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ORIGINAL ARTICLE

Jesper Max Tordsson · Lennart Göran Ohlsson
Lars Bertil Abrahmsén · Pia Jasmine Karlström
Peter Anders Lando · Thomas Nils Brodin

Phage-selected primate antibodies fused to superantigens for immunotherapy of malignant melanoma

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Abstract The high-molecular-weight melanoma-associated antigen, HMW-MAA, has been demonstrated to be of potential interest for diagnosis and treatment of malignant melanoma. Murine monoclonal antibodies (mAb) generated in response to different epitopes of this cell-surface molecule efficiently localise to metastatic lesions in patients with disseminated disease. In this work, phage-display-driven selection for melanoma-reactive antibodies generated HMW-MAA specificities capable of targeting bacterial superantigens (SAg) and cytotoxic T cells to melanoma cells. Cynomolgus monkeys were immunised with a crude suspension of metastatic melanoma. A strong serological response towards HMW-MAA demonstrated its role as an immunodominant molecule in the primate. Several clones producing monoclonal scFv antibody fragments that react with HMW-MAA were identified using melanoma cells and tissue sections for phage selection of a recombinant antibody phage library generated from lymph node mRNA. One of these scFv fragments, K305, was transferred and expressed as a Fab-SAg fusion protein and evaluated as the tumour-targeting moiety for superantigen-based immunotherapy. It binds with high affinity to a unique human-specific epitope on the HMW-MAA, and demonstrates more restricted cross-reactivity with normal smooth-muscle cells than previously described murine mAb. The K305 Fab was fused to the superantigen staphylococcal enterotoxin A (D227A) [SEA(D227A)], which had been mutated to reduce its intrinsic MHC class II binding affinity, and the fusion protein was used to demonstrate redirection of T cell cytotoxicity to melanoma cells in vitro. In mice with severe combined immunodeficiency, carrying human melanoma tumours, engraftment of human lymphoid cells followed by treatment with the K305Fab-SEA(D227A) fusion protein, induced HMW-MAA-specific tumour growth reduction. The phage-selected K305 antibody demonstrated high-affinity binding and selectivity, supporting its use for tumour therapy in conjunction with T-cell-activating superantigens.

Key words HMW-MAA · Phage display · Monoclonal antibody · Primate · Superantigen

J.M. Tordsson (✉) · L.G. Ohlsson · P.J. Karlström
P.A. Lando · T.N. Brodin
Active Biotech Research AB, SE-220 07 Lund, Sweden
e-mail: Jesper.Tordsson@activebiotech.com
Tel.: +4646-191255
Fax: +4646-191134

L.B. Abrahmsén
Pharmacia and upjohn AB, S-112 87 Stockholm, Sweden

Introduction

The incidence of malignant melanoma has increased more than twofold during the last 10 years. The most critical issue for a successful treatment of these patients is the time of diagnosis, since early stages of malignant melanoma can be efficiently cured by surgery, while survival is poor for patients with advanced disseminated disease for which no effective treatment is available [2]. Melanoma is one of the most immunologically active solid tumours, which suggests immunotherapy in advanced-stage disease or for adjuvant therapy.

Both cytotoxic T cells, which specifically lyse tumour cells in vitro, and antibodies against tumour-associated antigens can be detected in melanoma patients [45]. Single cases demonstrating natural regression of the primary tumour suggest involvement of host-mediated immune response mechanisms in melanoma malignancy [9]. Current immunotherapeutic approaches include methods intended to augment or alter the T-cell-based antitumour response and the use of monoclonal antibodies raised against melanoma-associated antigens for targeted delivery of effector molecules [22, 38].

The high-molecular-mass melanoma-associated antigen, HMW-MAA, is homogeneously and strongly expressed in the majority of melanoma cell lines and tissue specimens, but has a limited distribution in normal tissues

[36]. The antigen represents a well-documented immunological target molecule in applications for diagnosis/imaging and immunotherapy of malignant melanoma using naked antibodies [4, 23, 32, 36]. The use of anti-HMW-MAA antibodies for the delivery of potent effector molecules may offer an improved therapeutic potential, provided that systemic toxicity is not substantially increased by the introduction of such molecules [35].

Certain strains of *Staphylococcus aureus* produce immunostimulatory exotoxins such as the staphylococcal enterotoxin A (SEA). These proteins have been called superantigens because of their potent ability to activate, in an MHC-dependent fashion, a high frequency of T lymphocytes (defined by their T cell receptor Vβ gene family expression) to become cytotoxic and produce cytokines. In contrast to normal antigens, superantigens bind as unprocessed proteins outside the antigen-binding groove of the MHC II molecules on antigen-presenting cells [44].

To target an immune attack against tumour cells, we have genetically engineered tumour-reactive superantigens by the construction of a fusion protein between the superantigen SEA and a tumour-cell-binding Fab fragment. This fusion protein redirects T cell cytotoxicity and promotes the release of tumoricidal cytokines in the microenvironment of tumour cells recognised by the Fab [5]. To reduce systemic toxicity, the superantigen moiety was mutated in a region important for MHC II binding, SEA(D227A) [5]. This mutation does not alter the MHC-II-independent T-cell-activating capacity determined in vitro [41]. The use of different antibody specificities in the fusion proteins permitted lysis of various cell types, demonstrating the general applicability of superantigens for tumour therapy [5, 20, 26, 49].

The gene for the antibody fragment to be fused with the SEA gene can be cloned from murine-antibody-producing hybridoma cells. However, murine antibody Fab fragments may elicit neutralising human anti-(mouse Ig) antibodies when injected into humans. The diversity of antibody specificities generated in this way is also limited to those tumour-associated antigens that demonstrate immunodominance in the mouse. To circumvent these difficulties, phage display of antibody fragments is a powerful tool for selecting the specific antibodies and their encoding gene [21].

Antibody phage libraries from a variety of immunised animal species, and naive and semisynthetic sources of 10^{10} and beyond can be examined efficiently by the antigen-driven selection principle. Phage selection methods, based on the use of complex antigens such as cell suspensions, have been described [11, 21, 25]. Recently we reported a method to select antibody phage using tumour tissue sections [47]. Taken together these methods enable the use of various repertoires for identification of novel antibody specificities against cell-surface antigens expressed in vivo.

In this study, we have used an antibody phage library derived from a tumour-immunised cynomolgus monkey (*Macaca fascicularis*) for phage selection using melanoma cells and tissue sections. The approach was based on previous reports that primate antibody repertoires can be used successfully for the production of selective immune sera to tumour-associated antigens [8]. Several monoclonal cynomolgus antibodies were selected that were specific for the HMW-MAA. One of these antibodies, K305, which recognises a unique and selectively expressed epitope on the human HMW-MAA molecule, was fused as a cynomolgus Fab fragment to SEA(D227A). T cell lysis of melanoma cells and in vivo tumour reduction mediated by this antibody were demonstrated and suggest its applicability for immunotherapy of malignant melanoma.

