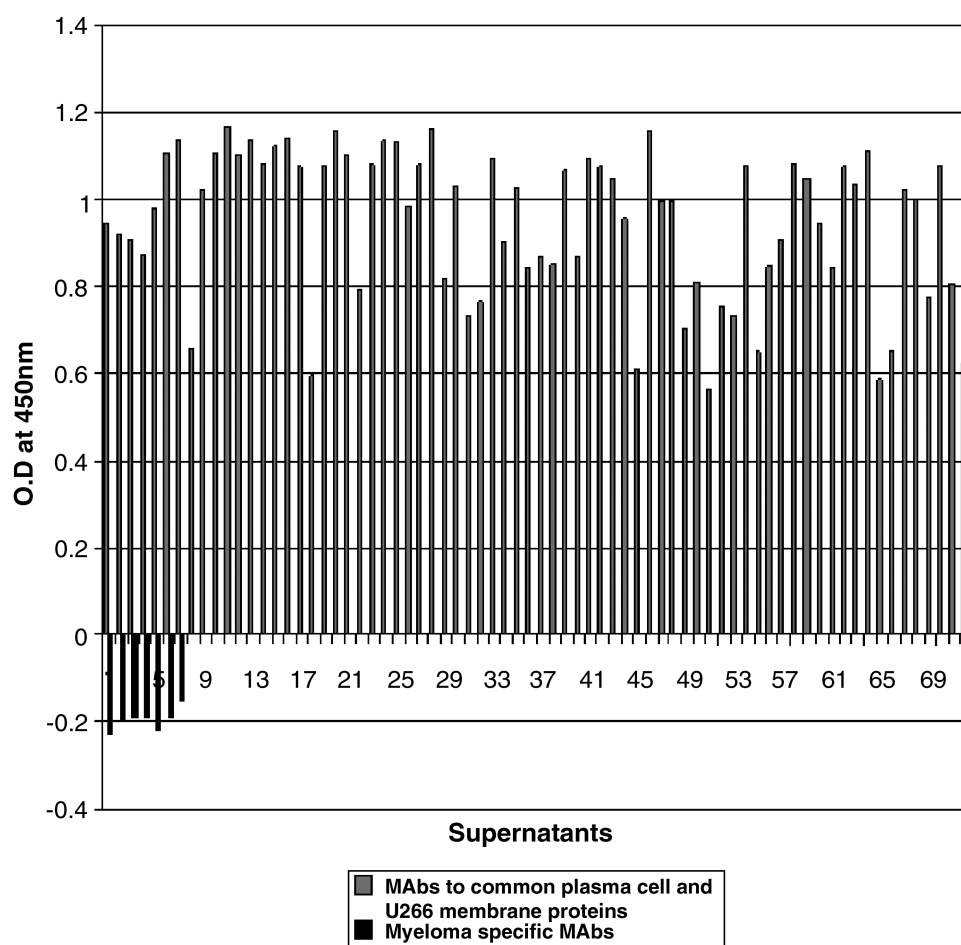


FIG. 1. ELISA screening results showing MAbs to common surface proteins of normal plasma and myeloma cells and MAbs to myeloma-specific surface proteins.

days 14 and 48, respectively. The formulae for fusion and specific efficiencies are as follows:

$$\text{Fusion efficiency} = \frac{\text{No. of wells with healthy heterokaryons}}{\text{Total no. of wells seeded}} \times 100$$

$$\text{Specific efficiency} = \frac{\text{No. of wells with positive secretory hybridomas}}{\text{No. of wells with healthy heterokaryons}} \times 100$$

For arriving at the specific efficiency, supernatants from the limiting dilution wells with sufficient metabolic activity were screened by ELISA for the presence of murine anti-U266 membrane proteins IgG. Membrane protein extract (20 µg) from the U266 cells (which were used as the immunogen) was coated onto ELISA plates for initial screening to ascertain the total number of positive clones. The refed medium (DMEM-HAT + 20% FBS + 20% CM) was used as blanks. Each positive supernatant from the initial screening was further ELISA tested with membrane extracts from non-malignant human plasma cells to identify myeloma-specific MAbS. Those supernatants with absorbance values of <1 for non-malignant plasma cell membrane extracts and >2 for myeloma membrane protein extracts were considered specific towards multiple myeloma membrane proteins.

Results

The protein concentration of whole membrane proteins of U266 obtained was found to be 387 µg/mL. The fusion efficiencies were estimated to be 45.42, 44.58, and 63.89% for the 20, 15, and 10 µg initial immunogen concentrations, respectively. 48 days post-fusion, a total of 150 clones were identified for screening and calculations of specific efficiencies. Initial ELISA-based screening helped identify 73 positive secretory clones (Fig. 1), of which the second set of fusions (initial antigen concentration of 15 µg) gave the highest number (54.54%). The specific efficiency for an initial immunogen concentration of 20 µg and 10 µg were found to be 52.54% and 33.33%, respectively. Of the 73 secretory clones, seven secreted MAbS specific to U266 membrane proteins. Five of these were from splenocytes obtained from the mouse initially immunized with 20 µg of the immunogen.

Discussion

Although *in vivo* immunizations typically require milligram quantities of antigen to be administered, we have shown here that microgram quantities administered by the routes of immunization we have chosen, as well as the immunization schedule we have followed, are sufficient to provide an adequate IgG immune response. From the specific efficiencies obtained, it is apparent that an initial antigen concentration of 15 µg administered intraperitoneally gave the best response. Overall we identified that almost one half of all the supernatants that were subjected to an initial ELISA-based screening showed sufficient levels of murine IgG. This is quite a significant number and this observation can be explained by the *in vivo* immunization approach along with the other conditions described in this study.

Although normal and malignant plasma cells share a large number of surface proteins, we could identify MAbS unique to

multiple myeloma. Considering that such unique specific markers are rather few for multiple myeloma, this study demonstrates that whole membrane extracts with solubilized proteins can provide us with useful MAbS by either intraperitoneal or subcutaneous primary immunizations, based on the immunogenic dose. It is also understood that the subcutaneous route for primary immunization required a higher antigenic dose compared to a lesser antigenic dose when primary administration is by the intraperitoneal route. This study demonstrates a method for the production of MAbS unique to cell surface proteins of human multiple myeloma. These antibodies have a potential range of applications not limited to imaging, diagnostics, and therapy.

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