Immune response of chimpanzee to purified melanoma 250 kilodalton tumor-associated antigen*

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Summary. Melanoma high molecular weight tumor-associated antigen (TAA), having a molecular weight of 250 kilodaltons (Kd), was purified from a crude cell membrane extract through a combination of lectin affinity, immunoadsorption, and high performance liquid molecular filtration chromatography. Compared to the starting extract, purified TAA was 600-fold higher in TAA activity per microgram of protein. Purified TAA was used to immunize a chimpanzee and the resulting antiTAA immune response was evaluated. Postimmune chimpanzee serum reacted in solid phase radioimmunoassay against purified TAA with a titer in excess of 100,000. In contrast, preimmune serum had a titer of <100 in the same assay. By immunoprecipitation analysis, we were able to demonstrate reactivity of the chimpanzee immune serum with a 250 Kd TAA in spent culture medium from melanoma cells metabolically labeled with ³⁵S-methionine and with iodinated purified 250 Kd TAA. Reactivity of the chimpanzee antiserum for the 250 Kd TAA was confirmed in blocking and reciprocal immunodepletion studies using murine monoclonal antibody 9.2.27. These studies suggest that the 250 Kd TAA defined by murine monoclonal antibodies may prove to be immunogenic in man and that manipulation of the immune response to this TAA might be used to the clinical benefit of the patient.

Introduction

Monoclonal antibody (MoAb) technology has permitted definition and molecular characterization of numerous human melanoma tumor-associated antigens (TAA) [3, 7, 9, 12, 15, 17, 19, 21, 23, 24, 25, 27, 32, 34]. Several of these TAA have recently been shown to serve as suitable targets for killing of melanoma cells by immune mechanisms, in vitro and in vivo, in model systems [2, 4, 8, 10, 13, 28]. A critical question which remains unanswered is whether any of these TAA, as defined by murine MoAbs, are immuno-

genic in the tumor-bearing patient and which, if any, can serve as appropriate targets in the clinical setting.

Our interest has centered around the use of TAA for immunotherapy of melanoma. For this therapy to be effective and efficient, it is vital that the TAA utilized be immunogenic for the patient and that the TAA be expressed by the patient's own tumor cells. The 250 kilodalton (Kd) TAA, initially defined by a rabbit antiserum [11] and later by MoAb 9.2.27 [25], is a cell membrane-associated antigen which serves as the core glycoprotein of a large proteoglycan molecule having a molecular weight in excess of 400 Kd [1]. This molecule is expressed by the majority of melanomas tested and has a limited distribution among normal tissues and tumors of nonmelanoma origin [31]. Because of these properties, it has been used as the target for immune-mediated destruction of human melanoma cells via antibody-dependent cell mediated cytotoxicity in vitro, and of human tumor xenografts in the congenitally athymic nude mouse [2, 28].

In this report, we present our results from studies in which we have evaluated the immunogenicity of highly purified 250 Kd melanoma TAA in chimpanzees. Because of the phylogenetic proximity of the chimpanzee to the human, this species has proven to be a valuable model in which to approximate human immune responsiveness to tumor antigens [30]. We demonstrate that upon appropriate presentation to the chimpanzee, purified 250 Kd TAA can be an immunogenic molecule. Potential clinical significance of this finding is discussed.

Materials and methods

Animals. A 6-year-old male chimpanzee (*Pan troglodytes*) designated Ossebaw (Os), was used for these studies. This animal had received no treatment of any kind prior to initiation of these studies. In addition, a female rhesus monkey (Jackie), previously immunized with whole melanoma cells, was used as a serum donor for the immunoprecipitation studies.