Materials and methods

Animals and immunisation procedures

Female mice (CB-17) with severe combined immunodeficiency (SCID) were obtained from Bommice, Ry, Denmark, and kept under pathogen-free conditions. The animals were acclimatised to laboratory conditions (20 ± 1 °C, 50 ± 5% relative humidity) for about 1 week before commencement of the experiments. The animals were subjected to visual inspection on arrival and housed in Macrolone cages (III) with ten animals in each cage. After randomising, the animals were used for the experiments. The mice were fed sterile pelleted rodent diet from Special Diets Services, Essex, UK and sterile water was always available ad libitum. Mice aged 8–12 weeks and with body weights of 17.0–26.0 g (Mean ± SEM = 20.6 ± 0.15 g) were used in this study. Cynomolgus monkeys (two individuals) were kept and immunised at the Swedish Institute for Infectious Disease Control (SIIDC), Stockholm. The monkeys were immunised s.c. with 2 ml crude mechanical suspension of 43 different metastatic melanoma tissues in phosphate-buffered saline (PBS) with 10% normal cynomolgus serum mixed with alum adjuvant. Booster doses were given on days 21, 35 and 49. The immune serum was collected on day 56. This large number of melanoma samples was used to select for an antibody response to commonly expressed melanoma-associated antigens.

All animals in the study were kept according to the Swedish legislation and the local ethical committee approved the animal studies.

Tissues and cells

Human tumours and normal tissue samples and peripheral blood mononuclear cells (PBM) from blood donors were obtained from Lund University Hospital and Malmö Academic Hospital, Sweden. The PBM were isolated by density centrifugation over a Ficoll-Paque cushion (Pharmacia, Uppsala, Sweden). The human melanoma cell lines FM3 and FM55 were kind gifts from Dr. Jesper Zeuthen (Copenhagen) whilst the FMEX and G361 cell lines were obtained from the American Type Tissue Culture Collection (ATCC). The cells were cultured in RPMI-1640 medium (Gibco, Middlesex, UK) supplemented with 2 mM L-glutamine (Gibco, Middlesex, UK), 0.01 M HEPES (Biological Industries, Israel), 1 mM $NaHCO_3$ (Biochrom KG, Berlin, Germany), 0.1 mg/ml gentamicin sulfate (Biological Industries, Kibbutz Beit Haemek, Israel), 1 mM sodium pyruvate (JRH Biosciences Industries, USA) and 10% heat-inactivated fetal bovine serum (FBS; Gibco Middlesex, UK) at +37 °C and 4% CO_2. The cells were repeatedly tested for *Mycoplasma* contamination with a Gene-Probe (San Diego, Calif.) *Mycoplasma* T. C. test.

Vector and phage library construction

A detailed construction of the scFv library, including a table of all primers, has been reported previously [47]. Briefly, immunoglobulin

λ and heavy-chain variable genes were amplified from cynomolgus lymph node cells by the polymerase chain reaction (PCR) and then assembled to scFv genes (also by PCR). Restriction sites (*Esp*3I) were added to the scFv genes by primer extension to permit insertion into the *Mlu*I-*Xho*I sites of the phagemid vector such that the scFv genes became fused to a truncated form of the phage gene III (residues 249–406). The size of the assembled melanoma library was approximately 3×10^7 primary transformants.

The phagemid library vector contained a *phoA* promoter for expression of the scFv-gIII fusion genes. An *Escherichia coli* heat-stable toxin II signal peptide and an amber stop codon between the scFv and gIII genes permitted the production and secretion of soluble scFv in non-suppressor strains. A sequence coding for the ATPAKSE detection tag, recognised by polyclonal rabbit antibodies, was inserted on the C-terminal side of the scFv gene.

Selection of the phagemid library

Both tissue sections and cell suspensions were used for the phage selections. For the tissue-based selections, cryosections were air-dried on slides, fixed in acetone at −20 °C for 10 min and rehydrated in 20% FBS in 50 mM TRIS pH 7.6/150 mM NaCl (TBS) for 20 min at room temperature. Library phage suspensions, 10^{10}–10^{11} in 100 µl 20% FBS, were preabsorbed on sections of normal spleen or small bowel overnight and then transferred to slides with melanoma tissue sections (the melanoma tumour samples used were from different patients in each round to promote selection towards frequently expressed tumour-associated antigens) for overnight incubation at 4 °C. The slides were given six 10-min washes by gentle agitation in 40 ml TBS in 50-ml Falcon tubes and two 5-min washes in 10 mM TRIS/1 mM EDTA/150 mM NaCl/6 mM $CaCl_2$/pH 8.0 (Genenase buffer). Phages were eluted with 400 µl, 33 µg/ml Genenase for 30 min after which 80 µl PBS 5% bovine serum albumin (BSA) was added. The washings and elution steps were all performed at room temperature.

For cell selections, library phage suspensions, 10^9–10^{10} in 100 µl PBS/1%BSA, were incubated with 3×10^6 cells for 1 h on ice. The cells were washed three times including a 10-min incubation period, using 2 ml PBS/1% BSA for each wash. The phages were eluted from the cells with 50 µl 33-µg/ml Genenase for 15 min. The cells were removed after centrifugation and 250 µl 1% BSA/PBS added to the supernatant. The phage titres were determined by infecting bacteria and counting colony-forming units using the bacterial strain *E. coli* DH5αF′. Phage rescue and culture of the phage library were according to standard techniques [47].

Production of SEA(D227A) fusion proteins

An expression plasmid vector, carrying a kanamycin-resistance gene and the *lac* promoter, was constructed for cassette insertion of scFv genes to obtain in-frame fusion with a flexible spacer of 18 residues followed by the mutant D227A of the superantigen staphylococcal enterotoxin A (SEA) [13]. A *Spe*I site 3′ to the *pho*A promoter and the *Xho*I site following the scFv were used to excise the signal peptide scFv-encoding fragment from the phagemid vector. Single colonies of *E. coli* UL635, transformed by electroporation with the expression vector containing the inserted scFv-SEA(D227A) genes, were transferred to 96-microwell plates (Nunc, Denmark) and grown for 17 h at 30 °C in 2 × YT medium (Yeast-extract Tryptone, sodium choloride) supplemented with 70 µg/ml kanamycin and 2% glucose. Small aliquots (5 µl) were transferred to plates with fresh medium containing antibiotics but without glucose, and cultured at 30 °C for 17 h. The microwell plates were centrifuged at 4000 rpm for 7 min and 100-µl samples of the supernatants were transferred to new plates with an equal volume of 1% BSA in each well. Additional induction using isopropyl thiogalactoside did not increase production of soluble fusion protein in this system (data not shown).

The fusion proteins were quantified in a sandwich-type enzyme-linked immunosorbent assay using rabbit anti-SEA antibodies for capture and biotinylated anti-SEA Ig as detector antibodies. The production of the K305Fab-SEA(D227A) and the recombinant control fusion protein C215Fab-SEA(D227A) was performed as previously described [12].