Melanoma cell lines. The M14 melanoma cell line which originated in the laboratory of Dr. Donald Morton [6] was obtained from Dr. Ralph A. Reisfeld of the Scripps Clinic and Research Foundation, La Jolla, California. This cell line was maintained in HB101 serum-free culture medium (Hana Biologics, Inc., Berkeley, Calif.) as a suspension culture. For metabolic labeling with ³⁵S-methionine, M14 was grown in RPMI-1640 medium (GIBCO) containing

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10% dialyzed fetal bovine serum (GIBCO, Grand Island, NY) containing 0.25 mM methionine, to which was added 1 mCi ^{35}S -methionine (New England Nuclear, Boston, Mass.), with a sp. act. of 800 Ci/mM. Labeling was performed for 16 h after which the culture supernatant was collected, concentrated, and dialyzed for 48 h against Dulbecco's phosphate-buffered saline (DPBS). For TAA extraction, melanoma cells (M14) cells were collected over a 2-month interval during normal passage of the cell line and frozen in membrane extract buffer (see below) until used.

Monoclonal antibodies. Murine MoAb 9.2.27 which is the antibody used to define the 250 Kd melanoma TAA [25] was a generous gift from Dr. Ralph Reisfeld of the Scripps Clinic and Research Foundation, La Jolla, California. Antibody 13-17 recognizes framework determinants of human Class II MHC antigens [15] and was kindly donated by Dr. Zenon Stepkowski of the Wistar Institute, Philadelphia, Pennsylvania. Antibody DuALL-2 recognizes framework determinants of human Class I MHC antigens (personal communication) and was provided by Dr. Richard S. Metzgar of the Duke University Medical Center, Durham, North Carolina. These latter two MoAbs were used to prepare immune adherence resins for specific depletion of the respective antigens from the TAA preparations and also for monitoring the TAA preparations for contamination by these antigens. Antibody 9.2.27 was used for monitoring TAA purification and for inhibition and reciprocal immunodepletion studies.

Purification of 250 Kd melanoma TAA. The procedure used to purify this TAA is presented in Fig. 1. Initially, a 4 ml packed volume of M14 melanoma cells was homogenized, using a Dounce homogenizer, in DPBS containing 0.01% CaCl_2 , 0.01 M phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, Mo.) and 0.1% aprotinin (Sigma) (membrane extract buffer). The cell membranes were purified by differential centrifugation and the final high speed cell membrane-containing pellet was resuspended in LCL buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 7.2, 0.01 M PMSF, 0.1% aprotinin) containing 0.1% Renex-30 (Accurate Chemical and Scientific Co., Westbury, NY). Following brief homogenization, the mixture was incubated at 4°C overnight. The extract was then centrifuged at 136,000 g for 1 h in an L5-50 ultracentrifuge equipped with a Ti 70.1 rotor. The supernatant was collected as a crude extract then passed through a gelatin-Sepharose resin (Bio-Rad Industries, Burlingame, Calif.) to specifically deplete fibronectin. The effluent was collected for further purification. LCL buffer containing Renex-30 was used throughout this procedure as the running buffer. Class I and II MHC antigens were depleted using appropriate murine MoAb immune adherence resins. Removal of these highly immunogenic moieties was monitored in solid phase radioimmunoassay (RIA) using the respective MoAbs. The effluent containing the TAA was next applied to a Lens culinaris lectin-Sepharose (LCL-S) resin (Sigma). The column was then extensively washed and the bound glycoproteins were eluted in LCL buffer containing 5% galactose. The 250 Kd TAA was selectively concentrated using this procedure. The eluted glycoprotein was dialyzed against 0.1 M NaCl containing PMSF, aprotinin, and Renex-30, and subsequently against HPLC buffer (0.1 M phosphate,

PURIFICATION OF 250Kd MELANOMA TAA

Fig. 1. Purification procedure for 250 kilodalton (Kd) melanoma tumor-associated antigen (TAA). Presence of 250 Kd TAA during this procedure was monitored using murine monoclonal antibody 9.2.27. Final HPLC step was performed twice

0.1 M NaCl, pH 7.2) containing PMSF, aprotinin, and Renex-30. Following dialysis, the material was concentrated and applied to a 2.6×60 cm TSK-3000 SW HPLC column (LKB Industries, Bromma, Sweden) and eluted with the HPLC buffer. The 250 Kd TAA was eluted at the void volume of this column as determined in our solid phase immunoassay using 9.2.27 to monitor for TAA activity. The fractions comprising the TAA peak were pooled, concentrated, and rechromatographed on the same resin and eluted as before. Fractions from this procedure were again assayed for TAA content and for Class I and Class II MHC antigens by solid phase RIA. TAA-containing fractions were pooled, concentrated, sterile filtered, and the protein content determined using the Bio-Rad protein assay. This material was then used for immunization purposes and for subsequent screening of chimpanzee hyperimmune serum [HIS] and preimmune serum.

Immunization protocol. Immunizations were spaced at bi-weekly intervals and serum samples were obtained following venipuncture immediately prior to immunization. Initially, the animal received a series of 6 biweekly immunizations, each consisting of 1 μg of purified TAA suspended in 4 ml of 0.9% NaCl. The initial challenge was emulsified in 2 ml complete Freund's adjuvant (GIBCO). Challenges were administered on each of the four extremities. After the initial series of challenges, the TAA was first bound to sterile LCL-S and extensively washed prior to administration. As explained in the *Results*, this was necessary because of the lack of response of the animal to the TAA in a soluble form.

Measurement of anti250 Kd TAA antibody. The antibody response of the immunized chimpanzee was evaluated using two RIAs. Reactivity with intact target cells was determined using a standard cell surface binding assay and solid phase RIA against purified TAA, as described in detail

elsewhere [31, 32]. Briefly, each of these assays utilizes either rabbit anti-human IgG and iodinated [16] Staph protein A (SpA) or iodinated affinity purified goat anti-human IgG (KPI Laboratories, Gaithersburg, Md.) to detect TAA-bound chimpanzee antibody. Human and chimpanzee immunoglobulins are highly similar and cross-reactive in this system, thereby permitting the use of anti-human IgG. For these studies, antigen was bound at varying concentrations from 0.05 µg/well to 20 µg/well in flexible 96-well polyvinyl chloride microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.).

Immunoprecipitation and sodium dodecyl sulfate – polyacrylamide gel electrophoresis. Reactivity of chimpanzee HIS with iodinated TAA and with metabolically labeled TAA from spent culture medium was evaluated by immunoprecipitation and, for metabolically labeled TAA, by subsequent sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Antibody was used in solid phase, bound to SpA-Sepharose as described by Galloway [11]. Precipitated antigens were analyzed using SDS-PAGE on 5% gels under reducing conditions.

Specificity of chimpanzee antiserum. Antibody blocking and immunodepletion studies were performed to confirm the specificity of the chimpanzee antiTAA serum. For blocking studies, 9.2.27, was used at a 1:5 dilution. Initially, either normal (preimmune Os) serum or Os antiTAA antiserum (100 µl diluted from 10^{-2} to 10^{-8}) was added to TAA-containing wells and incubated at room temperature for 2 h. After thorough washing with PCS-T, 9.2.27 was added to each well and to control wells preincubated with buffer only. After a further 2-h incubation, the plates were again washed with 5% chicken serum, 0.1% Tween 20 (PCS-T). Iodinated, affinity purified goat anti-mouse IgG was then added and the plates incubated for an additional 60 min. After repeated washing, the wells were cut from the plate and counted in a Prias Gamma Scintillation counter (Packard Instruments). Duplicate determinations were made for each point and the mean value taken for calculation of inhibition by the formula:

$$\% \text{Inhibition} = 1 -$$

$$\frac{\text{CPM bound 9.2.27} - \text{CPM bound experimental}}{\text{CPM bound 9.2.27}} \times 100,$$

where CPM experimental was the CPM bound via 9.2.27 where the wells had previously been "blocked" by either preimmune serum or HIS from Os and CPM bound 9.2.27 represented counts bound to solid phase TAA preincubated in buffer only.