Determination of the affinity of fusion protein binding to tissue sections and cells

The K305Fab-SEA(D227A) fusion protein (80 µg) was iodinated with 2 mCi $Na^{125}I$ according to the iodogen method. Cryosections (8 µm) were air-dried on multi-well slides, fixed in acetone at −20 °C for 10 min and rehydrated in 20% FBS. Iodinated K305Fab-SEA(D227A) in 20% FBS was added in twofold dilution series to the sections and incubated for 1 h. The sections were washed four times with TBS and dried before the bound radioactivity of individual wells was quantified in a gamma counter. For affinity determination, 30 000 FM3 cells/sample were incubated with iodinated K305Fab-SEA(D227A) at 100 µl/tube in a series of twofold dilutions in 1% BSA/PBS for 1 h and then washed three times in PBS before measurement of bound activity. Scatchard plots were constructed using the measured values for bound and total radioactivity.

Sequencing of antibodies

The K458, K460 and K743 scFv antibody genes inserted into the expression vector were sequenced in both directions by SEQUISERVE (Vaterstetten, Germany). Briefly, 1.0 pmol/reaction plasmid DNA and 10 pmol/reaction primer DNA were used for automatic sequencing on a PerkinElmer/Applied Biosystem model 373 A sequencer. The primers (20 base pairs each) annealed in the Lac promoter and in the SEA(D227A) gene, 39 bp upstream of the *Spe*I and 94 bp downstream of the *Sal*I insertion site of the scFv gene and the annealing temperatures were 60.1 °C and 64.9 °C, respectively. K305 and K461 scFv, were sequenced at Pharmacia and Upjohn AB, Stockholm, Sweden, using sequencing equipment from the same supplier.

Immunohistochemistry

All tissues were snap-frozen in 2-methylbutane pre-chilled in liquid nitrogen. Cryosections (8 µm) were air-dried on slides, fixed in acetone at −20 °C for 10 min and rehydrated in 20% FBS. Endogenous biotin was blocked with consecutive 15-min incubations with avidin and biotin (Vector Laboratories, Burlingame, Calif.), each diluted 1/6 in 20% FBS. If not otherwise specified, primary scFv-SEA (D227A) fusion proteins were incubated at a concentration of 5 µg/ml and mouse monoclonal antibodies at 1 µg/ml for 1 h at room temperature. Antibodies, avidin and biotin were all diluted in 20% FBS/TBS. Affinity-purified and biotinylated rabbit anti-SEA antibodies (5 µg/ml) and biotinylated rabbit anti-(mouse immunoglobulins) (Dako A/S, Denmark), diluted 1/300, were incubated for 30 min at room temperature followed by incubation for 30 min at room temperature with StreptABComplex HRP (Dako A/S, Denmark), diluted 1/110 in 50 mM TRIS pH 7.6. Between all steps the sections were washed three times in TBS. The staining reaction was developed for 8 min in 0.5 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (Saveen Biotech AB, Sweden) dissolved in TRIS pH 7.6 with 0.01% H_2O_2. After 10 min of counterstaining in 0.5% methyl green, the slides were rinsed for 10 min in tap water and gradually dehydrated in 70%–99% ethanol and xylene before mounting in DPX medium (Sakura Finetech, Calif.). The mouse antibodies specific for HMW-MAA, TP41.2 and 763.74, were a kind gift from Dr. Soldano Ferrone (New York Medical College and mAb 9.2.27 was obtained from PharMingen, (San Diego, Calif).

Flow cytometry

The melanoma cells FM3, FM55, FMEX-1 and G361 were dissociated with 0.02% w/v EDTA from the cell-culture flasks (Sarstedt

Inc. Newton, N.C., USA). Samples containing 3×10^6/50 µl, were incubated for 1 h at 4 °C with primary scFv-SEA(D227A) fusion proteins or cynomolgus serum, for 30 min with secondary biotinylated rabbit anti-SEA antibodies or anti-(human IgG) (crossreacting with cynomolgus IgG and used for detection of antibodies in serum of immunised animals southern Biotechnology, Al.), and finally for 30 min with fluorescence-isothiocyanate-labelled avidin. The reagents were diluted in 1% BSA/PBS, which was also used for washing cells twice after all incubation steps. Flow-cytometric analysis was performed in a FACSort flow cytometer (Becton Dickinson, Mountain View, Calif.).

Immunoprecipitation and sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE)

FM3 cells were iodinated by the use of a chloramide method as previously described [13]. Briefly, 50 µg 1,3,4,6-tetrachloro[*3a*] diphenylglycoluril was dissolved in methylene chloride and then air-dried in a glass tube. FM3 cells (13×10^6 cells in 0.5 ml PBS) and 0.5 mCi carrier-free ^{125}I were incubated for 10 min on ice with mild agitation. After two washes in PBS the cells were incubated in potassium iodide (50 µg/ml) in PBS, for 20 min on ice to reduce non-specific incorporation of ^{125}I and finally washed twice more in PBS. The iodinated cells were lysed in 2 ml sucrose/NP40 buffer (0.25 M sucrose/50 mM TRIS/1.5 mM $MgCl_2$/10 mM KCl/1% NP40/protease inhibitor cocktail tablets (Boehringer Mannheim, Germany) for 30 min on ice. The lysate was diluted by adding 15 ml PBS and ultracentrifuged for 1 h at 100 000 *g* at 4 °C. For immunoprecipitation, 10 µg primary antibody was incubated with 1 ml lysate for 1 h before the addition of 100 µg rabbit anti-SEA antibodies, which were incubated for 20 min and finally with 100 µl Sepharose protein G beads for 20 min. The beads were washed twice with 50 mM TRIS/500 mM NaCl/0.1% NP40. SDS-PAGE under reducing conditions, using a 4% TRIS/glycine polyacrylamide gel, and Western blotting were performed according to standard methods. Autoradiography using hyperfilm-MP (Amersham, Sweden) was performed for 1 week at −70 °C.

Cytotoxicity assay

Redirected T cell cytotoxicity (superantigen-antibody-dependent cellular cytotoxicity) was measured in a standard 4-h chromium-release assay employing ^{51}Cr-labelled FM3 or G361 cells as target cells and human T cells as effector cells, as previously described [1]. Percentage cytotoxicity was calculated as

$$100 \times \frac{\text{experimental release (cpm)} - \text{background release (cpm)}}{\text{total release (cpm)} - \text{background release (cpm)}}$$

Double-determinant assay for epitope mapping

A Nunc Maxisorb 96-well plate (Nunc, Denmark) was coated with 35 µl/well mAb 9.2.27 at 1 µg/ml or control antibody overnight at 4 °C. The unbound sample was removed and non-specific binding sites were blocked with 3% low-fat milk (Semper, Sweden) for 1 h at room temperature. The low-fat milk was removed, and incubation for 2 h at room temperature with the NP-40 extract of melanoma cells diluted 1/10 in 3% milk was performed. The plate was washed four times in PBS/0.05% Tween 20 after this and the subsequent steps. The scFv or Fab-SEA(D227A) fusion proteins at 30 µl/well diluted to 1 µg/ml, or cynomolgus serum diluted 1/50–1/100 000 were incubated for 1 h at room temperature. The secondary antibody, 1 µg/ml biotinylated rabbit anti-SEA antibodies or rabbit anti-(human Ig), was incubated for 1 h at room temperature, and finally horseradish-peroxidase-labelled extravidin (Sigma, St. Louis, Mo.) 1/1000 was added and incubated for 30 min at room temperature. The colour reaction was developed using 3,3′-diaminobenzidine (Sigma) according to the instructions of the supplier and read at 405 nm.

Tumour therapy in SCID mice

The experiments were performed as outlined [27]. Briefly, SCID mice were injected i.p. with 3×10^6 FM3 cells in 0.2-ml vehicle (PBS/1%Balb/c mouse serum) on day 1. The mice were injected i.p. with 3×10^6 PBM in 0.2 ml vehicle 4 days later. Between 1 h and 2 h after injection of lymphoid cells, all mice were injected i.v. with 100 µg test substance in 0.2 ml vehicle or with vehicle alone. Two additional i.v. injections of the test substance were given at 3-day intervals. The mice were sacrificed by cervical dislocation on day 49 and the number of tumours and the tumour mass were determined. Tumours weighing less than 5 mg were estimated to be 2 mg, tumours with a weight of more than 5 mg and less than 10 mg to be 7 mg and for tumours larger than 10 mg the actual weight was used. All tumours larger than 1 mg were counted. Each treatment cohort contained five to seven mice to permit comparison to other treatment cohorts treated simultaneously with the same batch of effector cells. Statistical significance was determined by the Mann–Whitney *U*-test using the program Sigma Stat.

Results

Immunisation of cynomolgus monkeys produced a high serum titre against human melanoma cells and HMW-MAA

Two cynomolgus monkeys (*Macaca fascicularis*) were immunised on days 0, 21, 35 and 49 with alum-precipitated crude suspensions of human melanoma tumour tissue. The immune sera collected on day 56 were tested for reactivity against melanoma cells and against the high-molecular-mass melanoma-associated antigen (HMW-MAA). The immune serum of both animals collected on day 56, but not the pre-immune serum, bound to surface antigens on FM3 human melanoma cells as determined by flow cytometry. Even at a dilution of 1:40 000, a minor shift in fluorescence could be detected compared to that of the pre-immune serum at the same dilution (Fig. 1A). A double-determinant assay was used to analyse serum reactivity against the human HMW-MAA. The immune serum could be diluted 25 000 times before reactivity reached the background level of the pre-immune serum (Fig. 1B). No reactivity was found when the melanoma extract or the capture antibody was omitted or when a capture antibody (mAb C215) was used against an epithelial antigen not present in the melanoma extract.

HMW-MAA specific scFv antibodies could be identified from a cynomolgus phage library selected using melanoma cells and tissue sections

The scFv antibody phage library was selected using cryosections of human tissues and melanoma cell lines. The yield of library phage (counted as colony-forming units) increased substantially after three repeated selections on cells or tissue sections (Table 1). Soluble proteins from 200 individual scFv antibody clones were produced from each of the selected libraries from the last two selection rounds. These antibodies were screened for

binding to melanoma tissue sections and melanoma cell lines by immunohistochemistry and flow cytometry. A secondary antibody directed against the immunological tag, ATPAKSE, was used for the detection in these assays. These screening systems permitted a preliminary evaluation of melanoma reactivity. Positive clones were then recloned in fusion with SEA(D227A). The latter format permitted both higher production yields in culture supernatants and a more sensitive detection on tissue sections using a rabbit antiserum against SEA.

Alternatively to the recloning of individual scFv, an aliquot of the selected library was recloned into the SEA(D227A) vector for a direct screening in this format. Several scFv-SEA(D227A) antibody-fusion proteins (Table 1) were identified that had an immunohistochemical staining pattern similar to the pattern described for murine antibodies against the HMW-MAA (see below). All of these antibodies, except K743 (third round), were identified from the second selection round.

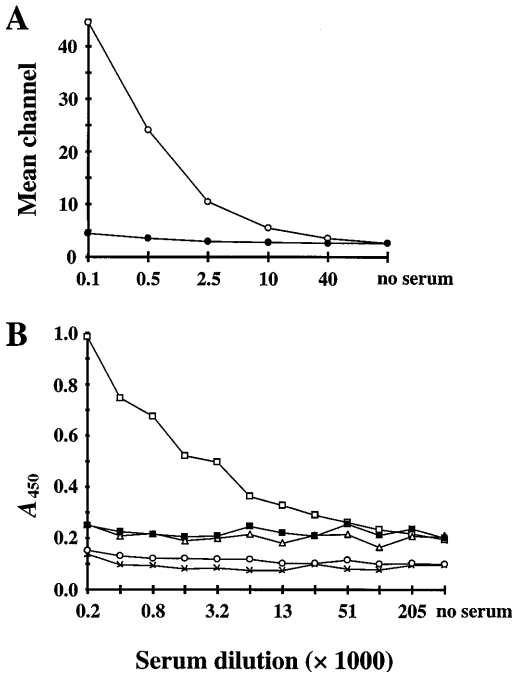


Fig. 1 **A** Flow cytometry demonstrating strong reactivity against human FM3 melanoma cells of a melanoma immune Macaca fascicularis serum collected on day 56 (○) compared to the preimmune serum (●). **B** Specific serum reactivity against the high-molecular-mass melanoma-associated antigen (HMW-MAA) determined by a double-determinant assay. The HMW-MAA was captured from a NP40 extract of FM3 melanoma cells with the monoclonal antibody 9.2.27 and then detected with the immune serum (□) or the preimmune serum (■). As further negative controls, either the irrelevant murine mAb C215 (△) or no antibody (○) was used for capture or the melanoma extract was omitted (×)

The phage-selected antibodies immunoprecipitate a 250-kDa chondroitinase-sensitive protein

The phage-selected antibodies [scFv/Fab-SEA(D227A) fusion proteins] were used for immunoprecipitation using an extract of ^{125}I-surface-labelled FM3 melanoma cells. The molecular masses of the precipitates were determined by SDS-PAGE. Three antibodies precipitated antigens with a molecular mass that distributed as a high-molecular-mass smear from a distinct 250-kDa band. Digestion of the precipitate with chondroitinase resulted in a single 250-kDa band with increased intensity. The molecular mass and the sensitivity to chondroitinase suggest that the antigens recognised are identical to the HMW-MAA precipitated in parallel by the murine mAb 9.2.27 (Fig. 2).