Reciprocal immunodepletion studies comparing Os preimmune serum and HIS with 9.2.27 were performed using antibodies bound in solid phase to SpA-Agarose (Sigma). Purified 250 Kd TAA was iodinated and equal amounts mixed with appropriate antibody or control absorbants. The mixture was rotated at room temperature for 1 h, after which the matrix was pelleted and the supernatant removed to a fresh antibody matrix. Upon completion of specific precipitation, determined by serial counting of precipitated antigen, the TAA-containing supernatants were mixed with fresh absorbants and precipitated once more to ensure complete depletion of the relevant TAA. Reciprocal immunoprecipitation was then performed using Os HIS and 9.2.27 and the final pellets washed and

quantitated. Preimmune Os serum was included in these studies to eliminate the possibility of nonspecific TAA depletion by Os HIS. Identical aliquots of labeled TAA were depleted using preimmune serum and subsequently depleted using either 9.2.27 or Os HIS.

Results

High molecular weight melanoma TAA was purified from a crude melanoma cell membrane extract using a combination of lectin affinity, immune adherence and HPLC (Fig. 1). As shown in Table 1, HLA-A, B, and HLA-Dr antigens were quantitatively removed using the appropriate immune adherence resins. TAA were not recovered in the eluates from these resins, suggesting lack of any covalent or noncovalent associations between the TAA and MHC antigens. TAA were selectively concentrated using LCL-S, eluted, and dialyzed against HPLC buffer. Material comprising the LCL eluate fractions was passed through the HPLC molecular filtration column, and the eluted fractions evaluated for TAA activity in solid phase RIA against MoAB 9.2.27. TAA activity was detected in those fractions comprising the first eluted protein peak, coincident with the void volume and material having apparent molecular weight of 240 Kd and greater (Fig. 2). It was also probable that some of the TAA activity in this preparation was contained in the high molecular weight proteoglycan form (>400 Kd) as this would also have eluted at the void volume of the HPLC column. No attempt was made to remove this material as our intent was to determine if this TAA, in either form, could be immunogenic when soluble and purified free of MHC antigens.

The protein content of the collected fractions was determined and TAA content per unit protein compared to that of the crude starting material. We arbitrarily assigned a TAA content of 1 unit/µg to the crude cell membrane extract and plotted a binding curve for fixed dilutions of 9.2.27 with increasing amounts of TAA in solid phase RIA (Fig. 3). Reactivity of 9.2.27 with the TAA at different stages of purification was then compared to this standard curve, and relative units of TAA determined. As shown in Table 2, the greatest purification of the TAA occurred in those steps which selected for properties of the TAA, namely, lectin affinity and high molecular weight. Thus, the material eluted from the lens culinaris lectin was enriched for TAA approximately 50-fold over the starting

Table 1. Antigen activity during purification

Antigen	HLA-Dr	HLA-A, B	TAA
Monoclonal antibody	13–17 (% ^a)	DuALL-2 (%)	9.2.27 (%)
Target			
Extract	1427 ^b (100.0)	2692 (100.0)	3746 (100.0)
DuAll-2 eluate	293 (20.5)	1023 (38.0)	201 (5.4)
13-17 eluate	528 (37.0)	170 (6.3)	189 (5.1)
Lens culinaris lectin (LCL) eluate	107 (7.5)	154 (5.7)	2810 (75.0)
HPLC	85 (5.9)	123 (4.6)	2587 (69.0)
Control (no TAA)	101 –	121 –	172 –

^a Antigen activity compared to that in starting extract

^b CPM ¹²⁵I anti-mouse IgG bound to target in solid phase radioimmunoassay 25 ng protein per well

Fig. 2. HPLC purification of melanoma TAA. Soluble melanoma glycoproteins were applied to a 2.6×600 mm TSK-3000 SW HPLC column in HPLC buffer, 0.1 M phosphate, 0.1 M NaCl, pH 7.2, with aprotinin, PMSF and Renex-30. The column was run at a flow rate of 1 ml/min and 1 ml fractions were collected. Following the initial elution of this column, TAA-containing fractions were pooled, concentrated and reapplied to the column under identical conditions. Shown in this figure is the final chromatogram from this column. Fractions having TAA activity (shaded area on figure) were pooled and used as the final TAA preparation for all subsequent experiments. Molecular weight standards for calibration of the column were: glutamate dehydrogenase (290 Kd), lactate dehydrogenase (140 Kd), enolase (67 Kd), adenylate kinase (32 Kd), and cytochrome C (12.4 Kd)