Epitope mapping of the selected antibodies

The binding of selected antibodies was epitope-mapped relative to each other and to murine anti-HMW-MAA mAb. The antibodies, FabK305, scFvK458, scFvK461 and FabK743 fused to SEA(D227A) were produced by fermentation and purified by affinity chromatography using immobilised polyclonal rabbit anti-SEA Ig (results not shown). The yields from 1-l fermentor cultures of the fusion proteins were 35 (K305), 47 (K458), 118 (K461) and 9.5 (K743) mg, respectively. HMW-MAA proteoglycan in the melanoma extract was captured with

Table 1 Library selections on cells and tissue sections. *HMW-MAA* high-molecular-mass melanoma-associated antigen, *ND* not determined

Negative/positive selection materials	Selection rounds	Library phage yield	Frequency of binders (%)	Identified HMW-MAA specificities
FM3 cells	1	6.8×10^{-6}	2.2	
	2	9.3×10^{-4}	53	K305
	3	1.1×10^{-1}	100	
M55M1 cells	1	2.0×10^{-5}		
	2	1.5×10^{-4}	11	None
	3	5.4×10^{-2}	7.5	
Spleen/melanoma tissue sections	1	1.6×10^{-6}		
	2	4.6×10^{-7}	24	K458, K460, K461
	3	1.3×10^{-3}	57	
Small-bowel tissue sections/FM3 cells	1	1.3×10^{-4}	ND	
	2	3.5×10^{-6}		K743
	3	1.3×10^{-3}		

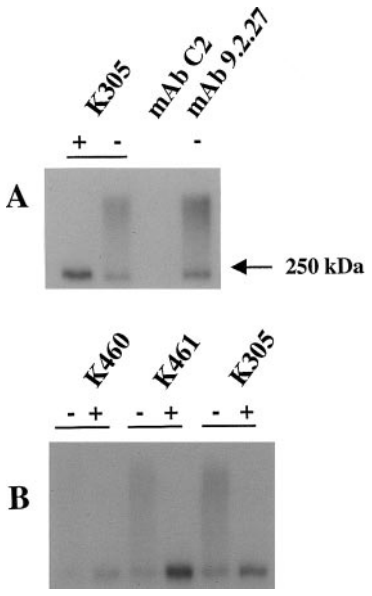


Fig. 2A, B Immunoprecipitation of HMW-MAA with phage-selected specificities from a 1% NP40 extract of FM3 melanoma cells, surface-labelled with ^{125}I. The molecular mass of the precipitates were determined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis on a 4% gel under reducing conditions. **A** Precipitation with the K305Fab-SEA(D227A) fusion protein. Enzymatic cleavage with chondroitinase (+) eradicated the high-molecular-mass product and increased the amount of the 250-kDa band. Precipitates using the murine mAb 9.2.27 recognising HMW-MAA or a murine negative control mAb C2 are shown for comparison. **B** SEA(D227A) fused to proteins K305, K460 and K461 precipitated proteins sensitive to chondroitinase, of identical molecular mass

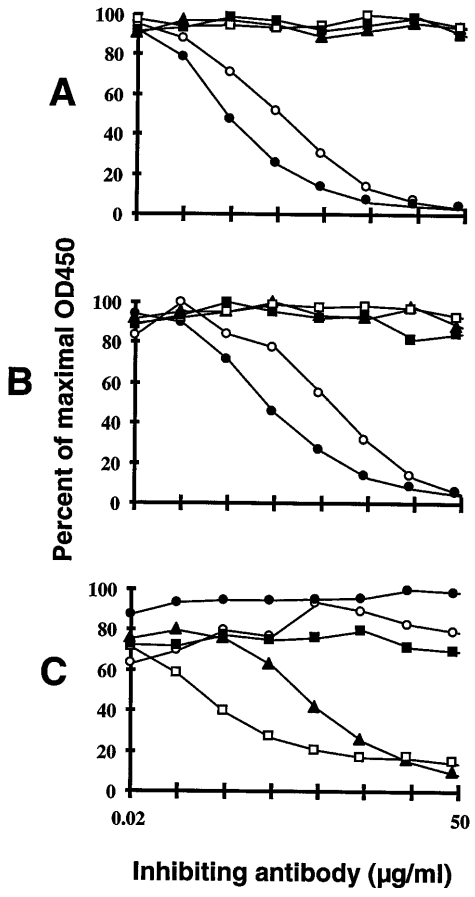


Fig. 3A–C Epitope mapping of scFv/Fab-SEA(D227A) fusion proteins. The HMW-MAA was captured by mAb 9.2.27 from the melanoma extract and detected with biotinylated scFv/Fab-SEA(D227A) fusion proteins. Binding to HMW-MAA of the biotinylated K305 Fab fusion proteins (**A**) K305 Fab (**B**) K461 scFv and (**C**) K458 scFv inhibited by the non-biotinylated fusion proteins K305 Fab (○), K461 scFv (●), K458 scFv (▲) or with the murine anti-HMW-MAA mAbs TP41.2 (□) or 763.74 (■). The fusion proteins K305 Fab and K461 scFv inhibited each other and K458 scFv was inhibited by the murine mAb TP41.2. Neither of the fusion proteins was inhibited by the mAb 763.74. No specific signal was achieved when the biotinylated negative control C215Fab-SEA(D227A) reagent was used (not shown)

mAb 9.2.27. The binding of biotinylated fusion proteins to the captured antigen was competed by non-biotinylated fusion proteins. Alternatively, K305Fab-SEA(D227A) was used as the capture reagent and murine antibodies were used to inhibit binding of the biotinylated fusion proteins to confirm the mapping results (not shown). K305 inhibited K461 (and vice versa) but not K458. The murine anti-HMW-MAA mAb TP41.2 or 763.74 did not inhibit K305 and K461 whereas TP41.2 inhibited the antibody K458. The antibody K743 did not demonstrate any detectable binding to the proteoglycan in this assay despite its identical immunohistochemical staining pattern. Thus, in this assay binding to two different epitopes on the HMW-MAA could be verified, defined by K305/K461 and K458 (Fig. 3).

Classification of antibodies based on homology to Vh and Vλ sequences

The cynomolgus antibodies fall into three groups based on the sequence similarity: (i) K305 and K461, (ii) K458 and K460 and finally (iii) K743. Comparisons with the closest human germline sequence of the variable light (λ) and heavy-chain genes demonstrated 82%–90% sequence identity on the nucleic acid level (Table 2). All of the antibodies had heavy chains with close homology to the VH3 family of human germline segments. Three human germline λ family homologues were represented by the selected antibodies, Vλ1, Vλ2 and Vλ3.

Demonstration of high affinity binding to cells and tissue sections by K305Fab-SEA(D227A)

Iodinated antibody K305Fab-SEA(D227A) was added in dilution series to FM3 cells, or to tissue sections of human malignant melanomas and the small bowel, to determine smooth-muscle reactivity. The affinity for melanoma tissue was in the range of 0.67–1.6 nM saturating $(2.2–6.4) \times 10^9$ sites/12-mm^2 section (exemplified in Fig. 4). The affinity for small-bowel tissue sections (smooth-muscle reactivity) was 0.82–1.3 nM. The affinity

Table 2 Nucleic acid identity of selected *Macaca fascicularis* antibody genes to human germline variable Ig genes

Antibody	VH (% identity)	VH family	VL (% identity)	V1 family
K305	DP-47 (89.1)	VH3	humlv 318 (91.7)	Vλ3
K461	DP-58 (90.1)	VH3	humlv 318 (90.3)	Vλ3
K458	DP-35 (89.1)	VH3	DPL1 (89.8)	Vλ1
K460	DP-58 (88.4)	VH3	DPL1 (89.8)	Vλ1
K743	DP-49 (82.3)	VH3	DPL13 (90.2)	Vλ2

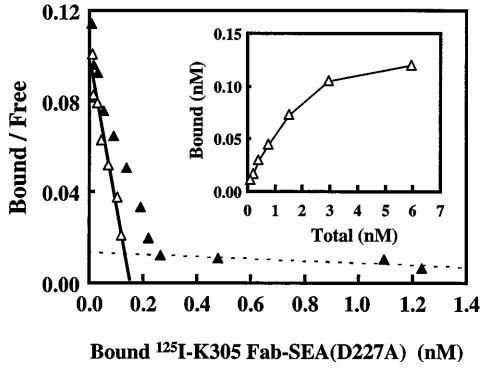


Fig. 4 Scatchard plot produced when tissue sections of metastatic melanoma were used for the determination of the antibody affinity. The affinity of the K305Fab-SEA(D227A) antibody determined in this experiment was 1.6 nM and the number of binding sites per section (3×4 mm) was 4.7×10^9

for HMW-MAA was confirmed in a cell-binding experiment (FM3 cells) demonstrating an affinity of 1.6 nM and 3×10^5 binding sites/cell (not shown).

Tumour and normal tissue reactivity of the fusion proteins

The phage-selected antibody K305 and the murine anti-HMW-MAA antibody mAb 9.2.27 strongly and homogeneously stained melanoma cells in seven out of eight biopsies of human malignant melanomas surgically removed from different patients. The predominant normal tissue reactivity found for theses antibodies was a moderate staining of smooth muscle, e.g. in the gut, and staining of the smooth-muscle layer in a fraction of blood vessel walls. In the colon and myometrium approximately one-third of the medium-sized blood vessels were negative, whereas two-thirds were weakly to strongly stained. Non-muscular vessels were negative. This pattern related to both arteries and veins for all antibodies tested, including K305, K461, K458, K743, TP41.2 and 9.2.27.

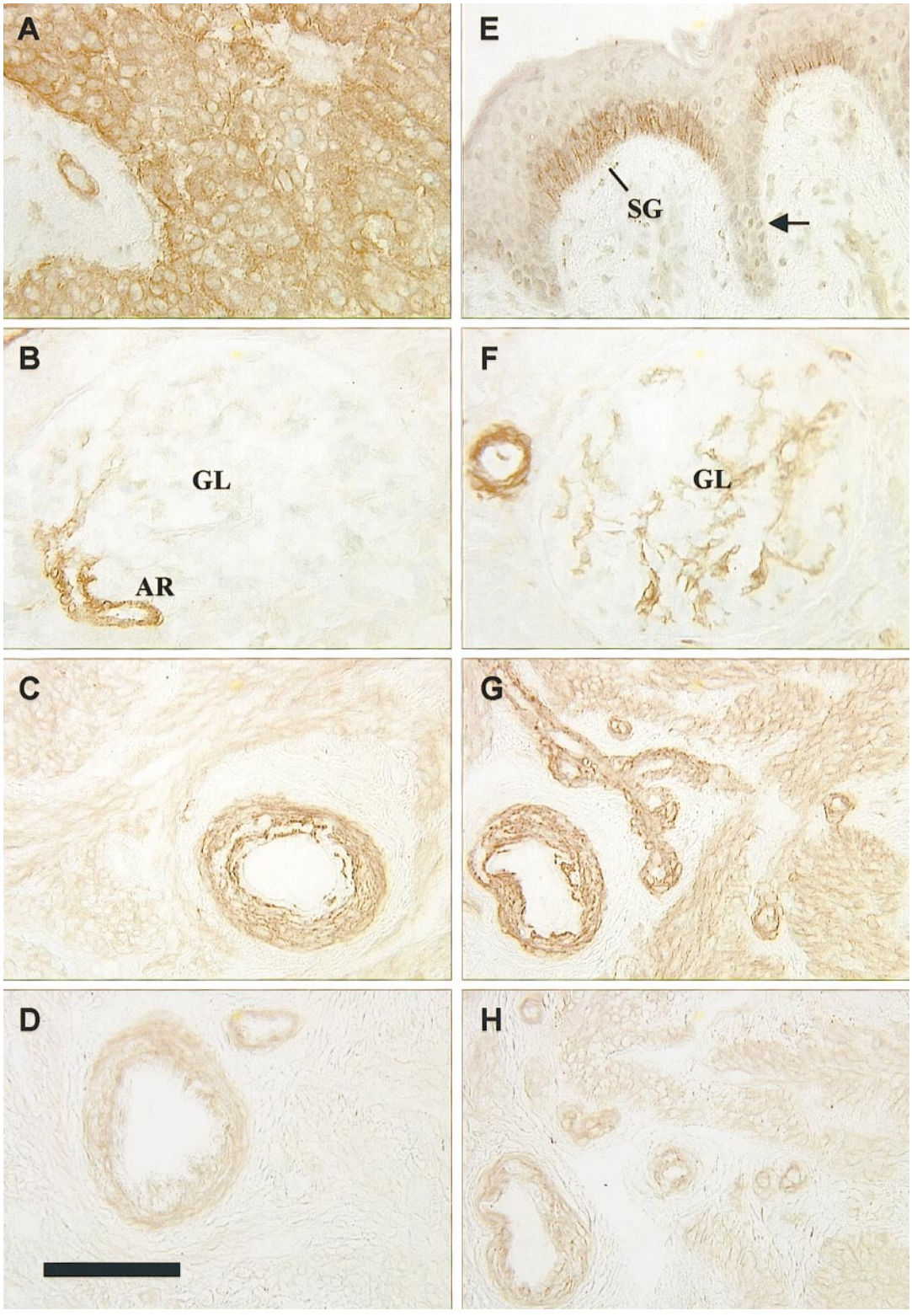
In contrast to K458, TP41.2 and 9.2.27, the K743, K305, and K461 antibody showed a clearly weaker staining of the uterine smooth muscle compared to a comparable subset of positively stained blood vessels (exemplified by K305 and 9.2.27 in Fig. 5) over a wide concentration range. This differential reactivity was most clearly demonstrated for the K743 clone (not shown). The murine mAb 9.2.27 also stained cells in the glomerulus, most likely mesangial cells, in contrast to K305, which only stained the glomerular arteriolar cuff (Fig. 5). Renal blood vessels (verified by staining of smooth-muscle actin) but not renal tubules (cytokeratin-positive) were stained by mAb 9.2.27 and K305. Normal tissue reactivity of K305 and mAb 9.2.27 also included a subpopulation of basal cells of the epidermis. Smooth muscle and blood vessels in cynomolgus colon were stained by the K743 clone (autoreactivity) and the mouse mAb 9.2.27, but not by K305, K461, K458 and TP41.2 (results not shown).