Fig. 3. Quantitation of TAA. Purified 250 Kd melanoma TAA was used in solid phase radioimmunoassay to establish a binding curve with monoclonal antibody 9.2.27. Binding of 9.2.27 was quantitated by the use of ^{125}I goat anti-mouse IgG. The linear relationship between TAA units and 9.2.27 binding was determined by linear regression analysis

Table 2. Purification and yield of 250 Kd TAA

Step	Units TAA/ μg	TAA yield ^a
1) Crude extract	1	100%
2) Immunodepletion ^b	1	82%
3) LCL eluate	62	75%
4) HPLC	614	69%

^a % of TAA from crude extract recovered

^b Gelatin-sepharose, DuALL-2 and 13-17 immune adherence

material while an additional 10-fold purification was achieved through HPLC chromatography. Procedural steps designed to specifically deplete contaminating antigens resulted in little change in TAA concentration. As presented in Table 2, we were able to achieve a purifica-

Fig. 4. Purified TAA analysis using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified melanoma 250 Kd TAA was radioiodinated, concentrated and then applied to a 5% SDS polyacrylamide gel under reducing conditions (lane 2). Lane 1 represents immunoprecipitate of purified TAA by monoclonal antibody 9.2.27. Trace contamination at approximately 40 Kd represented 20% of total activity on gel. Also evident is high molecular weight ($>440,000$ Kd) not able to enter gel. Molecular weight standards used were: myosin (200 Kd), phosphorylase B (92.5 Kd), albumin (69 Kd), ovalbumin (46 Kd), and carbonic anhydrase (30 Kd)

tion in excess of 600-fold using the procedure described. The final TAA preparation was determined to be free of contaminating Class I and Class II MHC antigens by solid phase RIA using MoAbs DuAll-2 and 13-17, respectively (Table 1). Purified TAA was iodinated and evaluated for purity by SDS-PAGE analysis using a 5% gel. As shown in Fig. 4, lane 2, essentially all of the radiolabeled material migrated with a molecular relative mass equivalent to a molecular weight of approximately 250 Kd. A very faint band of protein equivalent to a molecular weight of slightly less than 40 Kd was also detected. It was unclear whether this represented an actual contaminant in the TAA preparation, or a degradation product from the 250 Kd TAA resulting from the iodination or protolysis. We estimated this material to comprise not more than 20% of the total protein entering the gel. The immunoprecipitate of the labeled TAA obtained using MoAb 9.2.27 is shown in lane 1 (Fig. 4), demonstrating that the purified material was the TAA defined by this antibody.

The initial immunizations of the chimpanzee consisted of 1 μg purified TAA suspended in 0.9% NaCl, administered s.c. to the four extremities biweekly. Following 6 such immunizations, we were unable to demonstrate any significant rise in antibody reactivity with the purified TAA (data not presented). This observation strongly suggested that this TAA was not immunogenic, in the soluble form, at the dose we were administering. Rather than use greater amounts of TAA, we elected to attempt immunization of the chimpanzee with TAA in an insoluble form. For this purpose, we chose to bind the TAA to immobilized Lens culinaris lectin, thereby taking advantage of the affinity of the TAA for the lectin. Following subsequent

Fig. 5. Chimpanzee anti-250 Kd melanoma TAA IgG response. Preimmune chimpanzee serum (●—●) and chimpanzee HIS (○—○) were assayed against purified melanoma TAA in solid phase radioimmunoassay. Binding was quantitated using 125 I-labeled SpA.