K305Fab-SEA(D227A) mediate superantigen-antibody-dependent cellular cytotoxicity

Two human melanoma target cell lines were used to demonstrate redirection of T cell cytotoxicity to tumour cells by the K305Fab-SEA(D227A) fusion protein (Fig. 6A). Dose-dependent cellular cytotoxicity could also be achieved with SEA(D227A) alone or with SEA (D227A) fused to irrelevant antibodies (due to a weak interaction with MHC class II expressed by some melanoma cell lines, e.g. the FM3 line). However, this cytotoxicity was demonstrated at a 100-fold higher concentration and with a lower plateau value than the antibody-targeted K305Fab-SEA(D227A) cytotoxicity (Fig. 6A). The targeted cytotoxicity was (as expected) independent of superantigen presentation on MHC class II, since specific cytotoxicity was also demonstrated for MHC-II-negative G361 human melanoma cells (Fig. 6B).

Immunotherapy of established FM3 tumours in SCID mice with K305Fab-SEA(D227A)

We investigated the therapeutic properties of the fusion protein, K305Fab-SEA(D227A), in humanised SCID mice with progressively growing FM3 tumours inoculated intraperitoneally (i.p) 4 days previously. The therapy was initiated simultaneously by i.p. inoculation of human PBM and compared to the effects of vehicle (PBS) and a control fusion protein, C215Fab-SEA(D227A). A dramatic and statistically significant reduction ($P = 0.003$) of the tumour mass and number was achieved with the K305Fab-SEA(D227A) fusion protein as compared to the PBS control. It is important to note that the effect was significant stronger when compared to that of the control fusion protein (Fig. 7), thus demonstrating the dependence of specific antibody-mediated targeting for the observed therapeutic effect.



Discussion

In this study we report the identification and characterisation of phage-selected primate antibodies directed against two distinct epitopes on HMW-MAA. We also describe the potential for therapeutic use of a selected antibody K305 fused with superantigen by demonstrating T-cell-dependent growth inhibition of melanoma in humanised SCID mice. Commonly, tumour-associated

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Fig. 5A–H Immunoperoxidase staining of human tissues by the HMW-MAA-specific fusion protein K305Fab-SEA(D227A) (**A–E**) and by mAb 9.2.27, in (**F–H**). **A** Strong and homogeneous staining of a malignant melanoma tissue. **B** Staining of the glomerular arteriole (*AR*) but not within the glomerulus in contrast to the staining of glomerulus by mAb 9.2.27 shown in **F**. **C, D** K305Fab-SEA(D227A) staining of the uterine blood vessels but no staining of the uterine smooth muscle at either high concentration (20 μg/ml, **C**) or low concentration (0.08 μg/ml, **D**). **G, H** mAb 9.2.27 staining of uterine smooth muscle and blood vessels (0.02 μg/ml, **G**) and (0.005 μg/ml, **H**). **E** K305Fab-SEA(D227A) staining of the stratum germinativum layer (*SG*) of the epidermis. Areas in which the stratum germinativum layer invaginates into the papillary layer of the dermis (*arrow*) were not stained. *Bar* (**D**) = 100 μm

antigens are defined by murine monoclonal antibodies [19]. However, therapeutic use of murine mAb frequently leads to the development of human anti-(mouse Ig) antibodies in patients [24], which potentially neutralise the effector functions and enhance the serum clearance rate. Human monoclonal antibodies should ideally be used, but have, for various reasons [7], been difficult to produce by the hybridoma technique.

Chimeric antibodies with human constant domains or antibodies with murine complementarity-determining regions (CDR) grafted on human Vh/Vl frameworks often demonstrate reduced neutralising human antibody responses; however, responses to V region determinants have been demonstrated [24, 37]. An alternative approach is to use non-human primates for the generation of therapeutic antibodies [31], as primate antibodies show a high degree of homology and thus should not elicit a neutralising antibody response in man [29, 33].

Furthermore, specific immune responses to human tumour-associated antigens in non-human primates should be less restricted than in man and the reactivity to common species-specific tissue antigens should be much reduced as compared to murine responses [17, 43, 48].

The identification of primate antibodies to human specific (cynomolgus-negative) HMW-MAA epitopes included the clone K305, which demonstrated a restricted reactivity with uterine smooth muscle and kidney glomeruli when compared to murine mAb, supported previous findings of primate antibody responses restricted to tumour-associated antigens [8].

To be able to identify novel antibodies to epitopes on the cell-surface and in vivo expressed tumour-associated antigens, intact cells or suspensions of cells and tissues should preferentially be used as the immunogen. A suspension of pooled metastatic melanoma tissue samples in alum adjuvant was used to immunise the cynomolgus monkeys. The immune serum was found to be strongly reactive both with antigens expressed on the surface of human melanoma cells and almost equally strongly and specifically with the human HMW-MAA.

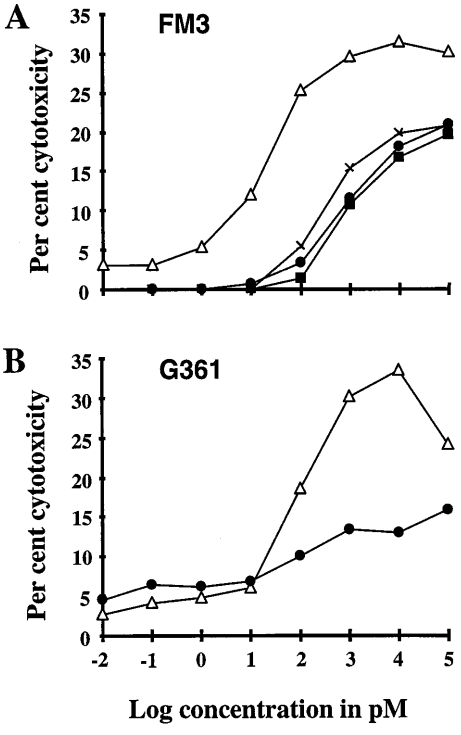

Fig. 6A, B Superantigen-antibody-dependent cellular cytotoxicity against human melanoma cells. A SEA-reactive T cell line (established from human peripheral blood lymphocytes stimulated with wild-type SEA, 12 pM) was added to the melanoma cells in an effector to target ratio of 30:1. **A** Lysis of MHCII$^+$/HMW-MAA$^+$/C215$^-$/C242$^-$ FM3 melanoma cells with K305Fab-SEA(D227A) (△) was efficient in a 100-fold lower concentration and reached a higher maximum cytotoxicity than SEA(D227A) (×) alone or the control fusion proteins C215Fab-SEA(D227A) (●) and C242Fab-SEA(D227A) (■). **B** The superantigen-mediated lysis directed by the antigen-specific antibody moiety was independent of MHC II, since MHC II$^-$/HMW-MAA$^+$/C215$^-$ G361 human melanoma could be lysed by K305Fab-SEA(D227A) (△) but not by the control fusion protein C215Fab-SEA(D227A) (●)

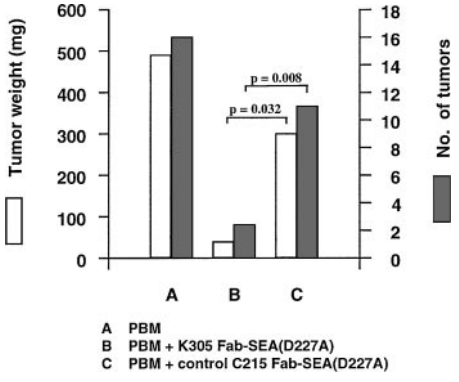

Fig. 7 Statistically significant growth reduction of established FM3 tumours grown in mice with severe combined immunodeficiency (SCID) treated with K305Fab-SEA(D227A). SCID mice with FM3 tumours grown i.p. for 4 days were grafted with human peripheral blood mononuclear cells (PBM) and, 2 h later, treated by i.v. injection of 100 μg test substance. The treatment was given three times with 3-days intervals. The tumour weight and number were calculated on day 49. *A* PBM + vehicle (1% Balb/c mouse serum in PBS), *B* PBM + K305Fab-SEA(D227A), *C* PBM + control C215Fab-SEA(D227A). One representative experiment out of three

This suggests that the HMW-MAA in the melanoma suspension was strongly immunodominant to the primate, which is supported by the finding of high anti-HMW-MAA serum antibody titre in a chimpanzee immunised with purified HMW-MAA [42].

The scFv antibody phage library was selected on human melanoma tissue sections and melanoma cell lines to generate enriched libraries against both the authentic in vivo phenotype and cell-surface-expressed antigens. A major advantage of this technology compared to the hybridoma technology is that the identification of novel antibodies and the cloning of their genes are simultaneously performed. This selection of genes encoding tumour-reactive antibodies permitted the rapid construction of superantigen fusion proteins to be evaluated for selectivity, tumour-targeting properties and therapeutic efficacy. The scFv format was suitable for the selection procedure and for screening of antibody specificities by immunochemical staining of tissue sections and cell lines. However, because of dimer formation of some library-selected scFv (results not shown) and the lower stability of scFv fragments [3], Fab-SEA fusion proteins of selected clones were constructed, produced by fermentation culture and purified. These were used for detailed immunohistochemical evaluation, for estimation of true binding affinity and in vivo experiments.

The K305Fab-SEA(D227A) fusion protein demonstrated a high binding affinity for its HMW-MAA epitope. The number of binding sites per cell differed by less than a factor of two from the number of binding sites determined with Fab generated from papain-cleaved murine mAb against the HMW-MAA [50]. The affinity determined when using tissue-expressed antigen in melanoma tissue sections was similar to the affinity determined when using cells. The number of available binding sites in a tissue section could also be calculated, to be used for estimation of the expression level and saturation density of the therapeutic target in vivo [10].

The efficacy of targeting antibodies to solid tumours has been suggested to be dependent on the antibody affinity and to peak between defined affinity thresholds [14, 39]. An antibody to the c-*erb*B-2 oncogene product with nanomolar affinity had a higher tumour to blood ratio and tumour retention when compared to a very-high-affinity (1.5×10^{-11}, reduced off-rate) and low-affinity (3.2×10^{-7}) variant with the same specificity (G.P. Adams, personal communication). Accordingly, the selected K305 antibody should be within the optimal affinity range for targeting and accumulation in tumour tissue. Higher-affinity variants (K461 and K460) to K305 and K458 may have been selected from the library, as indicated by the dose-inhibition curves and possibly by differences in the strength of signal in the immunoprecipitation data (Figs. 2, 3). However, for scFv, dimer formation varies and divalent formation could influence overall affinity [3].

The mapping of K305 and K461 to overlapping epitopes is supported by the high sequence similarity between the two antibodies. Likewise, high sequence similarity suggests that the two antibodies K460 scFv and K458 scFv bind to overlapping epitopes. Immunoprecipitation and epitope mapping could not directly link the K743 scFv antibody to HMW-MAA reactivity, but the characteristic tissue structures were identically stained in immunohistochemistry. Thus, two distinct epitope specificities are clearly linked to HMW-MAA reactivity. In addition our data possibly suggest a third epitope (recognised by K743 scFv).

The K305Fab-SEA(D227A) fusion protein had a very weak reactivity to human glomeruli and human uterine smooth-muscle cells over a wide concentration range. This suggests that the K305 epitope is expressed on an HMW-MAA variant with a more restricted normal tissue distribution. The possibility that the HMW-MAA molecule is heterogeneous has been suggested previously [15, 16, 51]. Renal tubules (verified by an antibody to cytokeratin) were not stained by the K305Fab-SEA(D227A) or the mAb 9.2.27 (in contrast to a previous report [15]). Blood vessel muscle cells (verified by anti-actin reactivity) in the kidney and elsewhere and intestinal smooth muscle were all stained with the selected scFv clones and all murine anti-HMW-MAA mAb.

The in vitro inhibition of melanoma cell growth [18] and adhesion to endothelial basement membranes [6] suggested a non-immunological as well as an immunological therapeutic potential for naked anti-HMW-MAA mAb. However, in clinical trials using naked or radiolabelled mAb, only minor and transient therapeutic results were observed despite high levels of specific uptake [28, 34]. Thus, antibody-mediated targeting of more potent effector molecules should be considered [30].

Superantigens have the capacity to induce powerful T-cell-mediated tumour killing and pro-inflammatory mechanisms when targeted by antibodies to the tumour site. Thus, this concept benefits from the use of antibody-defined epitopes on tumour-associated antigens for the retargeting of the T cell arm of the immune system proven to have the capability to eradicate tumour cells [46].

Targeting the HMW-MAA in melanoma patients by unlabelled or radiolabelled antibodies has not been associated with significant normal-organ-related accumulation or toxicity in normal tissues expressing the molecule, even at high antibody protein and radioactivity doses [34, 40]. This may be due to a limited accessibility of circulating macromolecules to HMW-MAA expressed by normal tissue. This work suggests that the use of potent effector molecules, such as the superantigen SEA(D227A), should be investigated to challenge the HMW-MAA as a useful target for efficacious and safe immunotherapy.

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