Fig. 6. Immunoprecipitation of 35 S-methionine-labeled protein form M14 spent culture medium. Lane 1. Jackie (monkey anti-melanoma), lane 2. Os preimmune, lane 3. Os HIS, lane 4. rabbit 6522 (antimelanoma). 5% gel. Standards, as per Fig. 4

immunizations with insoluble TAA, the animal produced a potent IgG antiTAA response, as presented in Fig. 5. When tested against purified TAA as used for immunization in solid phase RIA, preimmune serum from this animal had a titer of <100 . In contrast, serum collected following the second immunization using immobilized TAA had an antiTAA titer $>100,000$.

We evaluated the specificity of the chimpanzee HIS by immunoprecipitation and by blocking and reciprocal immunodepletion studies. For immunoprecipitation studies, we utilized spent culture medium from M14 melanoma cells metabolically labeled with 35 S-methionine, the original source in which the TAA was defined, and compared

reactivity of the chimpanzee HIS with other polyclonal antisera known to react with the 250 Kd TAA (Fig. 6). Rabbit antiserum 6522, originally used to define this TAA, reacts in a characteristic manner, precipitating fibronectin (molecular weight = 220 Kd) as well as the 250 Kd TAA (lane 4). Two additional species precipitated in a nonspecific manner in all samples. An identical pattern of reactivity was evident using a monkey antiserum raised against whole melanoma cells (lane 1). Earlier studies performed using this antiserum resulted in our first evidence that this TAA was immunogenic in primates when presented to the intact melanoma cell, and that purified TAA might be immunogenic in the chimpanzee. In contrast, the chimpanzee antiTAA HIS (lane 3) has a specific reactivity limited to the 250 Kd TAA. As with the other antisera, nonspecifically precipitated material was also evident. Preimmune chimpanzee serum (lane 2) showed no antiTAA activity. These immunoprecipitation results clearly demonstrated that the TAA used for immunization was of a high degree of purity and that the chimpanzee was able to respond to the TAA when presented in a suitable format.

Specificity of the chimpanzee HIS for the 250 Kd TAA was further evaluated in blocking and reciprocal immunodepletion studies using MoAB 9.2.27. In the blocking studies, serial dilutions of preimmune serum or HIS were added to solid phase TAA and the ability of such preincubation with chimpanzee serum to inhibit subsequent binding of 9.2.27 was determined. As shown in Fig. 7, chimpanzee antiTAA antiserum strongly inhibited the binding of 9.2.27 compared to preimmune serum. The inhibitory activity of the HIS was greater than 10^{-7} . In contrast, inhibition of 9.2.27 binding to TAA by preimmune serum never exceeded 12%, which represents background inhibition in this assay. Similar specificity of the chimpanzee HIS for the 250 Kd TAA was suggested by reciprocal immunodepletion studies. Equivalent amounts of iodinated purified TAA were sequentially depleted with aliquots of either solid phase 9.2.27 or chimpanzee HIS. When each sample had been totally depleted of specifically reactive material, reciprocal immunodepletion was performed. Chimpanzee

Fig. 7. Blocking of binding of monoclonal antibody 9.2.27 to purified TAA by chimpanzee serum. Preimmune chimpanzee serum (●—●) and chimpanzee HIS (○—○) were compared for their ability to specifically block the binding of 9.2.27 to the purified 250 Kd melanoma TAA in solid phase radioimmunoassay. TAA-coated wells were initially incubated with preimmune or HIS serum, then washed and incubated with 9.2.27 at $1/5$ dilution. Binding of 9.2.27 was then quantitated using 125 I goat anti-mouse IgG. Inhibition (blocking) was calculated as per the text

Table 3. Reciprocal immunodepletion of ^{125}I -TAA using chimpanzee hyperimmune serum (HIS) and monoclonal antibody 9.2.27

Depleting antibody ^a	CPM ^{125}I -TAA precipitated		
	Os HIS	Os preimmune serum	9.2.27
Precipitate			
1	19,028	1,108	6,724
2	3,108	987	8,648
3	2,552	1,011	3,044
4	824	923	1,076
5	831	820	1,021
Precipitate using 9.2.27	511	4,135	—
Precipitate using Os HIS	—	15,471	4,435

^a Os HIS, Os preimmune and 9.2.27 were bound to SpA-Sepharose for these studies

HIS completely depleted the TAA preparation of all antigen reactive with MoAb 9.2.27 (Table 3), a finding which again demonstrated the generation of an anti250 Kd TAA antibody in response to immunization. In contrast, 9.2.27 specifically depleted most, but not all, of the chimpanzee HIS reactive material from the TAA preparation. Such a finding suggests that the chimpanzee antiserum contained antibody against additional antigenic determinants present in the preparation. These could either represent actual contaminants not clearly demonstrated in the SDS-PAGE analysis, or partially degraded 250 Kd TAA no longer expressing the determinant reactive with 9.2.27. Similar depletion of separate aliquots of TAA using Os preimmune serum failed to significantly reduce the TAA content of these aliquots as defined by Os HIS and 9.2.27.

Discussion

In this study, we have attempted to purify and evaluate the immunogenicity of the 250 Kd melanoma-associated antigen in chimpanzee. Using a combination of immune adherence, lectin affinity, and high performance liquid molecular filtration chromatography, we have achieved a purification factor in excess of 600 for this TAA relative to the crude starting melanoma cell membrane extract. Additionally, we have been able to specifically deplete strongly immunogenic molecules such as Class I and Class II MHC antigens and fibronectin from the antigen preparation. Using this highly purified antigen preparation for immunization of a chimpanzee, we have demonstrated both humoral and cellular antiTAA immune responses to this TAA preparation, and have defined the specificity of the antibody response for 250 Kd TAA.

Although a number of murine monoclonal antimelanoma antibodies have been developed, little work has been performed to document whether any of these TAA represent potentially immunogenic molecules in the tumor-bearing host. This becomes an important consideration in view of the recent observations that certain murine MoAb-defined human melanoma TAA, most notably the 250 Kd TAA and GD3, are able to function as target molecules for antibody-mediated destruction of tumor [2, 4, 8, 10, 13, 28]. Thus, passive serotherapy for melanoma using appropriate murine monoclonal reagents is a possibility. However, such an approach is complicated by many con-

siderations including the host response against the mouse immunoglobulin and the questionable ability of host cellular elements to interact with murine antibody to effect tumor cell destruction [14]. Likewise, the use of immunotoxin or drug conjugates for therapy has associated complications which makes this less than an ideal approach [33].

As an alternative, the possibility exists for specifically manipulating the host's own immune reactivity against the TAA. In this way, problems associated with the introduction of heterologous serum proteins may be overcome. Most immunotherapy protocols attempt such manipulations by specific and/or nonspecific means [5, 18, 20, 22, 26, 29]. In such cases, however, the relevant target TAA for the therapy are ill-defined. As more information is gathered regarding the intricacies of the immune response and its regulation, it is becoming more apparent that the time and manner in which an antigen is presented and the properties of the antigen determine, to a large degree, the nature of the host immune response to that antigen. Thus, to maximize the benefit derived from immunotherapy, it is critical that we understand which TAA the patient is able to respond to and how best to present these TAA to the host.

Our approach to this problem has been to evaluate the immunogenicity of purified melanoma TAA in a chimpanzee model. We have chosen to study selected melanoma TAA defined by murine MoAbs for two reasons: specific monoclonal reagents reactive with these TAA are available to assist in TAA purification and analysis of the responses generated, and the TAA selected represented ideal potential targets for therapy because of their expression on the cell membranes of most melanomas examined. The critical question is then whether the TAA defined by murine MoAbs can also be immunogenic in the patient. In previous studies, we had immunized a number of nonhuman primates for the generation of antimelanoma antiserum. During the routine evaluation of one such antiserum, raised against whole, fresh melanoma cells, we observed reactivity against the 250 Kd TAA by immunoprecipitation followed by SDS-PAGE analysis (Fig. 6). This finding suggested that the TAA could be immunogenic when presented to the intact melanoma cell and served as the basis for the present study on the immunogenicity of the purified TAA.

The isolation of the TAA from melanoma cells in quantities sufficient for the present study has not presented a problem. However, should clinical trials ensue, it would be necessary to use an alternative source of TAA, such as fresh tissue, to insure adequate quantities of TAA. With this in mind, we have expanded the purification procedure described here to include the additional procedures of ion exchange and immunoaffinity chromatography. For the present study, we have been able to achieve a purification of TAA in excess of 600-fold over the starting material and specifically depleted several potent immunogens from the TAA preparation (Table 1). The purity of this preparation has been addressed in two ways. Following iodination of the final TAA preparation, we performed SDS-PAGE analysis (Fig. 4). The majority of the labeled material migrated in a manner identical to the 250 Kd TAA, as defined by MoAb 9.2.27. A trace amount of protein (<20%) was detected with a molecular weight <40 Kd. We were unable to determine whether or not this represented a true contaminant in the preparation or a de-

gradation product resulting from the iodination procedure. Second, we evaluated the resulting chimpanzee antiserum for its reactivity with spent culture medium from metabolically labeled melanoma cells (Fig. 6). Such medium contains the 250 Kd TAA as well as a multitude of other cellular constituents. It was evident that only the 250 Kd TAA was specifically precipitated from this material. In contrast, antisera raised against less pure melanoma preparations precipitated multiple components from the spent medium.

For our initial immunizations of the chimpanzee, we elected to use soluble TAA in saline. It was quickly apparent, however, that the TAA, in this mode, was not sufficiently immunogenic for the chimpanzee. This could have been a reflection of the small amounts of TAA administered or of the manner in which it was being presented to the recipient's immune system. Both of these possibilities were addressed by binding the TAA to LCL-S beads and using these as a solid support. The TAA was effectively concentrated and also presented in a matrix format. The chimpanzee responded rapidly to this immunization format with a potent IgG antiTAA response. The specificity of this response for the 250 Kd TAA was confirmed using immunoprecipitation and blocking studies with MoAb 9.2.27. Measurement of antiTAA cellular immunity, in this case, cell-mediated cytotoxicity, also suggested an immunizing effect of the TAA.

The finding that purified melanoma 250 Kd TAA is, under suitable presentation to the host, immunogenic in the chimpanzee, is an important prelude to similar studies in man. The immune responsiveness of the chimpanzee is highly similar to that of man and, as such, the chimpanzee represents the ideal animal model in which to approximate human antiTAA responsiveness. Given the present data and the observations indicating that the 250 Kd TAA can be an appropriate target antigen for immune destruction of tumor, it is conceivable that use of this TAA for therapy may prove clinically beneficial. Patients having growing tumor would be expected to be under continuous exposure to this TAA in some form as the result of surface shedding and cell death. Yet, the immune response potentially elicited by this TAA is obviously inadequate for eradication of the tumor. This could reflect either a very weak TAA-specific host response, inappropriate presentation of the TAA to the host, or TAA-specific suppressive responses. In such cases, presentation of the TAA to the host in a more highly immunogenic format might effectively stimulate antiTAA reactivity able to eradicate tumor. A more likely and immediate use for such purified TAA might be for adjunct immunotherapeutic purposes in patients rendered tumor-free by surgery. In such cases, the patients would be expected to have minimal endogenous exposure to TAA and might have heightened an appropriate responsiveness to TAA presented in an immunogenic format. It is also of some concern that this TAA is shed from intact tumor cells *in vitro*, thus raising the possibility that an immune response to this TAA might prove ineffective due to inhibition by soluble TAA. While this possibility cannot be directly addressed, it has, nonetheless been demonstrated that passive administration of antiTAA antibody in melanoma xenograft-bearing congenitally athymic mice, does have tumor inhibiting activity [2]. Such data suggests that the antibody administered does have the ability to bind to the target cells *in vivo*, in spite of the putative presence of

soluble TAA. The next phase of this study is to determine the optimal conditions for immunization of the chimpanzee with purified TAA and then to initiate human clinical trials.

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